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Cell Therapy, Tissue Engineering and Regenerative Medicine

**The role of circulating Monocyte subsets, Myeloid-Derived suppressor cells
and LOX-1 and PD-L1 expression in predicting Neoadjuvant treatment
response in Breast cancer patients**

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List of abbreviations:

BC Breast Cancer

CDK Cyclin-dependent kinase

DCIS Ductal Carcinoma In Situ

LCIS Lobular Carcinoma In Situ

IDC Invasive Ductal Carcinoma

ILC Invasive Lobular Carcinoma

ER Estrogen Receptor

PgR Progesterone Receptor

HER2 Human Epidermal growth factor
Receptor 2

TNBC Triple-Negative Breast Cancer

PRS Polygenic risk score

DFS Disease-free survival

NAC Neoadjuvant Chemotherapy

OS Overall survival

SOC Standard of care

pCR pathological Complete
Response

MMPs Matrix metalloproteinases

MDSCs Myeloid Derived Suppressor Cells

SLNB Sentinel lymph node biopsy

ALND Axillary Lymph Node Dissection

SERMs Selective Estrogen Receptor
Modulators

AIs Aromatase Inhibitors

ATRA All-trans retinoic acid

PMN-MDSCs Polymorphonuclear MDSCs

M-MDSCs Monocytic-MDSCs

NK Natural killer cells

ARG Arginase

NOS Nitric oxide synthase

ROS Reactive oxygen species

RNS Reactive nitrogen species

PDGF Platelet-derived growth factor

VEGF Vascular endothelial growth factor

TAN Tumor-associated neutrophil

MS Mass-spectrometry

EDTA Ethylenediaminetetraacetic
Acid
PBMCs: Peripheral blood mononuclear
cells

ddPCR Digital droplet PCR

FACS Fluorescence-Activated Cell Sorter

RD Residual disease

ECM Extracellular matrix

NSCLC Non-small cell lung cancer

LOX-1 Lectin-like oxidized low-density
lipoprotein (LDL) receptor-1

GM-CSF Granulocyte-macrophage colony-
stimulating factor

G-CSF Granulocyte colony-stimulating factor

M-CSF Macrophage colony-stimulating
factor

IDO Indoleamine 2,3-dioxygenase

1.Introduction

1.1 Breast cancer:

Breast cancer is the most commonly diagnosed cancer globally and the leading cause of cancer incidence among women, accounting for 11.7% of all cancer diagnoses in 2020, with an estimated 2.3 million new cases. Despite significant advancements in treatment, it remains the fifth leading cause of cancer-related deaths, claiming 685,000 lives annually. [2]

The incidence of breast cancer is strongly correlated with human development, with higher rates observed in countries with advanced healthcare systems and prolonged exposure to reproductive, hormonal, and lifestyle risk factors. However, over half of all cases occur in low- and middle-income countries, where limited access to early detection and treatment exacerbates the burden of disease. [1]

Early detection through mammographic screening has been pivotal in reducing mortality in developed countries. Risk factors such as age, reproductive history, and lifestyle contribute to its prevalence, with over a third of cases occurring in women over 70 in developed countries and higher proportion in younger women in less developed regions. [3] Addressing disparities in healthcare access and developing innovative, cost-effective detection strategies are essential to mitigate the global impact of breast cancer and improve outcomes worldwide. [4]

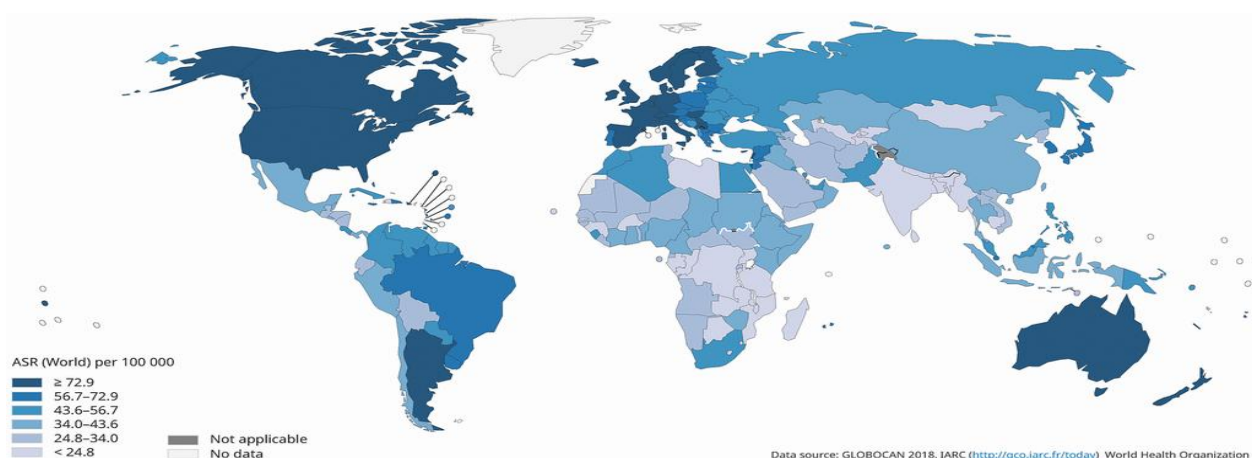


Figure 1: Global estimated incidence of breast cancer in 2018, females, all ages.[4]

1.2 Breast Anatomy and Cancer Development:

The breast is a glandular organ present in both males and females, with females having a higher proportion of glandular tissue compared to males. Structurally, the breast consists of fatty tissue (adipose tissue), 12–20 lobes further divided into lobules (milk-producing glands), and ducts that transport milk to the nipple. These components are supported by fibrous connective tissue and ligaments. The breast is also supplied by an intricate network of blood vessels, lymph vessels, and lymph nodes, as well as sensory nerves that contribute to its functional and sensory roles.

The female breast undergoes cyclic changes in response to hormonal fluctuations during the menstrual cycle, pregnancy, and lactation. Hormones such as prolactin, released during nipple stimulation, regulate milk production and can influence uterine contractions. This hormonal sensitivity makes the breast highly dynamic but also susceptible to pathological changes. [6] Breast cancer can spread outside the breast through blood vessels and lymph vessels.

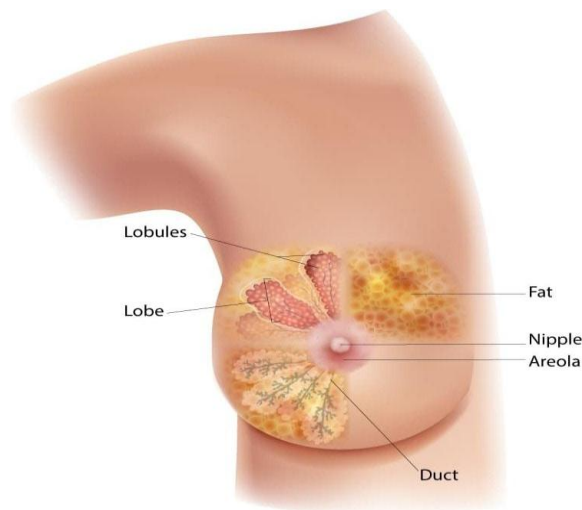


Figure 2: This shows the location of the lobules, lobe, duct, areola, nipple, and fat.[50]

1.3 Breast cancer risk factors:

Breast cancer arises from the uncontrolled growth of cells within the lobules, ducts, or surrounding tissue. Its development is influenced by a combination of genetic, hormonal, lifestyle, and environmental factors. Women with a first-degree relative diagnosed with breast

cancer are at a higher risk, with the presence of BRCA1 and BRCA2 mutations accounting for 2–9% of all cases.

Advances in next-generation sequencing (NGS) have identified additional BC-associated genes like PALB2, ATM, CHEK2, RAD51C, BARD1, and TP53, among others. These genes, when assessed together, can form a polygenic risk score (PRS) that helps stratify women by their likelihood of developing breast cancer. Despite empirical evidence showing that less than 10% of breast cancers are inherited, many women perceive genetics and family history as significant contributors, a belief often shaped by media coverage.

Age is another established risk factor, with breast cancer incidence doubling with each decade of life in postmenopausal women. High breast density also poses a significant risk, increasing susceptibility by up to fourfold compared to women with less dense breast tissue. Reproductive factors, such as early menarche, nulliparity, late first pregnancies, and lack of breastfeeding, alongside prolonged exposure to estrogen through hormone replacement therapy or oral contraceptives, are strongly associated with elevated breast cancer risk.

Environmental attributions, including exposure to pesticides, radiation, and air pollution, are frequently reported by breast cancer patients, though their perceived significance varies across studies. Additionally diet and the use of hormone replacement therapy are crucial in understanding breast cancer development.

This divergence between public perceptions and established risk factors underscores the importance of addressing misconceptions and enhancing awareness. Bridging these gaps is crucial for developing effective prevention strategies, fostering accurate risk assessment, and promoting early detection. [7]

1.4 Clinical and Histological Classification of Breast Cancer:

Breast cancer occurs in two broad categories: invasive and noninvasive. Some are slow-growing, while others are more aggressive. A number of factors influence how aggressive a tumor is, including its size, stage, etc. But the most aggressive types of breast cancer tend to be

inflammatory breast cancer and angiosarcoma of the breast, while ductal carcinoma in situ, lobular carcinoma in situ and phyllodes tumors tend to be more slow-growing.

Non-invasive Breast Cancer:

Non-invasive breast cancers, also known as carcinomas in situ, are abnormal cells confined within the milk ducts or lobules of the breast. The two main types are ductal Carcinoma in Situ (DCIS) which occurs when abnormal cells form within the milk ducts but have not spread beyond the duct walls and lobular Carcinoma in Situ (LCIS) that involves abnormal cells forming in the lobules (milk-producing glands) of the breast with a low potential for progression. [9]

Invasive Breast Cancer:

Invasive breast cancers cells that penetrate the boundaries of the ducts and lobules, infiltrating the adjacent adipose and stromal tissues of the breast. [10] It contains Invasive Ductal Carcinoma (IDC) that begins in the milk ducts and invades surrounding breast tissue and Invasive Lobular Carcinoma (ILC) that starts in the lobules and spreads into nearby breast tissue [8].

Molecular subtypes of breast cancer:

Breast cancer is categorized into molecular subclasses based on gene expression levels, cytogenic pathways, and genomic instability. Based on the molecular classification of breast cancer, as described in the review by Dixita Das in 2011, breast cancer can be categorized into 3 major subtypes: ER+ (Luminal A, Luminal B, HER2-enriched, and Triple-negative/Basal-like breast cancer (TNBC)[11]

Luminal Subtypes (A and B):

- Luminal A: tumors are estrogen receptor (ER) and/or progesterone receptor (PR) positive, HER2 negative, and have low proliferation rates marker Ki-67. They exhibit high expression of luminal epithelial genes and are associated with better prognosis and responsiveness to endocrine therapy. Prognosis and overall survival of luminal-A is greater than luminal-B subtype

- Luminal B: tumors are ER positive, often PR negative, HER2 positive, and show high proliferation (e.g., Ki-67). They have poorer prognosis, reduced response to endocrine therapy, and express fewer luminal genes

Luminal tumors express growth patterns similar to normal mammary gland epithelium. ER and PR receptors are key in the classification, with four subgroups based on their expression: ER+/PR+, ER+/PR-, ER-/PR-, and ER-/PR+. The double-positive (ER+/PR+) subtype responds better to endocrine therapies, while the double-negative (ER-/PR-) subtype has a worse prognosis and higher relapse rates. They are appropriate for chemotherapy if unresponsive for endocrine treatment therapy.

HER2-Enriched Breast Cancer: BC comprising 15% of cases, is marked by HER2 overexpression and rapid growth, It shows resistance to endocrine therapy but it responds well to targeted therapies (Anti-HER2 agents). **[12-14]** They have two subgroups can be distinguished: luminal HER2 (E+, PR+, HER2+ and Ki-67:15–30%) and HER2-enriched (HER2+, E-, PR-, Ki-67>30%)

TNBC: Triple Negative Breast Cancer (TNBC) is a heterogeneous disease characterized by the absence of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) expression based on immunohistochemistry. TNBC is typically observed in women who carry BRCA1 mutations. It is known for its distinct molecular profile, aggressive nature, and lack of targeted therapies. **[13]**

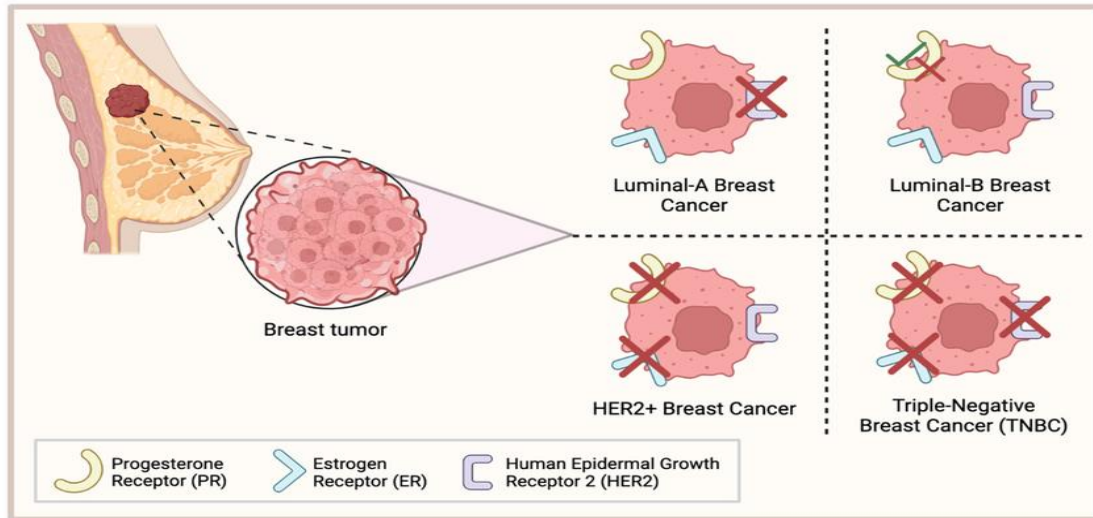


Figure 3: Classification of common breast cancers. Luminal-A breast cancer lacks expression of HER2 and low Ki67<20%; Luminal-B breast cancer is either PR+/- High Ki67>20%; HER2+ breast cancer lacks PR and ER expression High Ki67>30%; triple-negative breast cancer (TNBC) lacks expression of PR, ER, and HER2. [14]

1.5 Staging and grading of breast cancer:

In clinical practice, the TNM classification system is commonly used to describe the anatomical extent of breast cancer (BC), providing both prognostic and predictive information for treatment decisions [15]. The stages, ranging from 0 to 4, are determined by the tumor's size, type, and extent of penetration into breast tissues:

- **Stage 0:** It represents non-invasive breast cancer, where abnormal cells are confined to their origin site within the breast.
- **Stage I:** This stage marks the beginning of invasive breast cancer. The tumor is up to 2 cm, with no lymph node involvement.
- **Stage II:** Involves invasive BC that has spread to 1 to 3 axillary lymph nodes.
- **Stage III:** Characterized by invasive BC that has spread to 4 to 9 axillary lymph nodes or near the breastbone. This stage may include inflammatory breast cancer.

- **Stage IV:** This stage represents advanced, metastatic breast cancer. At this stage the cancer has spread beyond the breast and nearby lymph nodes to other organs such as the lungs, bones, liver, or brain. [6]

Table 1: Breast cancer stage scores associated with their typical TNM

stage	T	N	M
0	Tis	N0	M0
IA	T1	N0	M0
IB	T0	N1mi	M0
	T1	N1mi	
IIA	T0	N1	M0
	T1	N1	
	T2	N0	
IIB	T2	N1	M0
	T3	N0	
IIIA	T0	N2	M0
	T1	N2	
	T2	N2	
	T3	N1	
	T3	N2	
IIIB	T4	N0	M0
	T4	N1	
	T4	N2	
IIIC	Any T	N3	M0
IV	Any T	Any N	M1

1.6 Therapeutic option for Breast cancer:

In developing an optimal treatment strategy, multiple factors must be considered, including the including the stage and grade of the cancer, the presence of specific biomarkers, the patient's

overall health status, and individual preferences. [16]

1.6.1 Local therapy:

Local therapy refers to treatments that specifically target the tumor and surrounding tissues, with the primary objectives of removing or destroying the tumor, preventing local recurrence, and, when possible, preserving the appearance of the breast. It plays a pivotal role in the treatment of early-stage breast cancer, where the disease is typically confined to the breast or local lymph nodes. Local therapy consists primarily of *surgical interventions* and *radiation therapy*. [17]

Surgery: Most people begin their breast cancer treatment with surgery. There are different types of surgery for breast cancer, these include: [20-21]

- **Breast conserving surgery**: Breast-conserving surgery is an operation to remove the cancer and some normal tissue around it, but not the breast itself. This type of surgery may also be called lumpectomy, partial mastectomy, segmental mastectomy, quadrantectomy, or breast-sparing surgery [21]
- **Mastectomy**: Total mastectomy is surgery to remove the whole breast that has cancer. Some of the lymph nodes under the arm may be removed and checked for cancer. [17]
- **Surgery to the lymph nodes**: Lymph nodes may be removed either as part of the surgery to remove the breast cancer or as a separate operation. The two main types of surgery involved sentinel lymph node biopsy (SLNB) and axillary Lymph Node Dissection (ALND) [17-21] The status of the axillary lymph nodes plays a crucial role in determining the need for further treatments like radiation therapy or systemic therapies (chemotherapy, hormonal therapy). [16-21]

Radiation therapy: Is an effective cancer treatment that uses high-energy radiation to destroy or inhibit the growth of cancer cells. There are two primary types of radiation therapy: external and internal. **External radiation therapy** involves the use of a machine outside the body to direct radiation to the affected area, commonly used in the treatment of breast cancer. In contrast, **internal radiation therapy**, also known as brachytherapy, involves placing

a radioactive substance directly into or near the tumor using needles, seeds, or catheters. The specific type and method of radiation therapy depend on the type and stage of the cancer. [21-18]

1.6.2 Systemic therapy:

In addition to surgery to remove cancerous tissue and radiation therapy, breast cancer also is commonly treated with drugs that are taken orally or intravenously, as an infusion into a vein and they can be administered preoperatively (neoadjuvant), postoperatively (adjuvant), or both. [17, 21, 22]

Depending on the breast cancer subtype, systemic treatments are categorized into *chemotherapy, endocrine therapy, and targeted therapy*.

Endocrine therapy: Is a treatment for hormone receptor-positive breast cancer, primarily involving anti-estrogen drugs administered daily for at least five years, with extended durations considered for patients at higher risk of recurrence. This therapy targets estrogen-driven tumor growth by either inhibiting estrogen production or blocking its receptor activation.

Selective Estrogen Receptor Modulators (SERMs) such as tamoxifen and raloxifene bind to estrogen receptors and promote its degradation, inhibiting tumor growth in estrogen receptor (ER)-positive breast cancer. Tamoxifen, widely used as an adjuvant therapy, reduces breast cancer recurrence. [17] Additionally, Aromatase Inhibitors (AIs) suppress estrogen synthesis by inhibiting the aromatase enzyme, demonstrating superior efficacy in postmenopausal patients by blocking the conversion of androgens to estrogen.[23-21] Besides, CDK4/6 inhibitors and targeted therapies further enhance endocrine therapy, particularly in metastatic breast cancer. Endocrine therapy is used in both adjuvant and neoadjuvant settings to minimize recurrence risk and improve patient outcomes. [18]

Targeted therapy: Has revolutionized the treatment of breast cancer, particularly in HER2-positive and estrogen receptor-positive (ER+) subtypes. Key anti-HER2 agents, such as trastuzumab and pertuzumab, have been pivotal in targeting the HER2 receptor, with

trastuzumab blocking HER2 signaling and pertuzumab inhibiting HER2 heterodimerization. The combination of these therapies has demonstrated significant improvements in survival outcomes. For ER+ breast cancer, Tamoxifen works by competitively inhibiting estrogen receptor binding, while aromatase inhibitors reduce estrogen production, both effectively slowing tumor progression. CDK4/6 inhibitors are the key treatments for ER+ cancers in controlling cell cycle progression and metastasis. PARP inhibitors, like olaparib, are effective for patients with BRCA mutations, exploiting DNA repair deficiencies to promote cancer cell death. **[24-17-18]**

Chemotherapy: Is the treatment of breast cancer, particularly in early-stage and metastatic disease. It involves the use of cytotoxic agents to target rapidly dividing cancer cells, thereby inhibiting tumor growth and promoting cell death. Common chemotherapeutic agents for early-stage breast cancer include anthracyclines (e.g., doxorubicin) and taxanes (e.g., paclitaxel), which work by intercalating DNA, inhibiting DNA-associated enzymes, and disrupting microtubules, leading to mitotic inhibition and apoptosis. Chemotherapy can be administered either before (neoadjuvant) or after (adjuvant) surgery, depending on the patient's condition and cancer stage.

For metastatic breast cancer, chemotherapy is primarily used to control the disease, slow its progression, and alleviate symptoms. Despite advancements in targeted therapies, chemotherapy remains essential for certain aggressive subtypes, such as triple-negative breast cancer (TNBC), which lacks specific therapeutic targets. **[6-17]**

1.7 Neoadjuvant treatment (NAT) and pathological complete response (pCR):

Neoadjuvant treatment is a standard treatment approach for patients with newly diagnosed breast cancer, administered prior to surgery with the goal of achieving a pathologic complete response (pCR). Achieving pCR has been associated with improved long-term outcomes, including disease-free survival (DFS) and overall survival (OS). While NAT has not demonstrated a significant survival advantage over adjuvant chemotherapy, it offers several benefits, including tumor downstaging, improved breast-conserving surgery rates, and real-time assessment of tumor response to therapy.

The effectiveness of NAT varies by breast cancer subtype, with higher response rates observed in HER2-positive and triple-negative breast cancers (TNBC). Standard NAT regimens typically include anthracyclines and taxanes, with the addition of anti-HER2 agents such as trastuzumab and pertuzumab for HER2-positive disease. Recent studies have explored the use of novel targeted therapies, including immune checkpoint inhibitors and molecularly targeted agents, to enhance NAT efficacy. [25-26-27]

1.8 Physiological Role of Monocytes in Immunity:

Monocytes are essential for both innate and adaptive immunity, playing key roles in immune defense, inflammation, and tissue remodeling. They share functional and developmental similarities with macrophages and conventional dendritic cells (cDCs). Blood monocytes originate in the bone marrow (BM) from common myeloid progenitors (CMPs) and, once matured, enter the bloodstream, where they patrol for a few days in a transient inactive state. Their recruitment follows the leukocyte recruitment cascade, involving rolling, adhesion, and transmigration into inflamed tissues.

In humans, monocytes are classified based on CD14 and CD16 expression into three subsets: classical (CD14⁺⁺CD16⁻), intermediate (CD14⁺⁺CD16⁺) and non-classical (CD14⁺CD16⁺⁺). The CD16⁺ monocyte subsets are regarded as tumor-promoting due to their involvement in tissue remodeling, angiogenesis, and the production of cytokines that support these processes. Classical monocytes exhibited the highest levels of CD36, which is associated with scavenging and phagocytosis. The non-classical subset was the primary producer of tumor necrosis factor alpha (TNF α), while intermediate monocytes were characterized by elevated the expression of CCR5 and HLA-DR, which are important for antigen presentation. [47]

1.9 Myeloid-derived suppressor cells (MDSCs):

MDSCs are a heterogeneous population of cells derived from myeloid progenitors. These cells are characterized by their potent immunosuppressive activities, which allow them to play a critical role in regulating immune responses, particularly within the context of cancer. They classified into two main subsets based on their surface markers and lineage: Granulocytic (also known as polymorphonuclear, or PMN-MDSC) and monocytic (M-MDSCs). [29-31-32-34,Figure4]

The main characterization of two subtypes MDSC are:

PMN-MDSCs: CD33+, CD11b+,CD14-, CD15+, HLA-DR low/-

M-MDSCs : CD33+, CD11b+, CD14+, HLA-DR low/-

Lectin-type oxidized LDL receptor-1 (LOX-1) has also been proposed as a new marker to distinguish human PMN-MDSCs from non-immunosuppressive neutrophils. [31]

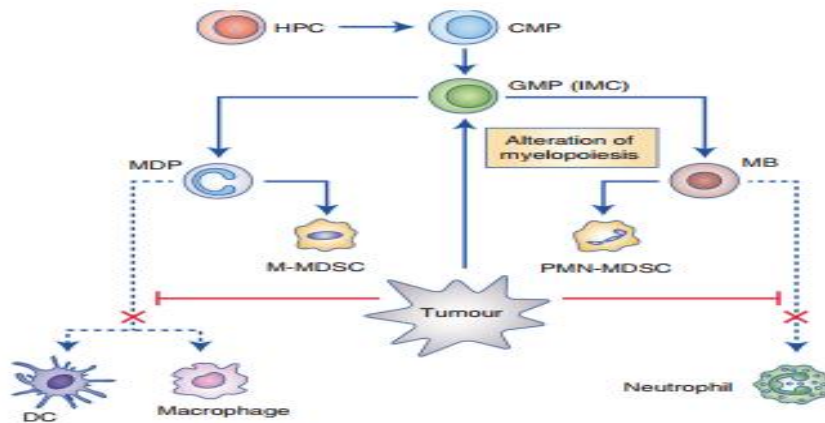


Figure4: Hematopoietic stem cells (HSCs) give rise to myeloid progenitors, which develop into MDSCs and other immune cells. Growth factors like GM-CSF and G-CSF regulate these processes. MDSCs are recruited to tumors and differentiate into PMN-MDSCs and other suppressive cells under specific conditions, influencing the tumor microenvironment [31]

1.9.1 MDSCs in Cancer:

In cancer, tumor-driven alterations in myelopoiesis impair proper differentiation, leading to the accumulation of monocytic (M-MDSCs) and polymorphonuclear (PMN-MDSCs) myeloid-derived suppressor cells. These MDSCs arise in response to tumor-secreted factors such as granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), and macrophage colony-stimulating factor (M-CSF), disrupting normal hematopoiesis and fostering an immunosuppressive microenvironment. [31] However under normal conditions, hematopoietic progenitor cells (HPCs) differentiate through CMPs into granulocyte/macrophage

progenitors (GMPs), which give rise to monocytic/dendritic progenitors (MDPs) or myeloblasts (MBs). These further develop into dendritic cells (DCs), macrophages, or neutrophils.

The suppressive functions of MDSCs are mediated through a combination of metabolic, enzymatic, and signaling mechanisms that collectively inhibit immune responses. One of the key pathways involves the production of reactive oxygen species (ROS) and nitric oxide (NO), which interfere with T cell receptor (TCR) signaling and induce oxidative stress, leading to impaired T cell proliferation and activation. Peroxynitrite (ONOO^-), a reactive nitrogen species generated by MDSCs, results in nitration of the TCR receptor and CD8 molecules, preventing their effective engagement with antigen-presenting cells (APCs). **[29-34]** Furthermore, MDSCs upregulate indoleamine 2,3-dioxygenase (IDO), depleting local tryptophan and generating kynurenine, leading to regulatory T cell (Treg) expansion and suppression of tumor-specific cytotoxic T lymphocytes (CTLs).

In addition to oxidative stress, MDSCs exert immunosuppressive effects by secreting anti-inflammatory cytokines, particularly interleukin-10 (IL-10) and transforming growth factor-beta ($\text{TGF-}\beta$). These cytokines promote the expansion of regulatory T cells (Tregs), which further dampen immune responses. Additionally, MDSCs facilitate the polarization of macrophages into an M2-like phenotype, characterized by high IL-10 and low IL-12 production, contributing to an immunosuppressive microenvironment. **[39,figure 5]** Another critical mechanism involves the metabolic depletion of essential amino acids required for immune cell function. MDSCs upregulate the expression of arginase-1 (ARG1), which breaks down L-arginine, a key nutrient for T cell proliferation and activation. Depletion of L-arginine leads to reduced expression of the TCR ζ -chain, impairing T cell signaling. Similarly, inducible nitric oxide synthase (iNOS) catalyzes the production of NO, which not only suppresses T cell function but also inhibits natural killer (NK) cell activity by inhibiting of JAK3 and STAT 5. **[29-31]** Additionally, MDSCs sequester cysteine, preventing T cells from acquiring this critical metabolite needed for activation.

Beyond direct suppression of immune cells, MDSCs interfere with T cell trafficking by downregulating L-selectin (CD62L) on naïve T cells, thereby impairing their ability to migrate to

lymph nodes where antigen presentation occurs. Under hypoxic conditions, which are common in the tumor microenvironment, MDSCs upregulate hypoxia-inducible factor 1-alpha (HIF-1 α), leading to increased expression of PD-L1 and ARG which binds to PD-1 on T cells, leading to their functional exhaustion. [30-31] This adaptation further enhances their suppressive activity while promoting angiogenesis through vascular endothelial growth factor (VEGF) secretion, indirectly supporting tumor progression. [30-34]

Adenosine production is another key immunosuppressive strategy employed by MDSCs. Adenosine signaling via the A2A receptor suppresses T cell proliferation and cytokine production, reinforcing an immunosuppressive environment. [31]

In addition to their direct immunosuppressive effects, MDSCs contribute to tumor growth and metastasis by enhancing angiogenesis, remodeling the extracellular matrix, and promoting the epithelial-mesenchymal transition (EMT) of tumor cells. The presence of MDSCs in the tumor microenvironment is associated with poor prognosis and resistance to various forms of therapy, including immunotherapy.

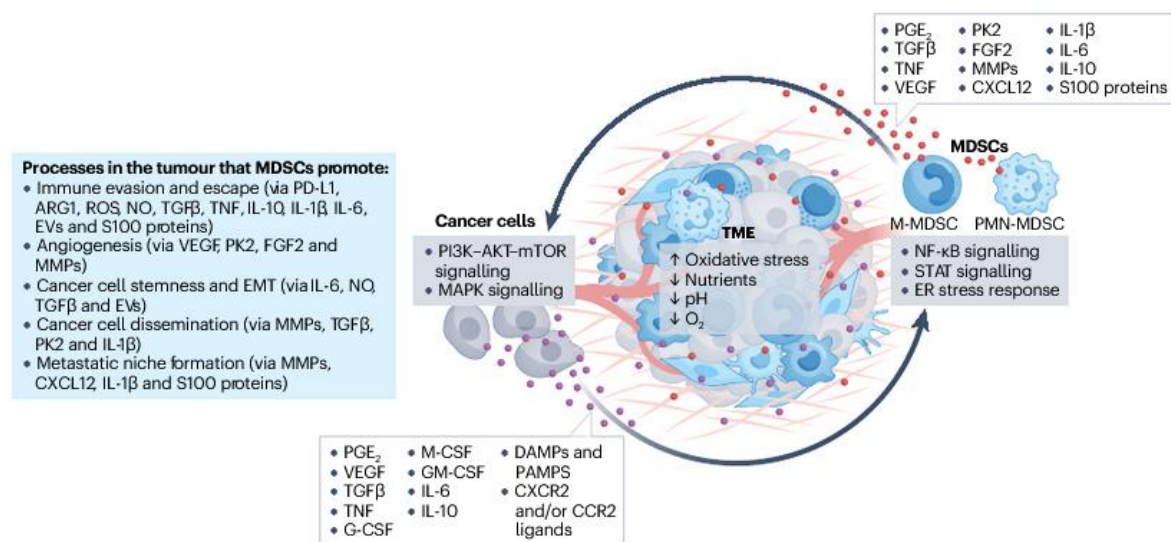


Figure 5: The Nurturing Relationship between MDSCs and Tumors: Myeloid-derived suppressor cells(MDSCs) are recruited, expanded, and activated by various factors in the tumor microenvironment (TME), such as hypoxia, nutrient shortages, acidity, and chronic inflammation. Tumor-derived growth factors (e.g., GM-CSF, M-CSF, G-CSF), cytokines (e.g., IL-6, IL-1 β , TNF, TGF β), chemokines (e.g.,

CXCR2, CCR2). MDSCs support tumor progression by suppressing anti-tumor immunity, promoting angiogenesis (via VEGF, PGE2, FGF2, PK2), enhancing cancer cell stemness and epithelial-to-mesenchymal transition (EMT) through IL-6 and TGFβ, and aiding cancer cell invasion and spread by secreting matrix metalloproteinases (MMPs). Additionally, MDSCs help form pre-metastatic niches through chemoattractants (e.g., CXCL12, S100 proteins) and contribute to metastasis via MMP and IL-1β production. [28]

1.9.2 MDSCs and Cancer Therapy:

The immunosuppressive functions of MDSCs present a significant challenge in treatment of cancer, particularly in the context of immunotherapy. Effective cancer therapy often requires the inhibition or depletion of MDSCs to restore the immunesystem's ability to combat tumor cells. Several strategies are being explored to target MDSCs in cancer therapy:

Chemotherapeutic Agents: Gemcitabine and 5-fluorouracil (5-FU) are two agents that effectively reduce MDSC numbers in tumor-bearing hosts. Their ability to specifically target MDSCs has made them a promising adjunct to immunotherapy, as their depletion enhances T-cell activation and restores immune surveillance. [30] Additionally, low-dose paclitaxel has been observed to promote MDSC differentiation into DC while simultaneously enhancing the activity of natural killer (NK) cells. These effects collectively contribute to a reduction in tumor-induced immunosuppression and improve therapeutic outcomes. [35]

Promoting MDSC Differentiation: Rather than simply depleting MDSCs, an alternative approach involves promoting their differentiation into mature, non-immunosuppressive myeloid cells. All-trans retinoic acid (ATRA), a vitamin A metabolite, has been extensively studied for its ability to induce the differentiation of MDSCs into macrophages and dendritic cells. [30-29] This effect is associated with reduced reactive oxygen species (ROS) production and enhanced antigen presentation, leading to improved anti-tumor immune responses. Another promising strategy involves targeting the ATP-converting ectoenzyme ENTPD2, which has been found to promote MDSC differentiation and improve responses to immune checkpoint inhibitors.

Targeting MDSC-Specific Pathways: MDSCs exert their immunosuppressive effects by producing various factors, including prostaglandin E2 (PGE2), nitric oxide (NO), arginase-1 (ARG1), and reactive oxygen species (ROS). Consequently, targeting these pathways offers a viable strategy for neutralizing MDSC function. Cyclooxygenase-2 (COX-2) inhibitors block PGE2 production, thereby impairing MDSC-mediated immune suppression.[29] Similarly, phosphodiesterase-5 (PDE-5) inhibitors, have been shown to reduce the production of NO and ARG1, restoring tumor-specific T-cell responses.[30] Additionally, activation of Toll-like receptor 8 (TLR8) has been reported to induce MDSC apoptosis via the Fas-FasL pathway while restoring interleukin-2 (IL-2) secretion by T cells. [36]

Tyrosine Kinase Inhibitors (TKIs): Tyrosine kinase inhibitors (TKIs) have emerged as promising agents in modulating the expansion and function of MDSCs, thereby enhancing the efficacy of immunotherapies. Notably, sorafenib, sunitinib and cabozantinib have been shown to decrease the differentiation of monocytes into functional MDSCs, reducing their suppressive capacity on CD8+ T cell proliferation and alleviating their immunosuppression. [37-38]

1.10 Lectin-like oxidized low-density lipoprotein (LDL) receptor-1 (LOX-1):

LOX-1, also known as OLR1 gene belongs to the C-type lectin superfamily on chromosome 12, is a transmembrane homodimer of 52 kDa expressed in endothelial cells, platelets, macrophages, smooth muscle cells, and cardio myocytes. It is responsible for oxLDL (oxidized LDL) recognition, triggering downstream pathways that intensify atherosclerosis via endothelial dysfunction, oxLDL uptake, and apoptosis. Activation of LOX-1 by oxLDL is implicated in the progression of atherosclerosis, myocardial fibrosis, and endothelial dysfunction. [40-41,figure6] More specifically, it stimulates the expression of adhesion molecules, pro-inflammatory and inflammatory signaling pathways (e.g. PI3K/AKT/GSK3 β) and molecules (IL-6, IL-8, IL-1 β), and pro-angiogenic proteins (NF-kB and VEGF, in vascular cells and macrophages). LOX-1 also plays an important role in the cancer onset, progression, and metastasis of various types of cancer (e.g., glioblastoma, osteosarcoma, prostate, colon, lung, pancreatic), including breast cancer. Recent studies evidence its function in VEGF induction, HIF-1 alpha activation, and MMP-9/MMP-2 expression, pushing up the neoangiogenic and epithelial-mesenchymal transition process. [42-

43-44] Additionally, reactive oxygen species (ROS) are also upregulated by binding of ox-LDL to LOX-1, an event that causes oxidative DNA damage and in vivo oxidation of lipids and proteins.

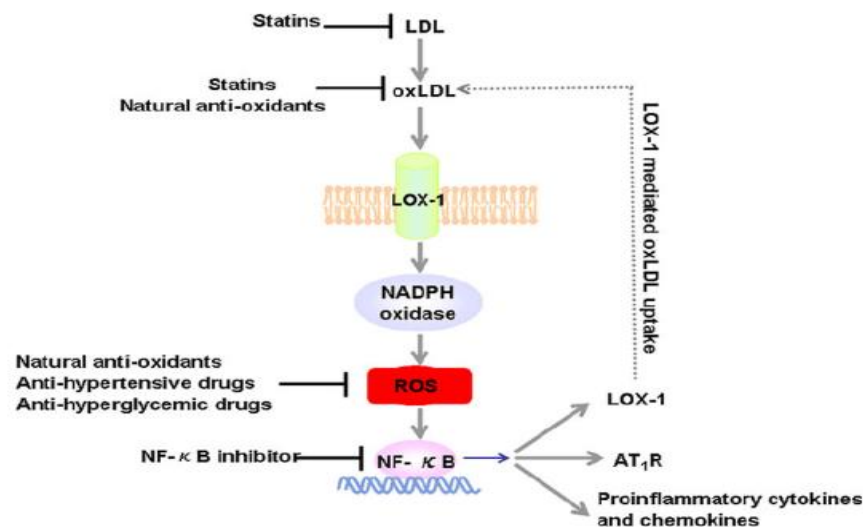


Figure 6: Activation of LOX-1 at a molecular level: LDL is oxidized within the vascular wall under atherogenic conditions to form oxLDL. The binding of oxLDL to LOX-1 activates the NADPH oxidase on the cell membrane that results in the quick increase of intracellular ROS formation. Increased ROS activates the redox-sensitive NF- κ B signaling pathway, generating three responses: (1) increases binding to LOX-1 promoter, therefore, increases LOX-1 expression and amplifies LOX-1-mediated oxLDL uptake; (2) enhances Angiotensin-II type 1 receptor expression; (3) [45]

Recent studies have demonstrated that LOX-1 is upregulated in lung cancer cells, and its expression is associated with a more aggressive tumor phenotype and poor prognosis in patients with non-small cell lung cancer (NSCLC). In particular, LOX-1+ CD15+ PMN-MDSCs have been identified as a potent immunosuppressive subset that promotes tumor progression by releasing high levels of arginase-1 (ARG1) and reactive oxygen species (ROS)—both of which suppress T cell-mediated immune responses. [48-49] Given that similar pro-tumorigenic PMN-MDSCs have been reported in breast cancer, it is plausible that LOX-1+ PMN-MDSCs could also serve as a diagnostic and prognostic marker in breast cancer and therapy resistance. Interestingly, Cassetta et al. reported that in cancer patients, profound PD-L1 expression was restricted to M-MDSCs and e-MDSCs, whereas LOX-1 expression was confined to PMN MDSCs. [46] Although direct

evidence in breast cancer is currently lacking, these findings from NSCLC studies suggest that similar mechanisms might be at play in breast cancer, making LOX-1+ PMN-MDSCs a potential target for further investigation.

2. Aim of the study

Myeloid-derived suppressor cells (MDSCs) have been found to play an important role in limiting immune responses in cancer. Higher circulating MDSC levels have been associated with greater tumor burden, poorer response to immunotherapy, and reduced overall survival. Optimal measurement of MDSC levels could provide clinicians with a useful prognostic and management tool, especially in the era of personalized medicine. In breast cancer, particularly across molecular subtypes such as ER+, HER2+, and triple-negative breast cancer (TNBC), MDSC subsets and associated markers may vary significantly, reflecting their contribution to immune evasion and treatment resistance.

The immunosuppressive function of MDSCs, along with altered monocytes populations, plays a critical role in tumor progression and resistance to therapy, particularly in patients undergoing neoadjuvant treatment (NAT). Therefore, evaluating MDSCs subtypes and their functional markers—such as PD-L1 and LOX-1—may provide valuable insight into treatment resistance mechanisms and help guide more personalized therapeutic approaches.

The purpose of this non-interventional observational study is to comprehensively evaluate monocytes and MDSCs in operable breast cancer patients in relation to tumor characteristics, treatment response, and immunosuppressive function. Specifically, the study aims to:

- Assess the lymphocyte-to-monocyte ratio (LMR) and characterize different monocyte subsets across breast cancer subtypes.
- Evaluate the distribution of MDSC subtypes (PMN-MDSC, M-MDSC) in relation to tumor grade, stage, and molecular classification obtained using FACS analysis on fresh (peripheral blood).
- Analyze the expression of PD-L1 on different subtype of MDSCs.
- Investigate LOX-1 expression on MDSCs, particularly granulocytic subsets, and its association with breast cancer subtypes and treatment outcomes.

3. Materials and methods

3.1 Patients' enrollment and sample collection

Peripheral whole blood samples were taken from patients who were eligible to receive NAT both at baseline and at the end of chemotherapy (before surgery). The study is design to enroll 74 patients in total.

Patients' recruitment was carried out only after informed consent sign and the fulfillment of the following inclusion criteria:

- Patients ≥ 18 years of age
- Breast cancer diagnosis
- Patients candidate for NAT
- Absence of side effects upon therapy administration
- Ability to understand and sign an informed consent

Exclusion criteria were represented by:

- Patients ≤ 18 years of age
- Patients not suitable for NAT
- Presence of metastases
- Inability to understand and/or sign an informed consent

In total, each sample comprises 25 ml of peripheral whole blood collected in EDTA tubes (BD Bioscience, Milan, IT) from the Oncology department of Azienda Ospedaliero-Universitaria (A.O.U.) Maggiore Della Carità, University of Eastern Piedmont, Novara. After collection, samples were processed within 4 hours from the 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 collected by diluting 10 ml of whole blood with 20 ml of physiological solution inside a 50 ml Falcon tube. Then, 15 ml of Ficoll-Paque solution (Lympholyte-H, Cedarlane, Netherlands) was added. The tube was then centrifuged for 20 minutes at 2200 G with an acceleration (AC) of 7 and break power (DC) of 0. By doing so, cells were divided by density gradient centrifugation, and PBMCs were collected from the interphase ring between diluted plasma and the separation medium, as shown in figure 4. Then, PBMCs were washed and counted in a Bürker chamber through a 1:10 dilution with TURK solution (previously diluted 1:10).

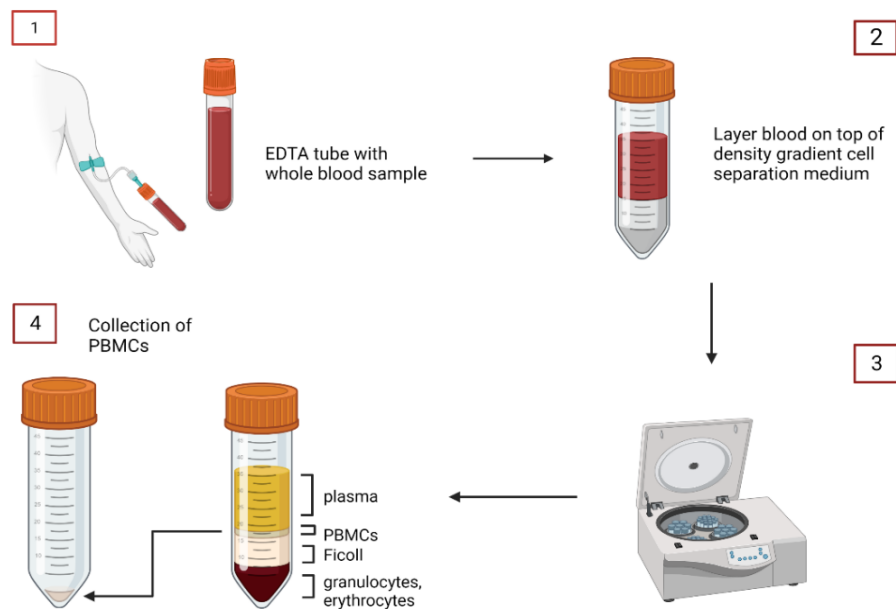


Figure 7: Schematic representation of gradient density centrifugation: (1) PBMC isolation start with blood collection from patients in an EDTA tube. (2) Blood is diluted with physiological solution to make it lighter and allow Ficoll stratification. Then, Ficoll is added at the bottom of the tube to create two distinct layers. (3) sample is centrifuged at 2200 rpm for 20 minutes, with acceleration 7 and brake 0. (4) After centrifugation, four layers can be distinguished. From the bottom to the top: red blood cells, Ficoll, PBMCs

and diluted plasma. Rpm Rotation per minute, PBMCs peripheral blood mononucleated cells. Designed by biorender

3.3 MDSCs staining:

From each sample, 1 million isolated PBMCs were stained in order to be analyzed at the Fluorescence-Activated Cell Sorter (FACS, BD Bioscience, Milan, IT). PBMCs staining was performed by incubating the cells for 15 minutes in dark conditions and at room temperature with the following surface markers: CD45 (BUV395), CD33 (APC), HLA-DR (BV605), CD14 (BUV737) and CD15 (BV510), CD16(PE-CY), LOX-1(PE) (all provided by BD Bioscience, Milan, IT) [table 2]. After a wash, cells were resuspended in 200ul of staining buffer (2%FBS, 98% PBS 1X) and immediately acquired by Flow Cytometer (BD Bioscience, Milan, IT). using the latest updated version of FlowJo software (BD Bioscience, Milan, IT). MDSCs subpopulations were classified and quantified according to Bronte et al.[5] as follows:

- M-MDSCs:** HLA-DR-/low, CD33 intermediate, CD15-, CD14+
- PMN-MDSCs:** HLA-DR-/low, CD33 intermediate, CD15+, CD14-, CD16+, LOX-1 +

Table 2: Antibodies MDSCs staining fluorophore

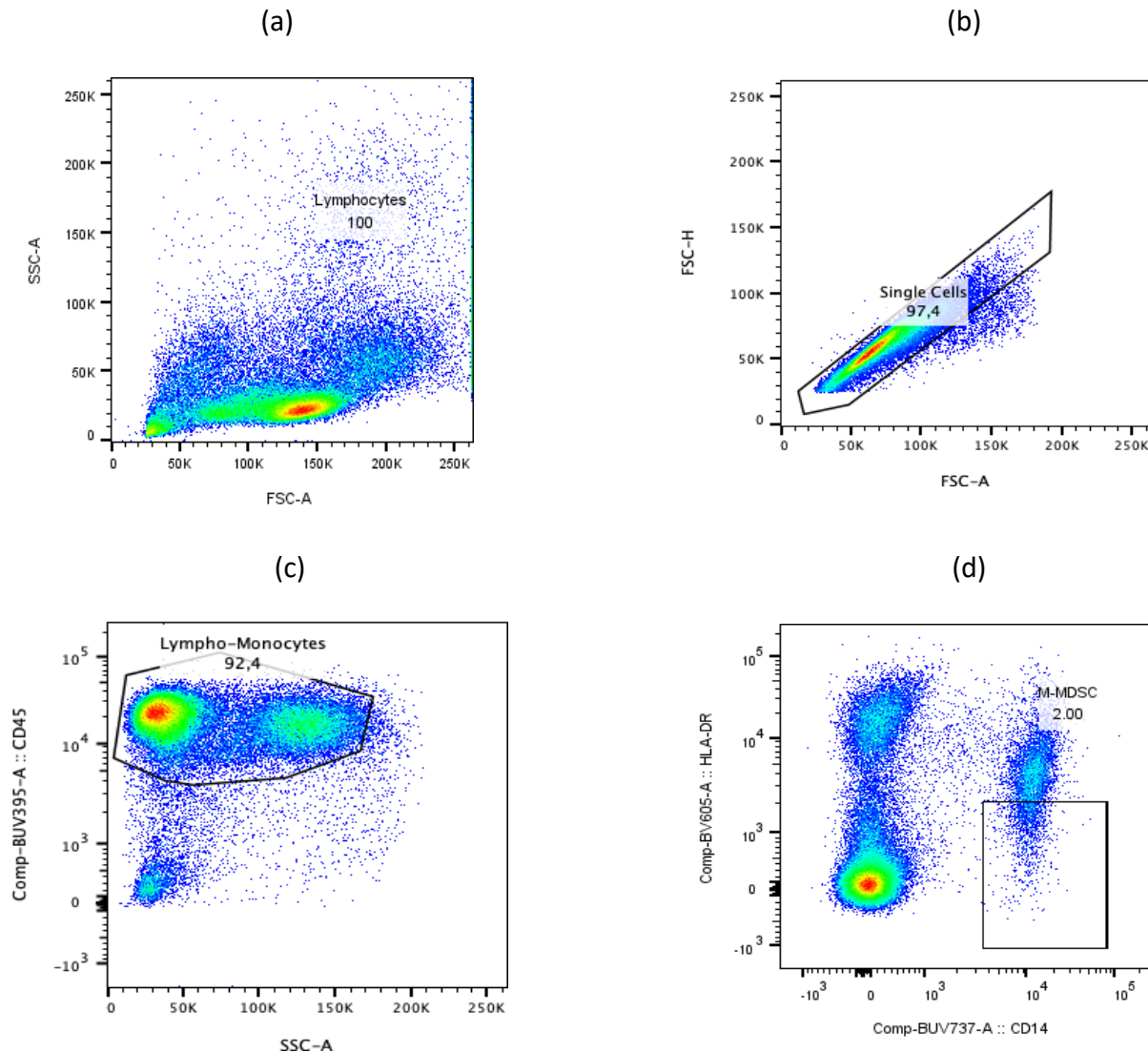
ANTIBODIES	FLUOROPHORES
CD45	BUV395
LOX-1	PE
HLA-DR	BV605
CD15	BV510
CD14	BV737
CD16	PE-CY
CD33	APC
PDL-1	BV421

3.4 Evaluation of Monocyte and MDSCs by FACS

3.4.1 MDSCs gating strategy:

Dead cells were excluded by using forward (FSC-A) and side (SSC-A) scatter. Then the doublets were removed with the parameters FSC-W (forward side scatter-width) and FSC-A (forward side scatter-area). To select lymphocytes and monocytes we looked at the CD45 expression: lymphocytes are bright, monocytes are positive. To distinguish the MDSCs subpopulations, HLA-DR, CD14, CD15 and CD33 are used.

In the plot with HLA-DR and CD14, monocytes (HLA-DR positive and CD14 positive) and M-MDSCs (HLA-DR low and CD14 positive) can be identified. For PMN-MDSCs in the lympho-monocytes' population, we looked at CD33 and CD15: they are CD33 intermediate and CD15 positive. (figure8)



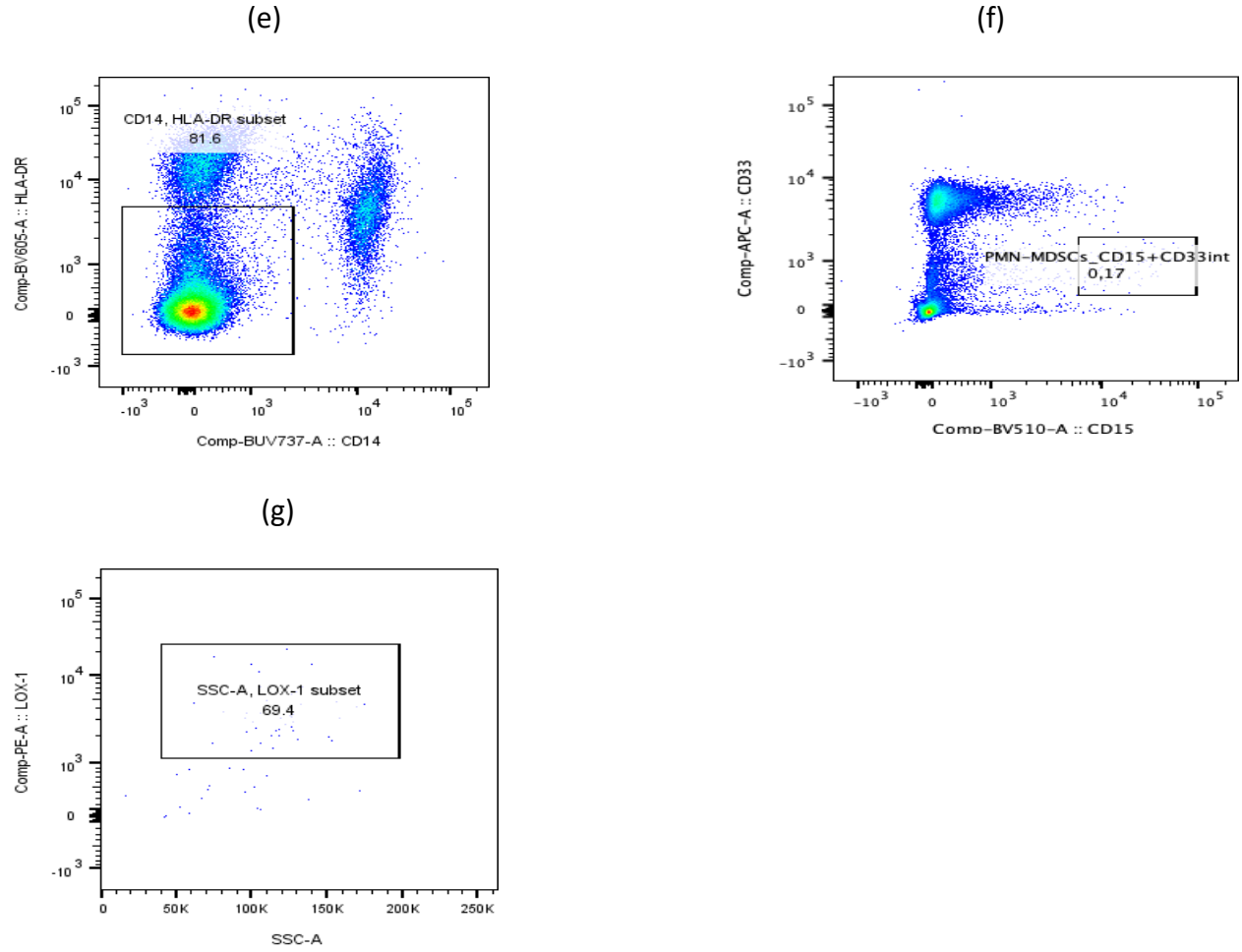


Figure 8: MDSCs gating strategy. (a) the initial gate was set on all viable lymphocytes and monocytes (100% of events) (b) Live cells are selected according to their morphology Duplets are removed with dimension parameters. (c) Lympho-monocytes population is selected among CD45 bright/positive cells. (d) M-MDSCs are selected in lympho-monocytes population as CD14⁺ and HLA-DR low/neg. (e) PMN-MDSCs are selected within cells negative for CD14 and HLA-DR (CD14⁻ HLA-DR⁻) (f) After they are gated as CD15⁺ and CD33^{int}; MDSCs myeloid derived suppressor cells; M-MDSCs monocytic myeloid derived suppressor cells; PMN-MDSCs polymorphonuclear myeloid derived suppressor cells; HLA-DR human leukocyte antigen-DR. (g) Expression of LOX-1 on PMN-MDSCs of the parent PMN gate was further assessed using SSC-A vs. LOX-1.

3.4.2 Monocytes gating strategy:

A panel for the identification of the three monocytes subpopulation was prepared. The 79 samples were investigated by flow cytometry using 100% of lasers power and the threshold was set up on FSC at 25000. The phenotypes for the identification of the monocytes were: classical monocytes (CD14⁺⁺ CD16⁻), non-classical monocyte (CD14⁻CD16⁺) and so-called intermediate monocytes (CD14⁺CD16⁺). To individuate the monocytes subpopulation, doublets were removed by using the parameters FSC-A vs FSC-H. After that, dead cells were eliminated using forward (FSC-A) and side scaeers (SSC-A) and then monocytes were gated by side scaeer (SSC-A) vs CD45, and to make sure that the only cells selected under this population were monocytes, CD16⁻,CD14⁻ were excluded. The three monocyte subpopulations were gated using CD16 vs CD14. (Figure 9)

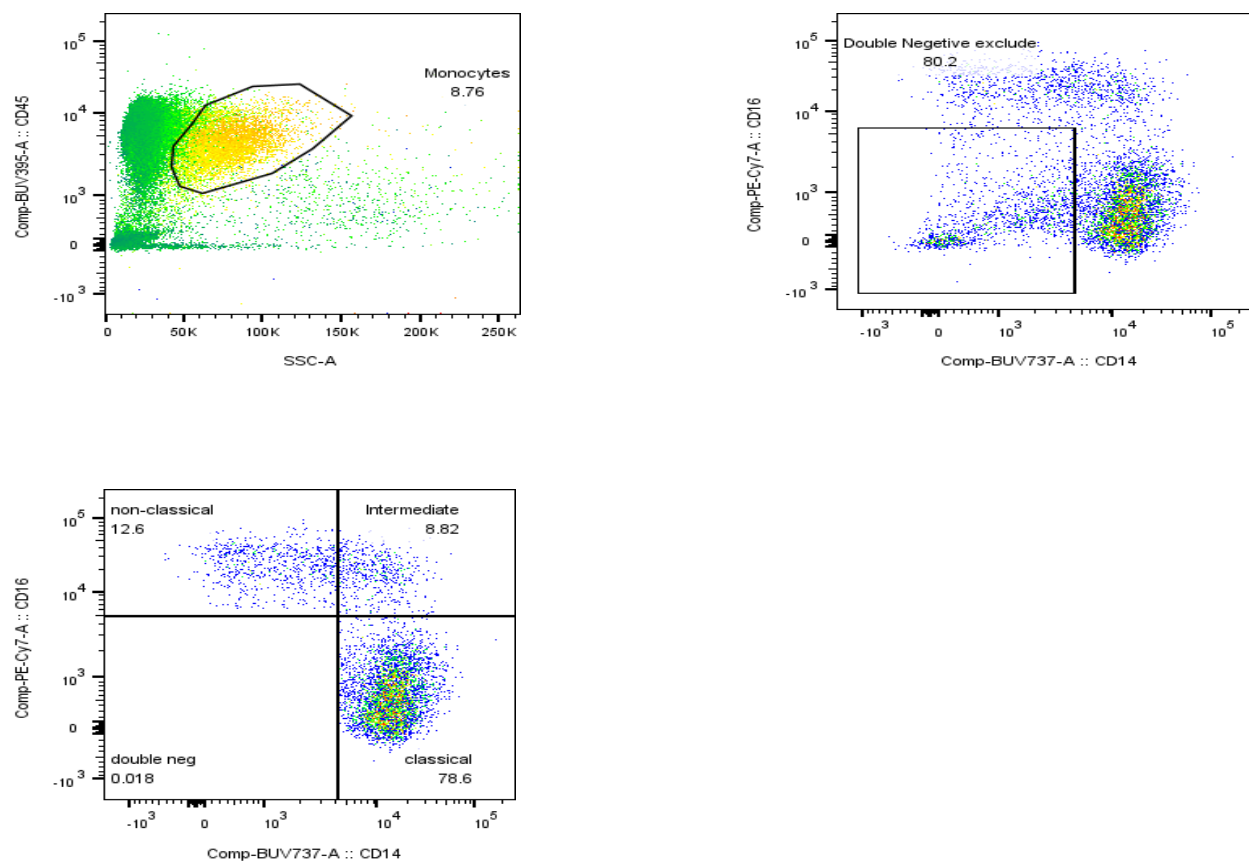


Figure9: Schematic representation of the monocytic gating strategy

3.5 Statistical analysis:

Normally distributed data were presented as mean and Standard Deviation (SD), whereas data following a non-normal distribution were presented as median and interquartile range (IQR). For statistical purposes, baseline characteristics were defined as categorical variables and reported as values and percentages. Statistical analysis was performed using non-parametric Mann Whitney U test, and Kruskal-Wallis test for comparison between more than two groups and Wilcoxon signed-rank test for before and after therapy patients. Tests were performed considering the median of the data. A two-sided P value < 0.05 ($\alpha = 0.05$) was considered statistically significant. Analyses were performed using R software and Graph Pad prism v.8.

4. Results

4.1 Patients baseline characteristics

Eighty-nine patients have been enrolled to the current date, and their baseline characteristics are resumed in Table 3.

Table 3: Patients characteristics

CHARACTERISTICS	STUDY POPULTAION NUMBER= 89	MEAN VALUE (RANGE)
MEDIAN AGE (IN YEARS)		54.5 (32.2-81.8)
MEDIAN BMI (RANGE)		24,6 (17,51-38,28)
CANCER FAMILIARITY		
YES	48 (53,93%)	
NO	24 (26,96%)	
NOT INDICATED	17 (19,1%)	
BRCA1/2 MUTATIONAL STATUS		
YES	1 (1,12%)	
NO	26 (29,21%)	
NOT INDICATED	62 (69,66%)	
HISTOLOGICAL GRADE		
G2	23 (25,84%)	
G3	34 (38,2%)	
NOT INDICATED	32 (35,95%)	
MOLECULAR SUBTYPES		
TNBC	23 (25,84%)	
HER2+	35 (39,32%)	
ER+	16 (17,97%)	
NOT INDICATED	15 (16,85%)	
MEDIAN KI-67 EXPRESSION (RANGE)		73.41% (10%-90%)
PGR EXPRESSION		10% (0%-100%)
TNM STAGING		
TX N2	1 (,12%)	
T1 N0	2 (2,2%)	
T1 N1	8 (8,98%)	
T1 N2	1 (1,12%)	
T2 N0	15 (16,85%)	
T2 N1	11 (12,35%)	
T2 N2	2 (2,2%)	
T2 N3	1 (1,12%)	
T3 N0	6 (6,74%)	
T3 N1	5 (5,61%)	
T3 N2	1 (1,12%)	
T3 N3	1 (1,12%)	
T4 N0	0 (0%)	
T4 N1	1 (1,12%)	
T4 N2	0 (0%)	
T4 N3	1 (1,12%)	
NOT INDICATED	33 (37,07%)	
PCR		
YES	30 (33,707%)	
NO	27 (30,33, %)	
NOT INDICATED	32 (35,95 %)	

4.2 Baseline Characterization of Myeloid and Monocyte Populations

4.2.1 Evaluation of Circulating Monocyte Subsets Across Breast Cancer Subtypes at baseline

To investigate immunological differences among breast cancer subtypes, we performed a baseline evaluation of circulating monocyte subsets in peripheral blood samples collected prior to treatment initiation. The analysis was conducted within a cohort of 72 patients, divided into three molecular subtypes: HER2+ (n=34), TNBC (n=23), and ER+ (n=15).

Monocytes were characterized and quantified using flow cytometry. As shown in figure 10, while slight variations in subset distributions were observed across the subtypes, statistical analysis revealed no significant differences at the baseline timepoint.

These findings suggest that the frequency of circulating monocyte subsets before therapy does not differ significantly among breast cancer subtypes. (table 4, figure 10)

Table 4: Summary of the distribution of monocyte subsets (in percentage) in ER+, HER2+, and TNBC groups at baseline. Abbreviations: SD = standard deviation; TNBC = triple-negative breast cancer.

	P value	P value	Pvalue	mean	mean	mean	SD	SD	SD
	HER2+ vs ER+	HER2+ vs TNBC	ER+ vs TNBC	HER2+	TNBC	ER+	HER2+	TNBC	ER+
Classical	0.8907	0.7427	0.2277	59.13	59.61	60.73	11.92	20.09	16.85
Intermediate	0.11	1.107	0.7979	11.83	10.69	11.73	7.62	9.409	8.422
Non-Classical	0.1345	0.3611	>0.9999	10.26	8.273	7.651	6.175	6.254	6.181

monocyte subsets at baseline

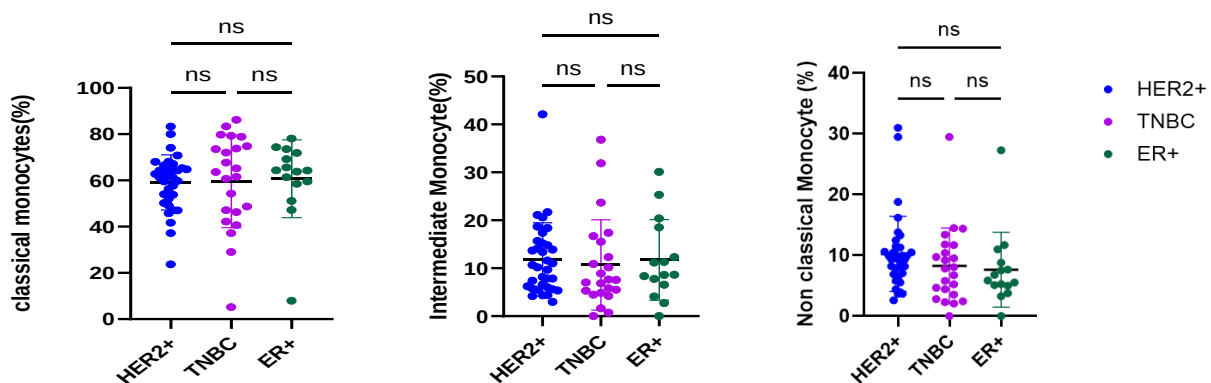


Figure 10: The plots show the relative frequency of circulating monocyte subsets at baseline in ER+, HER2+, and TNBC groups (Kruskal–Wallis test). Data are presented as means $\pm$ SD. ($p > 0.05$ = not statistically significant).

4.2.2 Assessment of MDSC Populations at baseline:

To evaluate the expression of MDSC levels in the three molecular subtypes of breast cancer, we investigated, by flow cytometry, the baseline levels of MDSC populations in 74 breast cancer patients. Specifically, the percentage frequencies of PMN-MDSCs and M-MDSCs were assessed in Group A (HER2+, n=35), Group B (TNBC, n=23), and Group C (ER+, n=16) using the Mann–Whitney test.

Initially, when analyzing the entire cohort irrespective of subtype, we observed a significantly higher frequency of M-MDSCs compared to PMN-MDSCs, suggesting a dominant role for the monocytic subset in the systemic immune suppression of breast cancer patients. Subsequently, this trend remained consistent when patients were stratified by subtype. In all three groups—HER2+, TNBC, and ER+—M-MDSCs were significantly more prevalent than PMN-MDSCs, reinforcing the observation that this subset is the predominant circulating MDSC population across different breast cancer phenotypes. (mean $\pm$ SD; p value in Table 6, Figure 12).

These findings highlight the overall enrichment of M-MDSCs in the blood of breast cancer patients at baseline, both globally and within specific subtypes.

Table 6: Summary of the expression of MDSC subgroups (in percentage) in all breast cancer patients, stratified by PMN-MDSC and M-MDSC populations. Abbreviations: SD = standard deviation; PMN-MDSC = polymorphonuclear MDSC; M-MDSC = monocytic MDSC.

	P value	mean	mean	SD	SD
		M-MDSC	PMN-MDSC	M-MDSC	PMN-MDSC
Overall	<0.0001	2.242	0.1462	1.668	0.2191
HER2+	<0.0001	2.133	0.1746	1.079	0.2202
TNBC	<0.0001	2.506	0.07184	2.49	0.09613
ER+	<0.0001	2.009	0.2056	1.233	0.3308

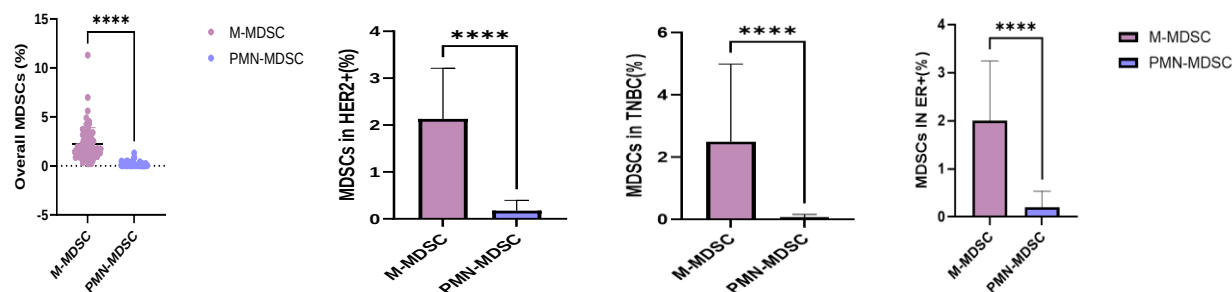


Figure 12: The plot shows the relative cell frequency of circulating MDSC populations of all patients, which has been stratified in subtypes TNBC, HER2+, Luminal (Mann-Whitney Test). Data presented as means $\pm$ SD (p value $< 0,05$ = not statistically significant; **** $> 0,05$).

Furthermore, to explore the relationship between MDSC levels and tumor grade, we performed an analysis comparing the frequencies of PMN-MDSCs and M-MDSCs in patients with Grade 2 ($n=23$) and Grade 3 ($n=34$) regardless to the subtype of breast cancer.[Figure 13, table 7,8]

In Grade 2 tumors, we evaluated M-MDSC and PMN-MDSC levels across the three main breast cancer subtypes: HER2+, ER+, and TNBC. However, due to the limited number of TNBC patients in this grade, TNBC data were excluded from the subtype comparison. In both HER2+ ($n=14$) and ER+ ($n=6$) cases, M-MDSCs remained more frequent than PMN-MDSCs, consistent with the overall trend observed in the full cohort.[table 7, Figures 14]

A similar analysis was conducted for Grade 3 tumors, where M-MDSC and PMN-MDSC frequencies were compared across HER2+ ($n=13$), TNBC ($n=15$), and ER+ ($n=6$) groups. In this case, sufficient patient numbers allowed evaluation in all three subtypes and A similar result was observed in M-MDSCs that show significantly higher frequencies than PMN-MDSCs across all subtypes, with statistically significant differences observed in every comparison ($p < 0.05$ or lower, depending on group; Table 8 and Figures 16).

Table 7: Frequencies of PMN-MDSCs and M-MDSCs (mean $\pm$ SD, %) in breast cancer patients with Grade 2 tumors, stratified by molecular subtype (HER2+ and ER+) using the Mann–Whitney test. TNBC cases were excluded due to insufficient sample size.

Abbreviations: SD = standard deviation; PMN-MDSC = polymorphonuclear myeloid-derived suppressor cell; M-MDSC = monocytic myeloid-derived suppressor cell; TNBC = triple-negative breast cancer.

	P value	mean	mean	SD	SD
Grade2		M-MDSC	PMN-MDSC	M-MDSC	PMN-MDSC
Overall	<0,0001	1.943	0.1881	1.057	0.3208
HER2+	<0.0001	2.046	0.1562	0.9395	0.2318
ER+	0.0087	1.39	0.321	0.5998	0.5281

Overall Grade Comparison

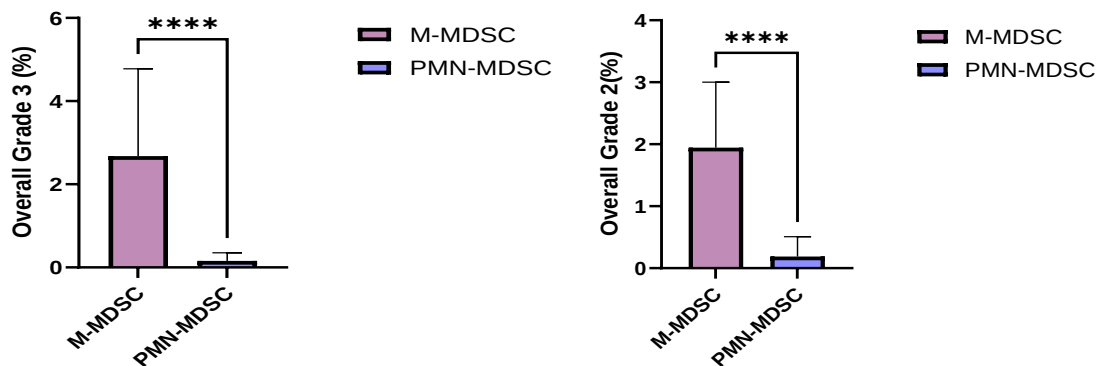


Figure 13: The plot shows the overall comparison of circulating PMN-MDSC and M-MDSC frequencies between Grade 2 and Grade 3 breast cancer patients, independent of molecular subtype. It was performed using the Mann–Whitney test ($p < 0.05$ considered significant).

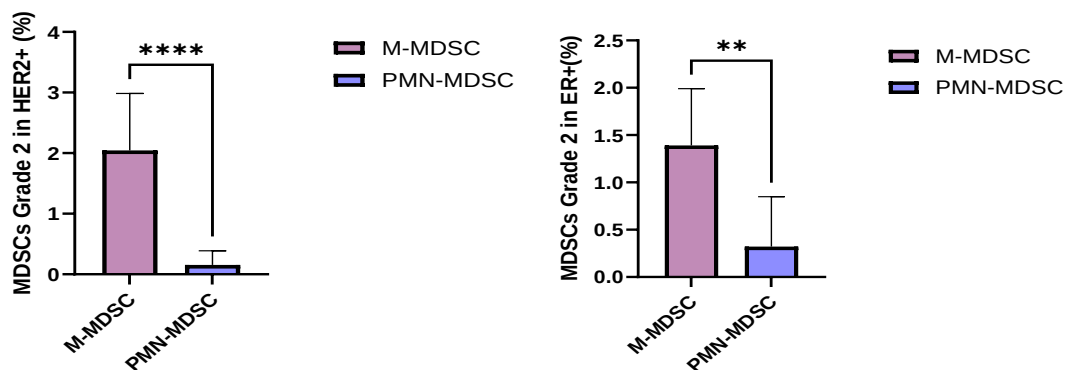


Figure 15: The plots show the relative frequencies of circulating PMN-MDSCs and M-MDSCs in breast cancer patients with Grade 2 tumors, stratified by molecular subtype (HER2+ and ER+). TNBC was excluded due to insufficient sample size.

Data are presented as mean $\pm$ SD. Statistical analysis was performed using the Mann–Whitney test ($p < 0.05$ considered significant).

Table 8: Frequencies of PMN-MDSCs and M-MDSCs (mean $\pm$ SD, %) in breast cancer patients with Grade 3 tumors, stratified by molecular subtype (HER2+, ER+, and TNBC). Statistical comparisons were performed between PMN-MDSC and M-MDSC frequencies using the Mann–Whitney test.

Abbreviations: SD = standard deviation; PMN-MDSC = polymorphonuclear myeloid-derived suppressor cell; M-MDSC = monocytic myeloid-derived suppressor cell; TNBC = triple-negative breast cancer.

	P value	mean	mean	SD	SD
Grade3		M-MDSC	PMN-MDSC	M-MDSC	PMN-MDSC
Overall	<0.0001	2.678	0.157	2.099	0.1939
HER2+	<0.0001	2.484	0.2571	1.054	0.2504
TNBC	<0.0001	2.818	0.07523	2.921	0.1143
ER+	0.0022	2.747	0.1445	1.533	0.1221

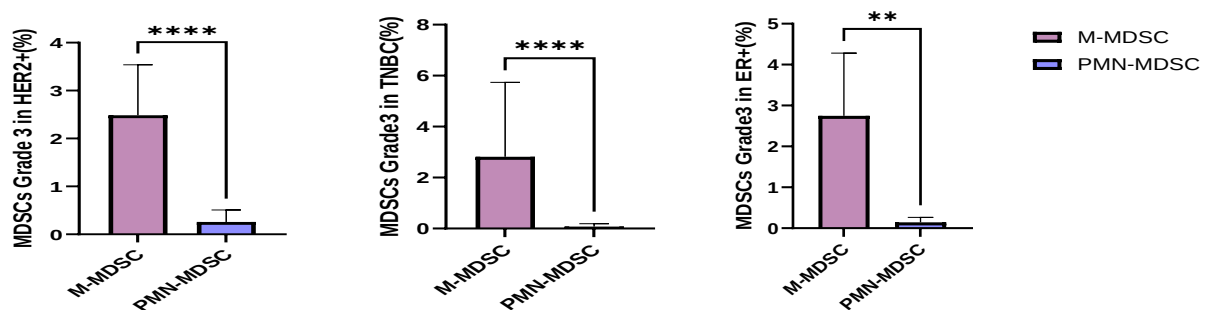


Figure 16: The plots show the relative frequencies of circulating PMN-MDSCs and M-MDSCs in breast cancer patients with Grade 3 tumors, stratified by molecular subtype (HER2+, ER+, and TNBC). Data are presented as mean $\pm$ SD. Statistical analysis was performed using the Mann–Whitney test ($p < 0.05$ considered significant)

In addition, we also evaluated whether MDSC frequencies differed between Grade 2 and Grade 3 tumors within each breast cancer subtype. Specifically, we performed separate analyses for PMN-MDSCs and M-MDSCs, comparing their frequencies between grades in HER2+ and ER+ patients. Due to an insufficient number of TNBC cases, the TNBC subgroup was excluded from both analysis.

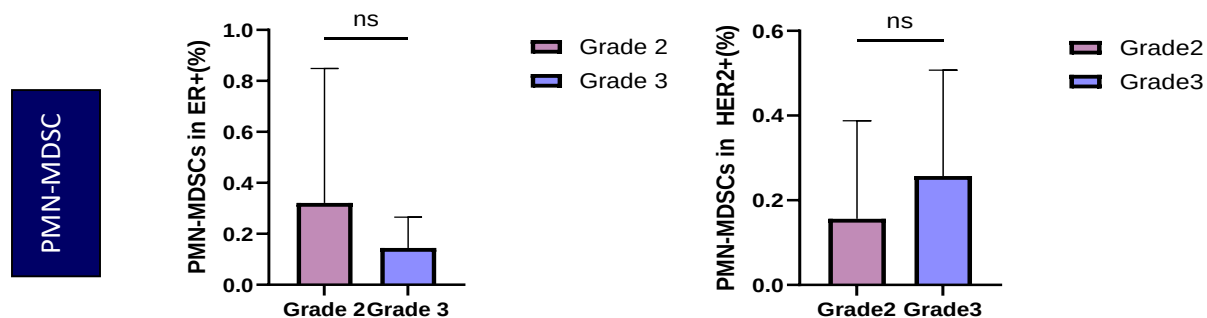
For the PMN-MDSC analysis, the sample sizes were as follows: HER2+ (Grade 2: n = 14; Grade 3: n = 13) and ER+ (Grade 2: n = 6; Grade 3: n = 6). For the M-MDSC analysis, the same distribution was maintained: HER2+ (Grade 2: n = 14; Grade 3: n = 13) and ER+ (Grade 2: n = 6; Grade 3: n = 6).

For both MDSC subpopulations, the comparisons between Grade 2 and Grade 3 within HER2+ and ER+ subtypes revealed no statistically significant differences. This was consistent across both PMN-MDSCs and M-MDSCs, suggesting that tumor grade alone does not substantially influence the circulating levels of either MDSC subset in these molecular subtypes at baseline.(Table 9, figure17)

Table 9: Summary of PMN-MDSC and M-MDSC frequencies (mean $\pm$ SD, %) in HER2+ and ER+ breast cancer patients stratified by tumor grade (Grade 2 and Grade 3). Statistical comparisons were performed between PMN-MDSCs and M-MDSCs within each group using the Mann–Whitney test. Abbreviations: SD = standard deviation; PMN-MDSC = polymorphonuclear myeloid-derived suppressor cell; M-MDSC = monocytic myeloid-derived suppressor cell.

	P value	Mean(Grade2)	Mean(Grade3)	SD(Grade2)	SD(Grade3)
PMN-MDSC, HER2+	0.1978	0.1562	0.2571	0.2318	0.2504
PMN-MDSC, ER+	0.5887	0.321	0.1445	0.5281	0.1212
M-MDSC, HER2+	0.3255	2.046	2.484	0.9395	1.054
M-MDSC, ER+	0.2403	1.39	2.747	0.5998	1.533

PMN-MDSC Frequencies in Grade 2 and Grade 3



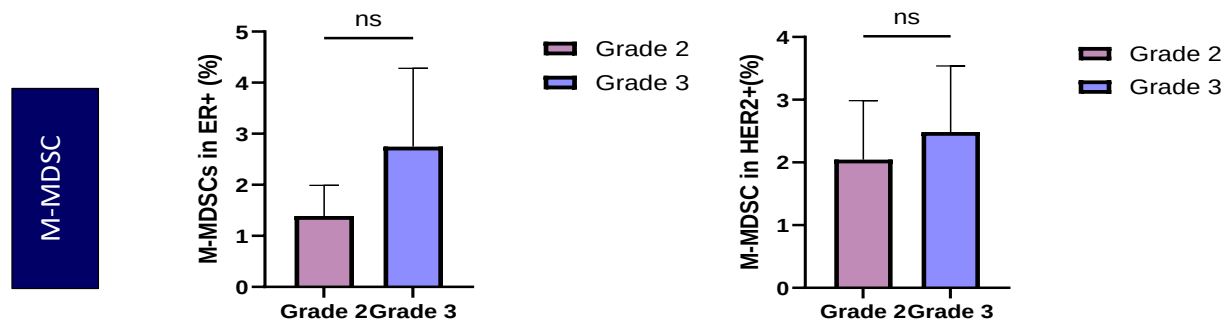


Figure 17: The plots show the frequencies of PMN-MDSCs and M-MDSCs in breast cancer patients, stratified by tumor grade (Grade 2 and Grade 3) and molecular subtype (HER2+, ER+). TNBC was excluded due to insufficient sample size. Data are presented as mean $\pm$ SD. Statistical comparisons were performed using the Mann–Whitney test ($p < 0.05$ considered significant).

4.2.3 Evaluation of LOX-1 Expression in Circulating PMN-MDSCs Across Breast Cancer Subtypes at baseline

We analysed the expression of LOX-1, a marker commonly associated with PMN-MDSCs in breast cancer patients at the baseline timepoint, within two different populations: first in the broader lympho-monocyte population (Figure 17, A), and then specifically within the PMN-MDSC subset (Figure 17, B).

In Figure 18 A, LOX-1 expression in PMN-MDSCs was evaluated across breast cancer subtypes at baseline, including HER2⁺ ($n = 33$), TNBC ($n = 23$), and ER⁺ ($n = 16$) patients within the Lymphocyte-monocyte population. A statistically significant difference was observed between HER2⁺ and TNBC groups ($P = 0.0066$), with HER2⁺ patients showing higher levels of LOX-1⁺ PMN-MDSCs. No significant differences were found between HER2⁺ and ER⁺ or between TNBC and ER⁺ groups. These findings suggest a potential enrichment of immunosuppressive LOX-1⁺ PMN-MDSCs in HER2⁺ breast cancer, which may contribute to a distinct immune landscape in this subtype.

In Figure 18 B, LOX-1 expression was evaluated more specifically within the PMN-MDSC gate, in a slightly smaller cohort (HER2⁺: $n = 31$, TNBC: $n = 21$, ER⁺: $n = 15$). Although a similar trend in expression levels was noted, the differences did not reach statistical significance. This suggests

that the broader lympho-monocyte gate may capture transitional or pre-suppressive myeloid populations that also express LOX-1, particularly in ER+ cases.(table 10)

Table 10: Summary of LOX-1 expression (in percentage) in circulating PMN-MDSCs across ER+, HER2+, and TNBC breast cancer patients at the baseline timepoint. Abbreviations: SD = standard deviation; PMN-MDSC = polymorphonuclear myeloid-derived suppressor cell; TNBC = triple-negative breast cancer.

	P value	P value	P value	P value	mean	mean	mean	SD	SD	SD
		HER2+ vs ER+	HER2+ vs TNBC	ER+ vs TNBC	HER2+	TNBC	ER+	HER2+	TNBC	ER+
PMN-MDSC, LOX1+(The frequency of Lympho-mono)	0.0072	>0.9999	0.0066	0.0986	0.09909	0.02924	0.1339	0.1637	0.08403	0.255
PMN-MDSC, LO1+(The frequency of PMN)	0.9147	>0.9999	>0.9999	>0.9999	45.3	45.64	42.17	26.74	30.23	31.06

(A)

(B)

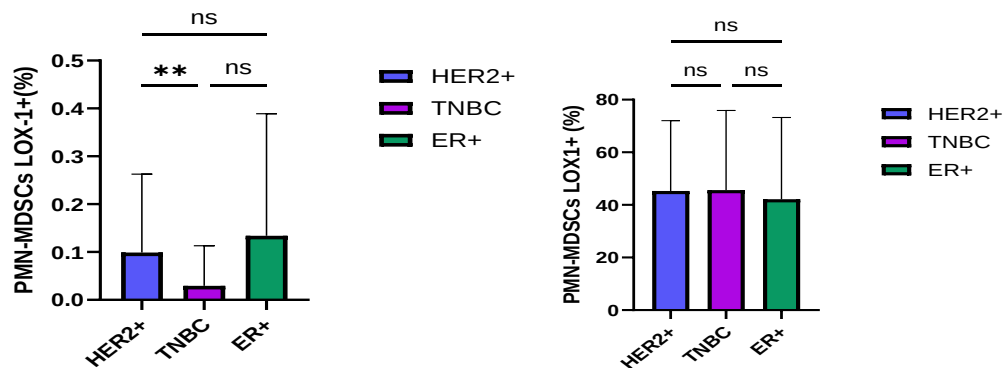


Figure 18: The plots show the relative frequency of LOX-1 expression in circulating PMN-MDSC (in lympho-monocytes[A] and PMN-MDSCs population[B]) at baseline in HER2+, ER+, and TNBC breast cancer patients (Kruskal–Wallis test). Data are presented as means $\pm$ SD ($p < 0.05$ considered significant; ns = not statistically significant).

4.3. Association of Myeloid Populations during pre-NAT phase and its correlation with pCR achievement

4.3.1 Correlation of Monocyte Subpopulations With Pathological Complete Response (pCR) and Residual Disease (RD)

To evaluate whether the overall frequency of circulating monocytes correlates with pathological response to neoadjuvant therapy, we analyzed the total monocyte population in breast cancer patients stratified by response status (pCR vs RD). The analysis included a total of 55 patients, with 29 patients achieving pCR and 26 presenting with RD.(figure 19)

We further stratified the data by molecular subtype to assess potential subtype-specific associations. In the HER2+ subgroup (pCR = 17, RD = 11), the TNBC subgroup (pCR = 8, RD = 9), and the ER+ subgroup (pCR = 5, RD = 6), monocyte frequencies were compared between responders and non-responders.

Across all comparisons—both in the overall cohort and within each breast cancer subtype—no statistically significant differences were observed in total monocyte levels between the pCR and RD groups (Mann–Whitney U test). These findings suggest that total circulating monocyte frequency at baseline is not predictive of response to neoadjuvant treatment in this patient cohort.(table 11)

Table 11: Summary of the total monocyte frequency (in percentage) in breast cancer patients stratified by treatment response (pCR vs RD) and molecular subtype (HER2+, TNBC, ER+). Abbreviations: pCR = pathological complete response; RD = residual disease; SD = standard deviation; TNBC = triple-negative breast cancer.

	P value	Mean(pCR)	Mean(RD)	SD(pCR)	SD(RD)
Overall	0.0571	10.32	7.948	4.993	4.249
HER2+	0.7201	9.405	9.085	3.498	4.964
TNBC	0.762	9.978	7.692	7.647	3.616
ER+	0.2468	10.72	6.25	5.93	3.697

Monocyte population at baseline: PCR and RD

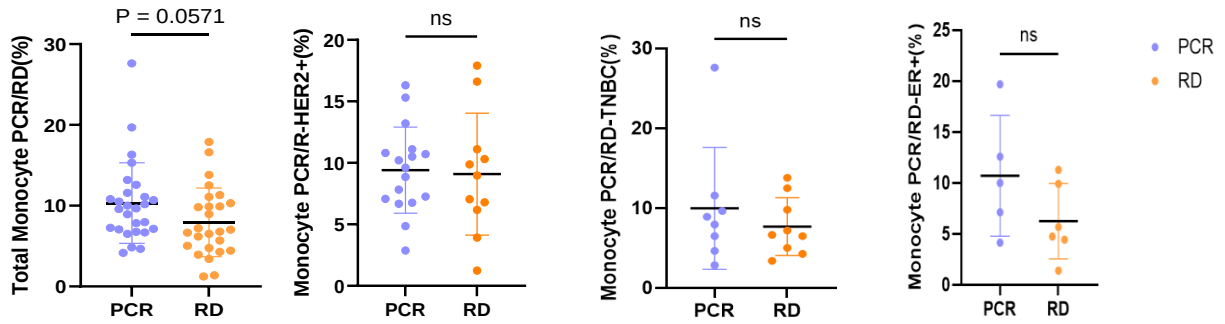


Figure 19: The plots show the relative frequency of total monocytes in breast cancer patients stratified by pathological response (pCR vs RD). Comparisons were made for the overall cohort and within molecular subtypes.

Data are presented as mean $\pm$ SD. No statistically significant differences were observed in total monocyte levels between pCR and RD groups across all categories (Mann–Whitney U test).

Furthermore, we explore the relationship between circulating monocyte subsets and treatment response, we evaluated the frequencies of classical, intermediate, and non-classical monocytes in patients stratified by pathological complete response (pCR) and residual disease (RD) within each breast cancer subtype. In the HER2+ cohort (pCR = 16, RD = 11), no statistically significant differences were observed between pCR and RD groups across all monocyte subsets. Similarly, in the ER+ group (pCR = 6, RD = 6), monocyte subset frequencies did not significantly differ between outcome groups.

However, in the TNBC cohort (pCR = 8, RD = 9), a significant increase in intermediate monocytes was observed in patients who achieved pCR compared to those with RD, while classical and non-classical subsets showed no significant differences. These findings suggest a potential association between intermediate monocytes and favorable treatment response in TNBC, whereas such associations were not evident in HER2+ or ER+ subtypes.(table 12, figure 20)

Table 12: Summary of the expression of monocyte subsets (in percentage) of interest in breast cancer patients, stratified by molecular subtype (HER2+, TNBC, ER+) and by treatment response (pCR vs RD). Abbreviations: SD = standard deviation; pCR = pathological complete response; RD = residual disease.

subtype	Monocyte subset	P value	Mean(PCR)	Mean(RD)	SD(PCR)	SD(RD)
HER2+	Classical	0.0963	64.04	54.5	10.54	13.08
	Intermediate	0.342	12.61	13.78	9.512	5.41
	Non classical	0.585	9.407	11.29	6.274	7.962
TNBC	Classical	0.9629	59.64	58.56	18.44	24.73
	Intermediate	0.0152	18.32	6.649	11.79	5.263
	Non classical	0.2359	10.95	6.358	8.586	4.804
ER+	Classical	0.5887	64.43	66.07	8.764	10.47
	Intermediate	0.4848	15.27	11.06	7.175	10.4
	Non classical	0.9372	6.213	5.157	2.542	3.173

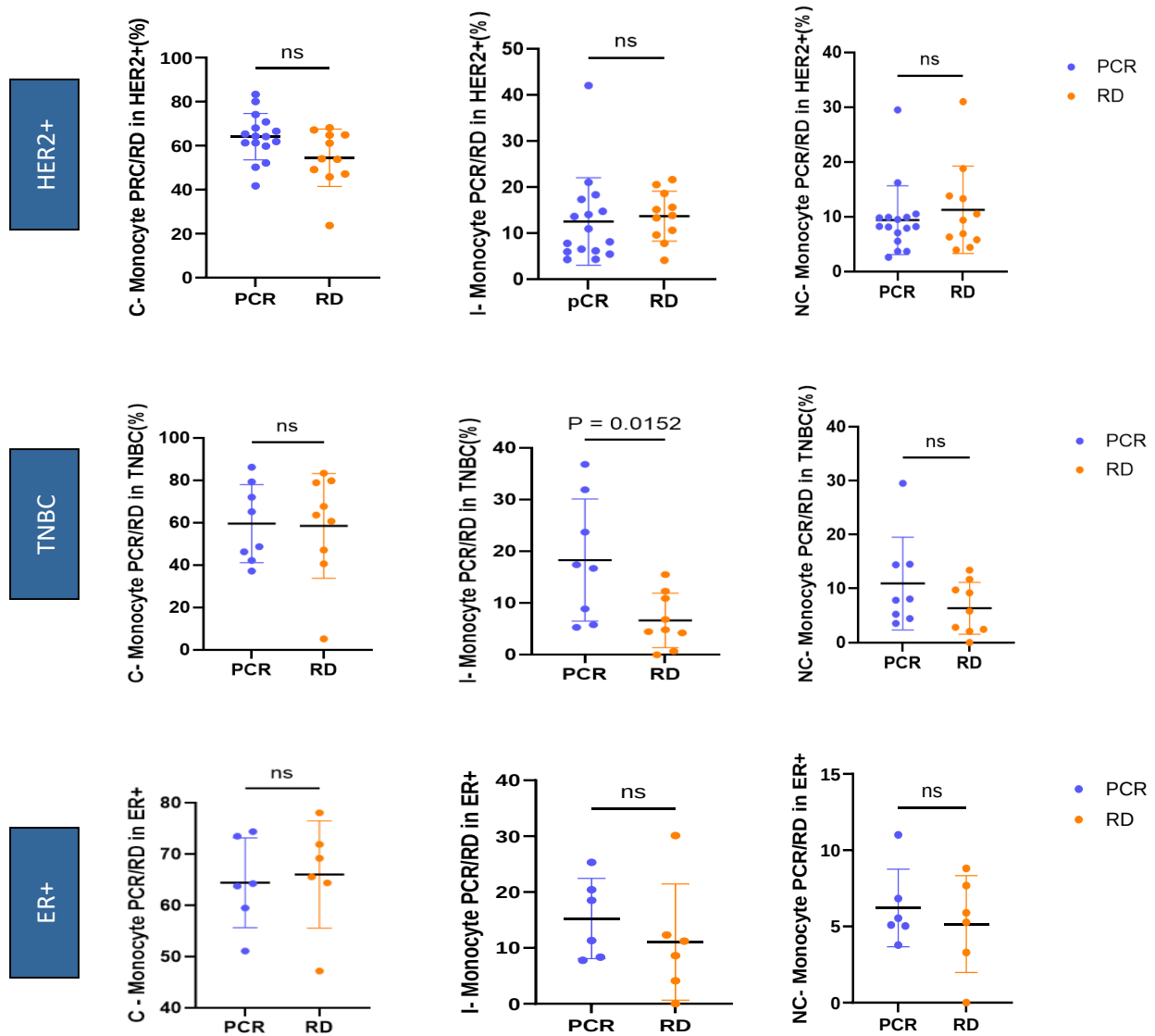


Figure 20: The plots show the relative frequency of circulating MDSC subpopulations in breast cancer patients, stratified by molecular subtype and treatment response (pCR vs RD).

Data are presented as mean $\pm$ SD. Statistical analysis was performed using the Mann–Whitney U test.

In addition, to assess the potential predictive value of the lymphocyte-to-monocyte ratio (LMR) at baseline, we compared LMR levels between patients who achieved a pathological complete response (pCR) and those with residual disease (RD), stratified by molecular subtype of breast cancer.

In the HER2+ subgroup (pCR = 16, RD = 11), TNBC subgroup (pCR = 8, RD = 9), and ER+ subgroup (pCR = 5, RD = 7), baseline LMR values were analyzed using the Mann–Whitney U test. Across all three subtypes, no statistically significant differences in LMR were observed between pCR and RD groups.(table 13, figure21)

These findings suggest that the baseline lymphocyte-to-monocyte ratio does not appear to distinguish between responders and non-responders to neoadjuvant therapy in HER2+, TNBC, or ER+ breast cancer patients within this cohort.

Table 13: Summary of the lymphocyte-to-monocyte ratio (LMR) in HER2+, TNBC, and ER+ breast cancer patients, stratified by pathological response (pCR vs RD). Abbreviations: LMR = lymphocyte-to-monocyte ratio; pCR = pathological complete response; RD = residual disease; SD = standard deviation; TNBC = triple-negative breast cancer.

	P value	Mean(pCR)	Mean(RD)	SD(pCR)	SD(RD)
HER2+	0.2122	4.453	3.46	1.317	2.355
TNBC	0.743	4.835	5.392	0.9211	1.914
ER+	>0.9999	4.142	4.12	1.14	1.559

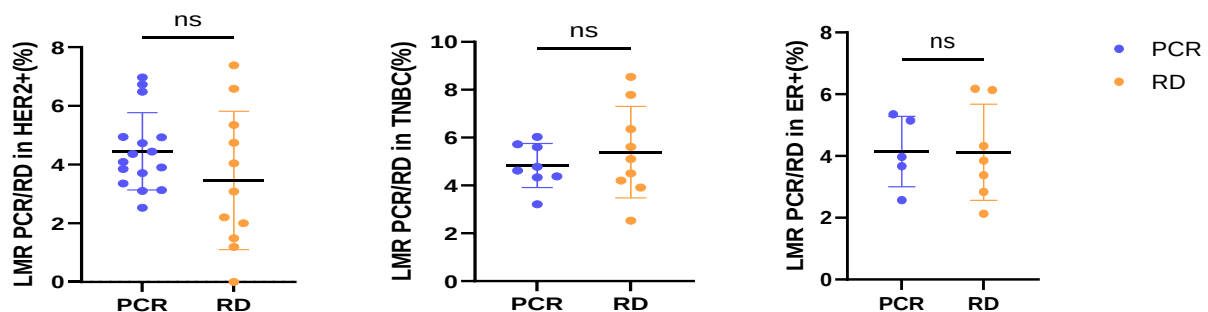


Figure 21: The plots show the baseline lymphocyte-to-monocyte ratio (LMR) in breast cancer patients stratified by pathological response (pCR vs RD) across three molecular subtypes: HER2+, TNBC and ER+. Data are presented as mean $\pm$ SD. No statistically significant differences were observed between groups (Mann–Whitney U test).

4.3.2 Correlation Between Baseline MDSC Subsets and Pathological Outcome (PCR and RD) Following Neoadjuvant Therapy in Breast Cancer

To investigate the potential predictive value of MDSC subtypes at baseline in breast cancer patients undergoing neoadjuvant therapy, we compared the relative frequencies of monocytic (M-MDSC) and polymorphonuclear (PMN-MDSC) populations between patients achieving a pathological complete response (pCR) and those with residual disease (RD), stratified by molecular subtype.

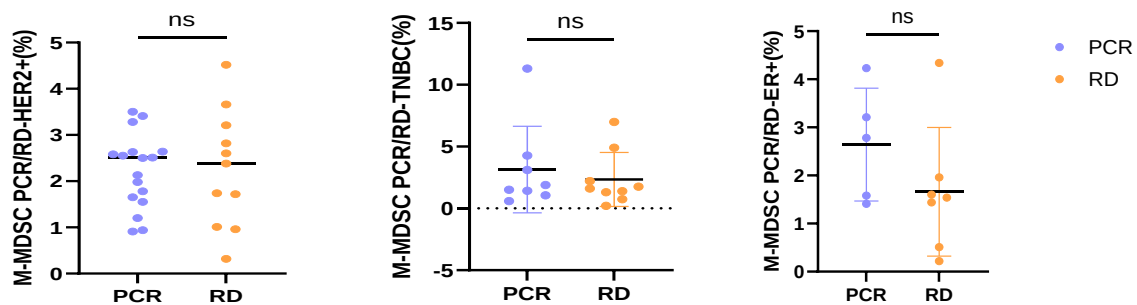
In the HER2+ group (M-MDSC: pCR = 17, RD = 11; PMN-MDSC: pCR = 17, RD = 11), no statistically significant differences were found between pCR and RD patients for either MDSC subtype. Similarly, in the TNBC subgroup (M-MDSC: pCR = 8, RD = 9; PMN-MDSC: pCR = 8, RD = 9) and ER+ subgroup (M-MDSC: pCR = 5, RD = 7; PMN-MDSC: pCR = 5, RD = 7), the frequencies of M-MDSCs and PMN-MDSCs were not significantly different between response groups.(table 14, figure 22)

These results suggest that baseline levels of M-MDSCs and PMN-MDSCs are not associated with response to neoadjuvant treatment across any of the major breast cancer molecular subtypes.

Table 14: Summary of the distribution of M-MDSC and PMN-MDSC subpopulations (in percentage) in HER2+, TNBC, and ER+ breast cancer patients at baseline, stratified by pathological response (pCR vs RD). Data are expressed as mean $\pm$ standard deviation. Abbreviations: SD = standard deviation; pCR = pathological complete response; RD = residual disease; PMN-MDSC = polymorphonuclear myeloid-derived suppressor cells; M-MDSC = monocytic myeloid-derived suppressor cells; TNBC = triple-negative breast cancer.

Patients	PCR/RD	PMN-MDSC		M-MDSC		Pvalue PMN-MDSC	P value M- MDSC
		mean	SD	mean	SD		
HER2+	PCR	0.2459	0.2687	2.22	0.8043	0.0887	0.9263
	RD	0.126	0.1717	2.267	1.265		
TNBC	PCR	0.07518	0.1441	2.144	3.504	0.3841	0.743
	RD	0.07711	0.0764	2.344	2.184		
ER+	PCR	0.164	0.1242	2.642	1.174	0.3434	0.343
	RD	0.2818	0.4931	1.659	1.338		

M-MDSC population at baseline: PCR and RD



PMN-MDSC population at baseline : PCR and RD

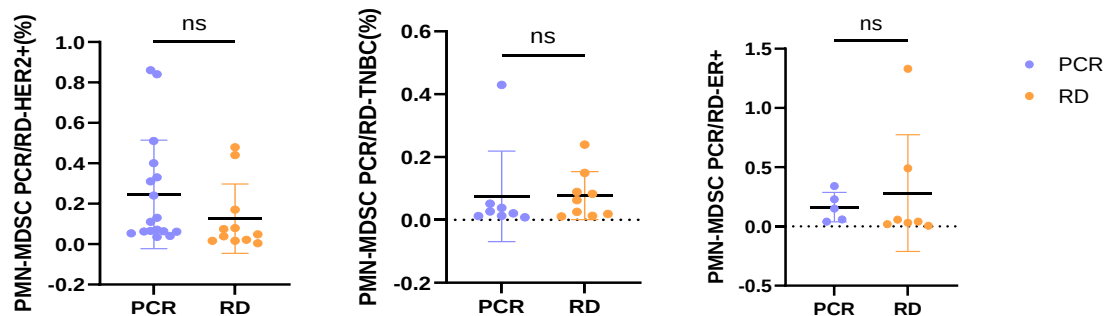


Figure 22: The plots show the relative cell frequency of circulating M-MDSC and PMN-MDSC subpopulations in breast cancer patients stratified by pathological response (pCR vs RD) within HER2+, TNBC, and ER+ subtypes. Data are presented as mean $\pm$ SD. No statistically significant differences were observed between groups (Mann-Whitney U test).

4.3.3 Evaluation of PMN-LOX1⁺ Expression Across Breast Cancer Subtypes and Its Association with pCR/RD Response

To explore the relationship between PMN-LOX1⁺ expression and therapeutic response in breast cancer patients, we evaluated the frequency of LOX-1⁺ cells within both the lymphocyte-monocyte (LM) population and the PMN-MDSC compartment. The analysis was performed across all patients (n = 52 in LM; n = 52 in PMN) stratified by pathologic complete response (pCR) and residual disease (RD) status following NAT. While no statistically significant difference was observed in the LOX-1⁺ frequency within the LM compartment (p = 0.0838) (figure 23, A), a trend toward significance was noted in the PMN-MDSC compartment (p = 0.0584)(figure 23, E), suggesting a potential association between higher PMN-LOX1⁺ levels and poor therapeutic outcome.

Further subgroup analysis was conducted for each molecular subtype of breast cancer—HER2+, TNBC, and ER+—in both the LM and PMN compartments. For HER2+ patients (PCR = 16, RD = 14), TNBC patients (pCR = 8, RD = 9), and ER+ patients (pCR = 5, RD = 7), no significant differences in LOX-1 expression were found between pCR and RD groups, neither in the LM nor in the PMN populations. Despite the lack of statistical significance, these data offer important insight into the immunosuppressive landscape shaped by LOX-1⁺ MDSCs in the tumor microenvironment and its potential influence on treatment resistance.

Table 15: Summary of the expression levels of LOX-1⁺ cells within the lymphocyte-monocyte and PMN-MDSC populations, stratified by breast cancer subtype (HER2+, TNBC, ER+) and pCR/RD outcome. Abbreviations: SD = standard deviation; PMN = polymorphonuclear; LM = lymphocyte-monocyte; pCR = pathologic complete response; RD = residual disease.

	P value	mean	mean	SD	SD
		PCR	RD	PCR	RD
Overall (In LM populatin)	0.0838	0.1257	0.1298	0.1816	0.2782
HER2+	0.0693	0.1547	0.06657	0.2158	0.1017
TNBC	0.3326	0.06265	0.01153	0.1409	0.01578
ER+	0.404	0.1336	0.2046	0.1006	0.3738
Overall (In PMN-MDSC population)	0.0584	57.04	40.91	27.92	27.34
HER2+	0.4292	50.49	37.56	31.88	24.14
TNBC	0.5358	57.11	44.69	32.23	23.27
ER+	0.1758	68.85	40.91	27.92	27.34

PMN-MDSC LOX1+ population at baseline: PCR and RD

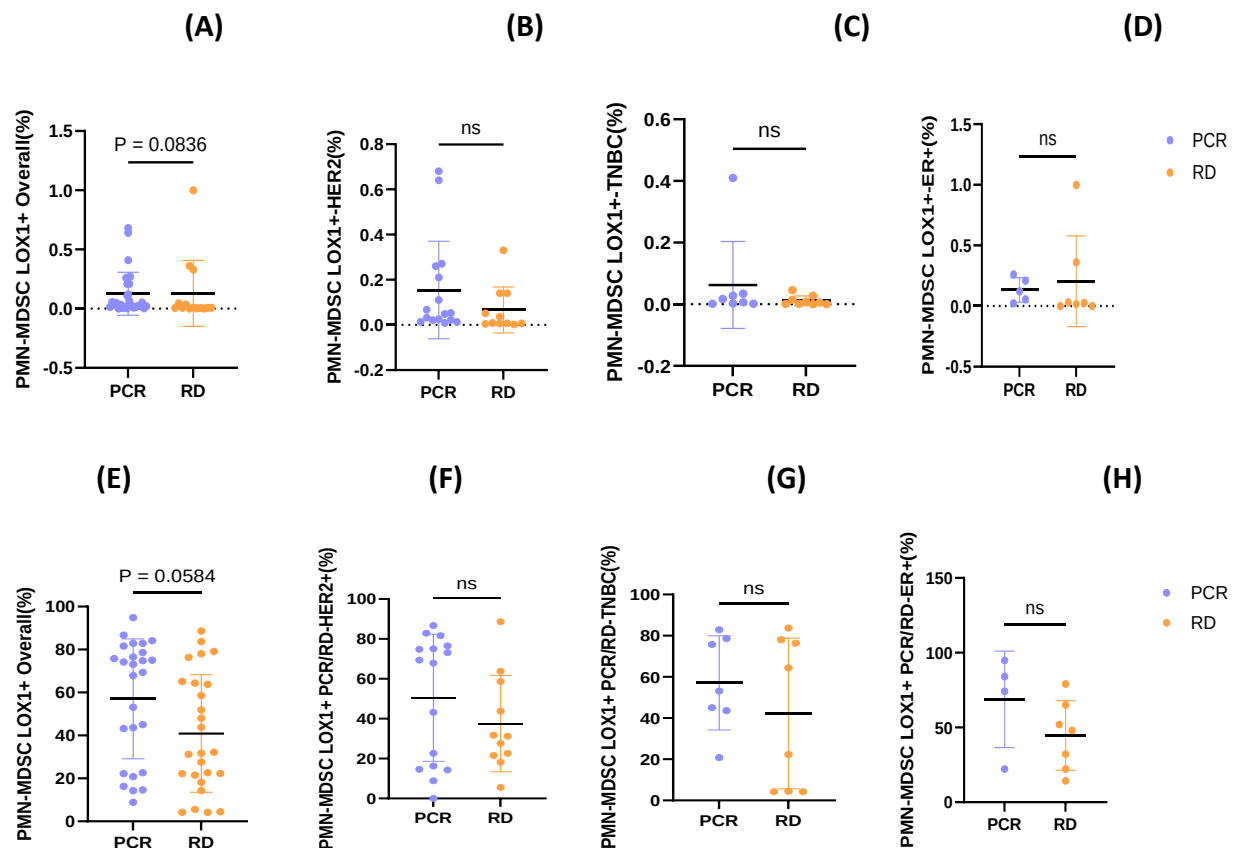


Figure 23: The plot shows the relative frequency of LOX-1⁺ expression within the PMN-MDSC compartment of breast cancer patients at baseline, stratified by response to therapy (pCR vs RD) and molecular subtype.

Panels A–D represent LOX-1⁺ cell frequency within the lymphocyte-monocyte population: overall (A), HER2⁺ (B), TNBC (C), and ER⁺ (D). Panels E–H represent LOX-1⁺ cell frequency within the PMN-MDSC population: overall (E), HER2⁺ (F), TNBC (G), and ER⁺ (H). (Mann–Whitney U test)

4.3.4 Univariate Logistic Regression Analysis of Myeloid Subpopulations and pCR Achievement

To evaluate the predictive potential of circulating myeloid populations in relation to treatment response, a univariate logistic regression analysis was performed to assess the association between each immune subset and pathological complete response (pCR) in breast cancer patients. The analysis included PMN-MDSCs, M-MDSCs, LOX-1⁺ PMN-MDSCs, total monocytes, and the three major monocyte subsets (classical, intermediate, and non-classical).

Among all evaluated subsets, non-classical monocytes were the only population significantly associated with pCR, with an odds ratio (OR) of 5.7 (**p = 0.0266**). This indicates that higher baseline frequencies of non-classical monocytes are positively associated with achieving pCR following neoadjuvant chemotherapy.

No significant associations were found for total monocytes, classical or intermediate monocytes, PMN-MDSCs, M-MDSCs, or LOX-1⁺ PMN-MDSCs. (*Figure 24*)

These findings highlight non-classical monocytes as a potential predictive biomarker of treatment response in early breast cancer and warrant further validation in larger patient cohorts.

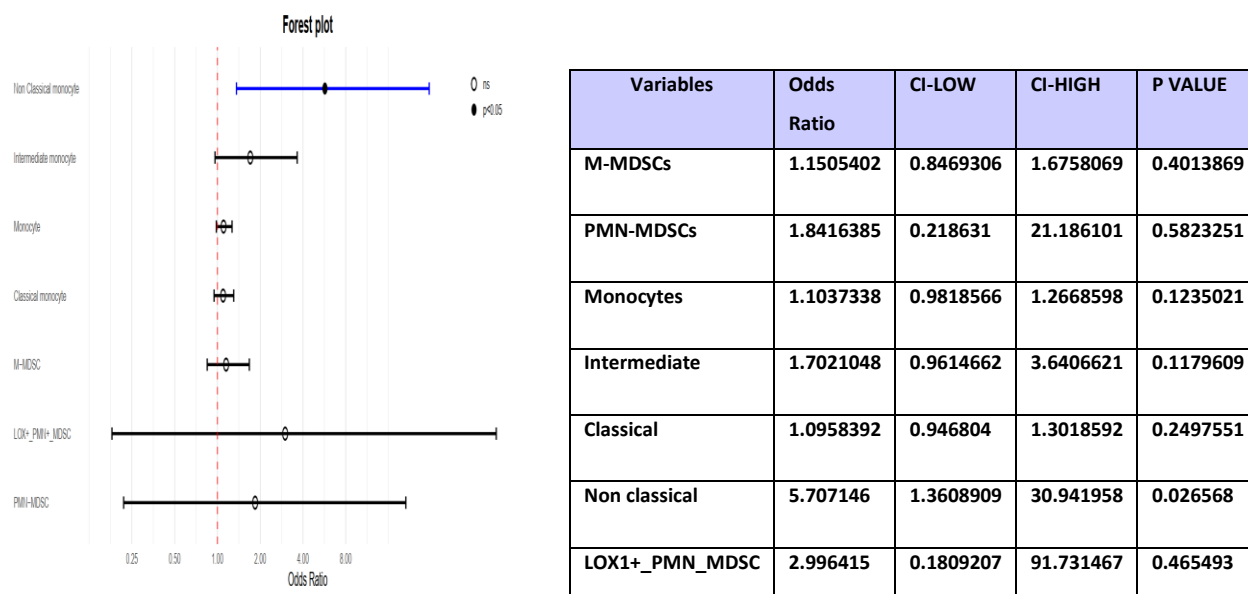


Figure 24: Univariate logistic regression analysis evaluating the association between circulating myeloid cell subsets and pathological complete response (pCR) in breast cancer patients.

4.4 Assessment of Circulating Myeloid and Monocyte Subsets Before and After Neoadjuvant Chemotherapy in Breast Cancer Patient

4.4.1 Dynamic Changes in Monocyte Subsets during the Pre-NAT and Post-NAT

To evaluate the effect of neoadjuvant chemotherapy on circulating monocyte populations, we examined the frequency of classical, intermediate, and non-classical monocyte subsets at baseline (pre-NAT) and after completion of treatment (post-NAT) in breast cancer patients (n = 50). While a numerical decrease in intermediate monocytes and slight variations in classical and non-classical subsets were observed after therapy, these changes did not reach statistical significance (p = 0.475 for classical, p = 0.4526 for intermediate, and p = 0.1714 for non-classical). This suggests that neoadjuvant chemotherapy did not significantly alter the distribution of circulating monocyte subsets in this patient cohort (Figure 26, Table 17).

Table 17: Summary of the distribution of monocyte subpopulations (in percentage) in breast cancer patients at baseline (pre-NAT) and after treatment (post-NAT). Data are stratified into classical, intermediate, and non-classical monocytes.

Abbreviations: SD = standard deviation; NAT = neoadjuvant treatment.

	P value	Mean(Pre)	Mean(Post)	SD(Pre)	SD(Post)
Classical	0.475	61.79	61.66	16.48	15.14
Intermediate	0.4526	14.06	8.207	8.978	5.829
Non classical	0.1714	7.603	9.032	6.362	4.548

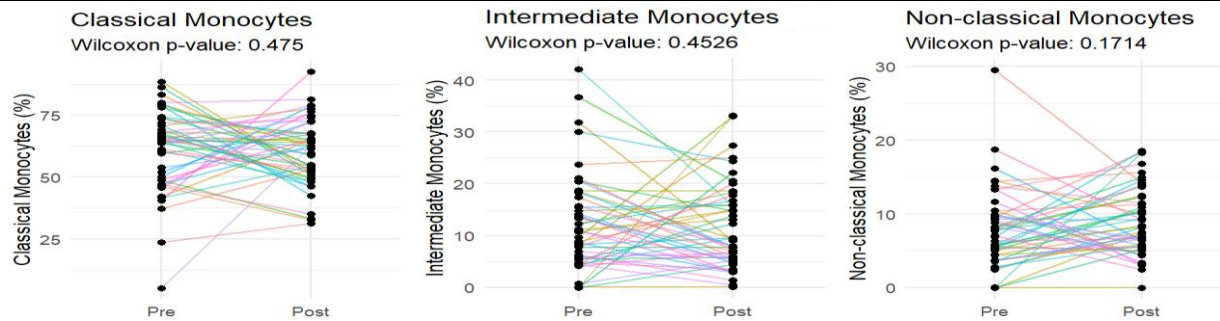


Figure 26: The plots illustrate the relative frequencies of overall circulating classical, intermediate, and non-classical monocyte subsets before and after neoadjuvant chemotherapy (pre-Nat vs. post-NAT). Statistical analysis was performed using the Wilcoxon matched-pairs signed-rank test. Data are presented as means $\pm$ SD. No statistically significant differences were observed between pre- and post-treatment time points.

4.4.2 Evaluation of MDSC in Breast Cancer Subtypes Before and After Neoadjuvant Treatment

To explore the dynamic changes in myeloid-derived suppressor cell (MDSC) populations following neoadjuvant chemotherapy (NAT), we analyzed both PMN-MDSC and M-MDSC subsets at baseline (pre-NAT) and after treatment completion (post-NAT). In the overall patient population (M=MDSC n=41, PMN-MDSC n= 35)), no statistically significant differences were observed in either M-MDSC or PMN-MDSC frequencies.

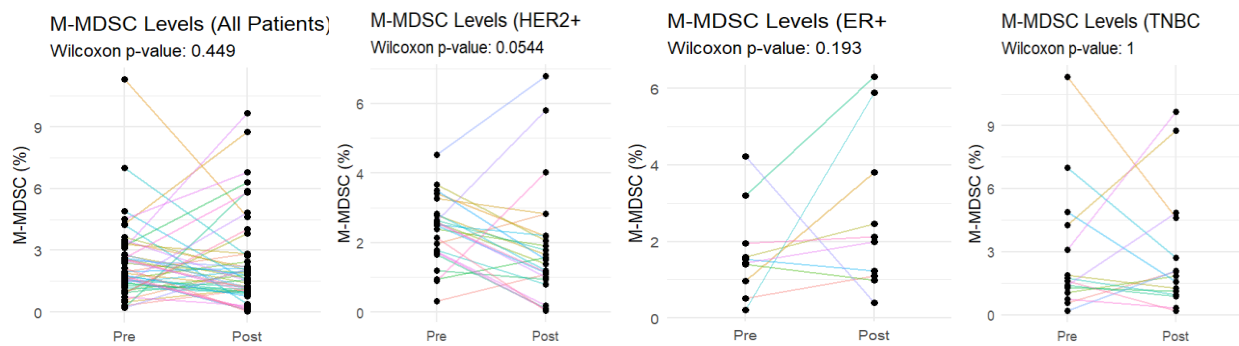
We next stratified patients by breast cancer subtype to evaluate potential subtype-specific MDSC responses. For the M-MDSC subset, no significant differences were detected in HER2+ (n=25), TNBC (n=15), or ER+ (n= 10) groups between pre- and post-NAT timepoints.

Similarly, we explore potential subtype-specific changes in PMN-MDSC levels. No statistically significant differences were observed between pre- and post-neoadjuvant treatment samples in the TNBC (n = 14), HER2⁺ (n = 21), or ER⁺ (n = 12) subgroups. (Table 18, Figure 27).

Table 18: Summary of PMN-MDSC and M-MDSC mean frequencies ($\pm$ SD) before and after NAC in breast cancer patients, stratified by molecular subtype. A statistically significant increase in PMN-MDSCs was observed in TNBC patients after treatment.

	P value	Mean(Pre)	Mean(Post)	SD(Pre)	SD(Post)
Overall (M-MDSC)	0.4486	2.477	2.116	1.988	2.274
HER2+	0.0544	2.373	1.692	1.067	1.826
TNBC	1	2.778	2.611	1.856	2.882
ER+	0.193	2.223	2.4	1.5	2.366
Overall (PMN-MDSC)	0.1343	0.1367	0.08651	0.2098	0.1596
HER2+	0.727	0.2479	0.1855	0.9851	0.2234
TNBC	0.06491	0.06762	0.2608	0.1168	0.3014
ER+	0.193	0.1047	0.2899	0.1134	0.5112

M-MDSC population patients: PRE and POST



PMN-MDSC population patients: PRE and POST

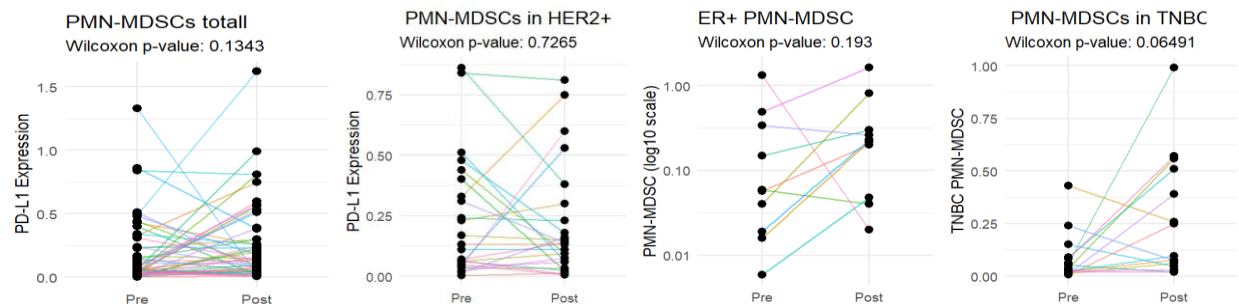


Figure 27: The plots display the relative frequencies of circulating M-MDSC and PMN-MDSC subpopulations in breast cancer patients before (PRE) and after (POST) neoadjuvant chemotherapy (NAT)

Data are presented as mean $\pm$ SD. Statistical analysis was performed using the Wilcoxon matched-pairs signed-rank test.)

4.4.3 Evaluation of PMN-LOX1⁺ Cells Before and After Neoadjuvant Therapy

To explore the effect of neoadjuvant chemotherapy (NAT) on the immunosuppressive myeloid compartment, we evaluated the frequency of LOX-1⁺ cells within PMN-MDSCs and the broader lympho-monocyte population at baseline (pre-NAT) and after treatment (post-NAT).

Subtype-specific analysis of LOX-1⁺ PMN-MDSCs revealed differential modulation across breast cancer subtypes following neoadjuvant treatment. In the HER2⁺ subgroup ($n = 21$), a significant reduction in LOX-1 expression was observed post-treatment ($p = 0.0421$). Similarly, TNBC patients ($n = 12$) showed a pronounced decrease in LOX-1⁺ PMN-MDSCs after therapy ($p = 0.00854$). In contrast, no significant change in LOX-1 expression was detected in the ER⁺ subgroup ($n = 14$, $p = 0.969$) (Figure 28A–C). These findings indicate that the immunosuppressive LOX-1⁺ PMN-MDSC population is more sensitive to chemotherapy-induced modulation in HER2⁺ and TNBC subtypes compared to ER⁺ tumors. When stratified by breast cancer subtype, distinct patterns emerged. Within the PMN-MDSC population, a significant decrease in LOX-1⁺ cell frequency was detected in ER⁺ patients ($n = 8$, $P = 0.0078$). However, HER2⁺ ($n = 19$) and TNBC ($n = 12$) subtypes did not demonstrate significant changes. (figure 28 B-D)

In the lympho-monocyte population, both HER2⁺ ($n = 16$) and ER⁺ ($n = 8$) patients showed no significant differences between timepoints, while in TNBC patients ($n = 11$) a mild increase in LOX1⁺ cells was noted but did not reach statistical significant ($p = 0.05195$), suggesting a potential shift toward an immunosuppressive profile, although statistical significance was not reached. (figure 28 F-H). These results suggest that LOX-1 expression in PMN-MDSCs may be selectively modulated by chemotherapy, particularly in TNBC patients, highlighting the potential subtype-specific dynamics of immunosuppressive cell populations during treatment.

Table 19: Summary of LOX-1⁺ PMN-MDSC frequency (in percentage) in breast cancer patients before and after neoadjuvant therapy, stratified by molecular subtype and analyzed within two populations: PMN-MDSCs and lymphocyte-monocyte (L/M) gate.

Values are presented as mean $\pm$ standard deviation (SD) for both pre- and post-treatment groups. Statistical analysis was conducted using the Wilcoxon matched-pairs signed-rank test.

	P value	Mean(Pre)	Mean(Post)	SD(Pre)	SD(Post)
Overall (In LM populatin)	0.2056	0.1423	0.08559	0.2254	0.1559
HER2+	0.1928	0.1242	0.05536	0.1843	0.06669
TNBC	0.537	0.05168	0.1478	0.1194	0.262
ER+	0.4258	0.1938	0.06329	0.315	0.0671
Overall (In PMN-MDSC population)	0.779	60.98	38	23.76	21.4
HER2+	0.0421	54.45	33.59	27.55	21.81
TNBC	0.00854	663.03	46.81	21.11	22.8
ER+	0.9687	68.16	35.26	17.78	15.86

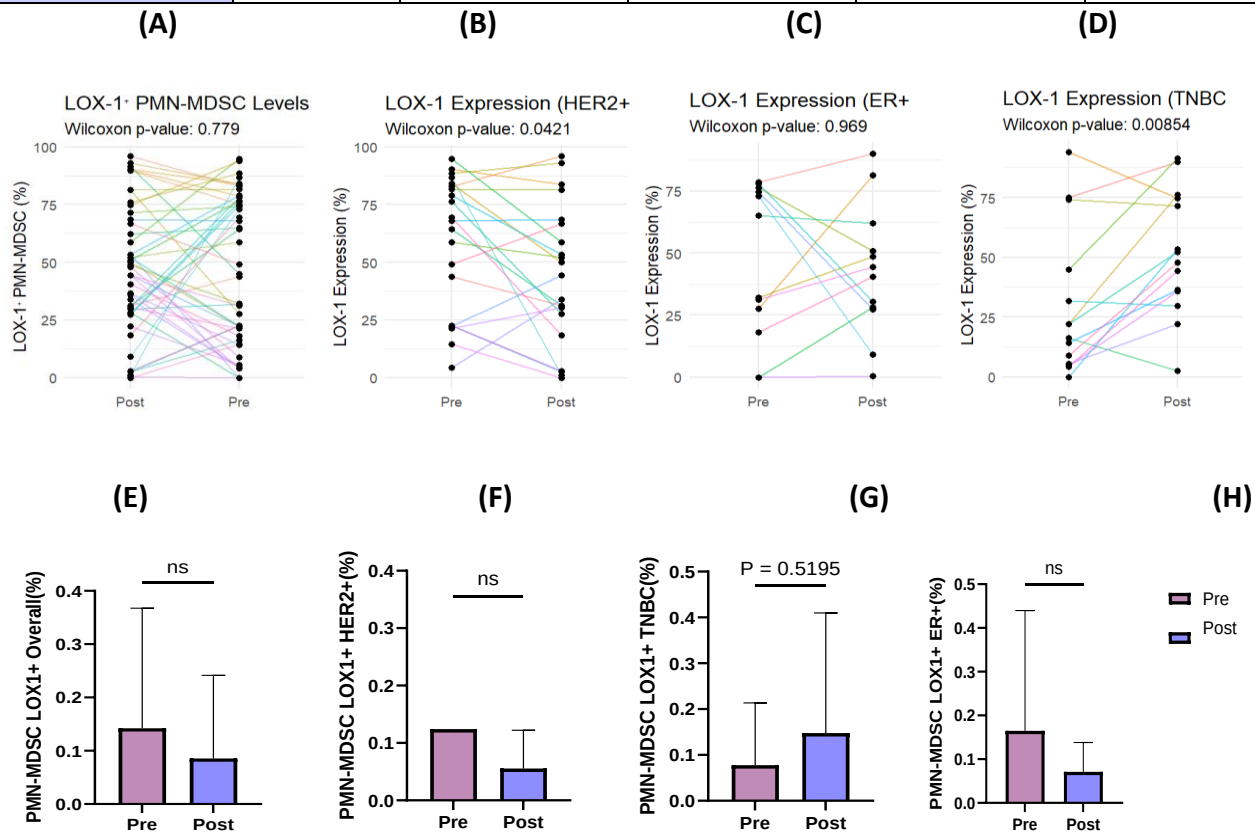


Figure28: The plots show the relative frequency of LOX-1⁺ expression within the PMN-MDSC and lymphocyte-monocyte compartments of breast cancer patients before (Pre) and after (Post) neoadjuvant treatment.

Panels A–D correspond to LOX-1⁺ expression within the PMN-MDSC population: overall (A), HER2⁺ (B), TNBC (C), and ER⁺ (D). Panels E–H represent LOX-1⁺ expression within the lymphocyte-monocyte gate: overall (E), HER2⁺ (F), TNBC (G), and ER⁺ (H). Statistical comparisons were performed using the Wilcoxon matched-pairs signed-rank test. Data are shown as mean ± SD. A P value < 0.05 was considered significant.

4.5 Assessment of Circulating Myeloid and Monocyte Subsets Before and After Neoadjuvant Chemotherapy in Breast Cancer Patient who achieve Pathological Complete Response

4.5.1 Dynamic Changes in Circulating Monocyte Subsets (Classical, Intermediate, and Non-Classical) in pCR Patients

To assess the impact of neoadjuvant chemotherapy on circulating monocytes in patients who achieved pathological complete response (pCR), we performed a Wilcoxon matched-pairs signed-rank test to compare pre- and post-treatment values. In the overall pCR group (n = 28), no significant difference was observed in total monocyte frequency before and after therapy (p = 0.991).

When stratified by breast cancer subtype, no differences were observed. In the HER2⁺ subgroup (n = 16), there was no significant change in monocyte levels post-treatment (p = 0.669). Likewise, ER⁺ patients (n = 4) showed no significant difference (p = 0.875). In TNBC patients (n = 8), an increase in monocyte frequency was noted after treatment; however, this change did not reach statistical significance (p = 0.0781). Collectively, these findings suggest that circulating monocyte levels remain relatively stable following therapy in patients who achieve pCR, regardless of molecular subtype (Figure 29A–D)

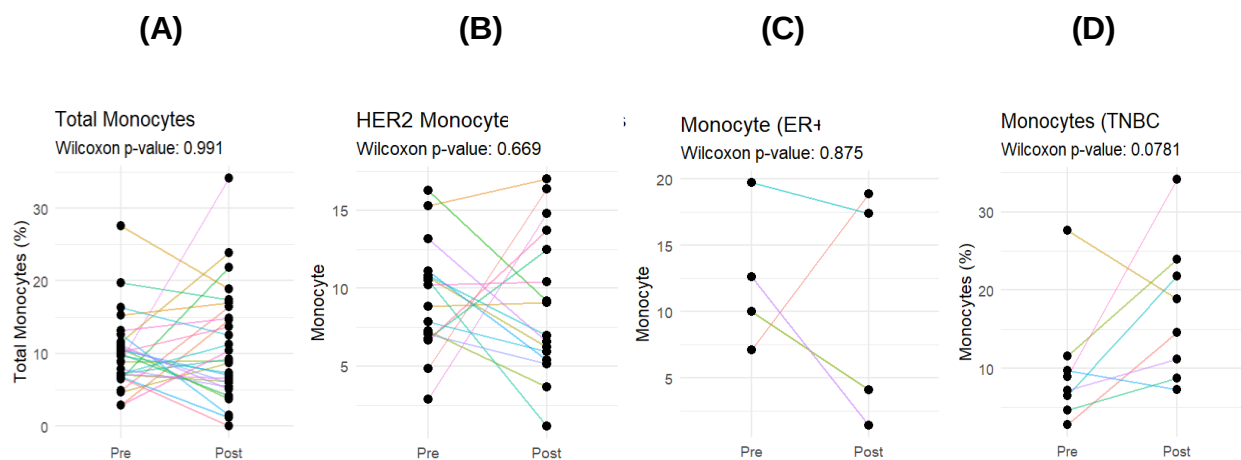
To further investigate the dynamics of circulating monocyte subsets in patients who achieved pathological complete response (pCR), we investigated classical, intermediate, and non-classical monocyte frequencies before and after neoadjuvant chemotherapy using the Wilcoxon matched-pairs signed-rank test.

For **classical monocytes**, no statistically significant differences were observed between pre- and post-treatment time points in the total pCR cohort (n = 26, p = 0.1528). Subtype-specific analysis

similarly revealed no significant changes in HER2⁺ patients (n = 18, p = 0.5226) and TNBC patients (n = 6, p = 0.5625). The ER⁺ group could not be evaluated due to insufficient patient numbers.(Figure 29 E-G)

For **intermediate monocytes**, no significant differences were detected in the total pCR cohort (n = 26, p = 0.3272). Subtype analysis showed no significant changes in HER2⁺ patients (n = 18, p = 0.3927) and TNBC patients (n = 6, p = 1). Again, the ER⁺ group was not assessed due to limited sample size.(Figure29 H-J)

For **non-classical monocytes**, the total pCR group showed no significant differences pre- and post-treatment (n = 26, p = 0.2022). Consistently, no significant changes were observed in HER2⁺ (n = 18, p = 0.865) and TNBC (n = 6, p = 0.3125) subgroups. ER⁺ analysis was not performed for this subset.(Figure 29 K-M). Collectively, these findings indicate that none of the circulating monocyte subsets (classical, intermediate, or non-classical) were significantly modulated by neoadjuvant chemotherapy in patients who achieved pCR, regardless of molecular subtype (Figure 29E–M).



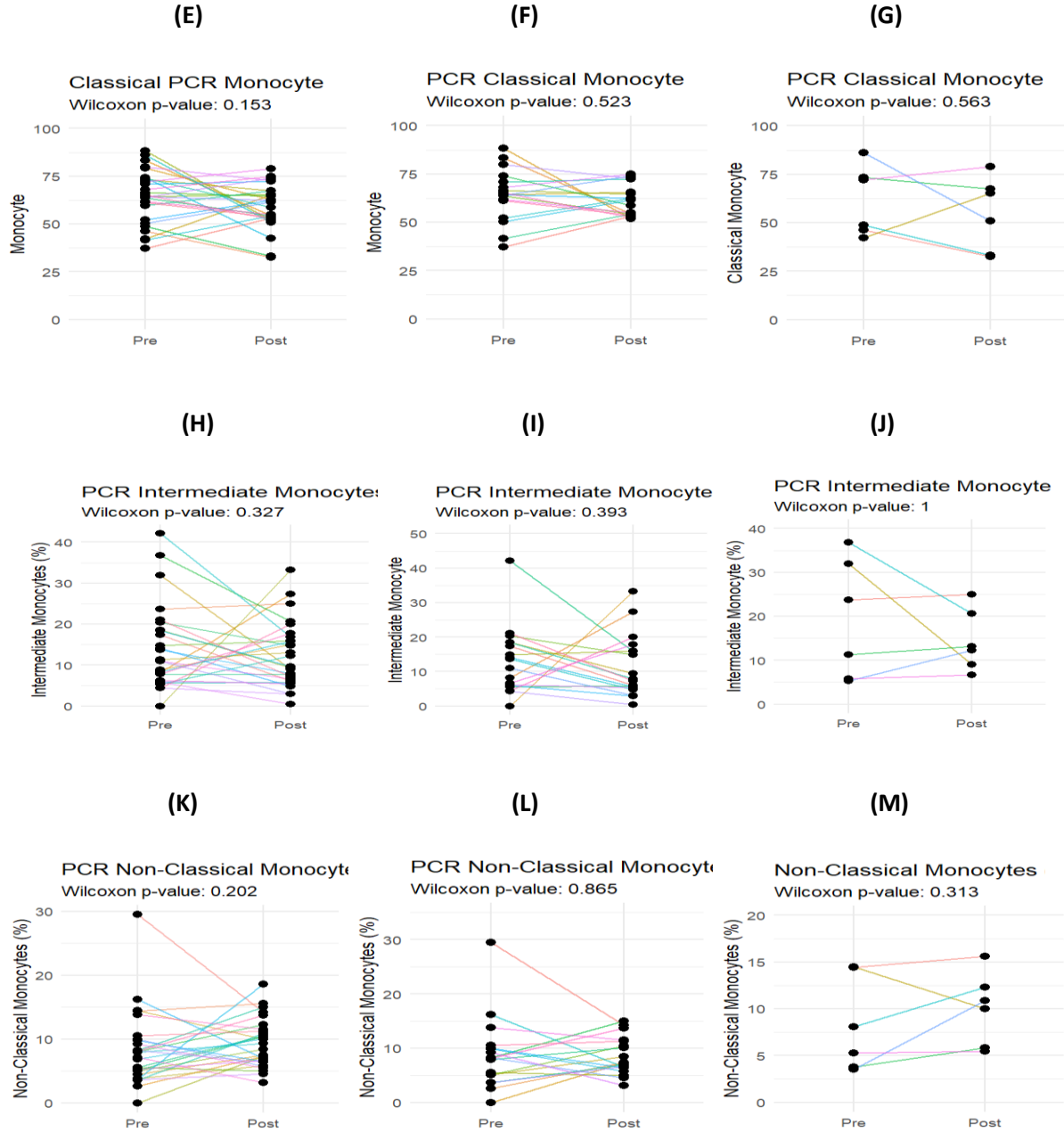


Figure 29: The plots show the relative frequency of total monocytes and their subsets (classical, intermediate, and non-classical) in breast cancer patients who achieved pathological complete response (pCR) before (Pre) and after (Post) neoadjuvant treatment. Panels A–D correspond to total monocytes: overall (A), $HER2^+$ (B), ER^+ (C), and TNBC (D). Panels E–G represent classical monocytes: overall (E), $HER2^+$ (F), and TNBC (G). Panels H–J represent intermediate monocytes: overall (H), $HER2^+$ (I), and TNBC (J). Panels K–M correspond to non-classical monocytes: overall (K), $HER2^+$ (L), and TNBC (M). Statistical comparisons were performed using the Wilcoxon matched-pairs signed-rank test.

4.5.2. Dynamic Changes in Circulating M-MDSCs and PMN-MDSCs Before and After Neoadjuvant Chemotherapy in pCR Patients

To explore how neoadjuvant chemotherapy affects circulating MDSC populations in patients who achieved pathological complete response (pCR), we compared the frequencies of monocytic (M-MDSCs) and polymorphonuclear (PMN-MDSCs) subsets before and after treatment using the Wilcoxon matched-pairs signed-rank test. Overall, no significant changes were observed in M-MDSC levels after therapy ($n = 28$, $p = 0.4254$). Subtype analysis confirmed the absence of significant differences in HER2^+ ($n = 16$, $p = 0.2312$), ER^+ ($n = 4$, $p = 1$), and TNBC ($n = 8$, $p = 0.7422$) groups.

Similarly, PMN-MDSC frequencies remained stable following treatment ($n = 28$, $p = 0.4461$). No significant differences were observed when patients were stratified by subtype: HER2^+ ($n = 16$, $p = 0.6701$), ER^+ ($n = 4$, $p = 0.625$), and TNBC ($n = 8$, $p = 0.1484$). These results indicate that MDSC subset frequencies were not substantially altered by therapy in patients who achieved pCR.

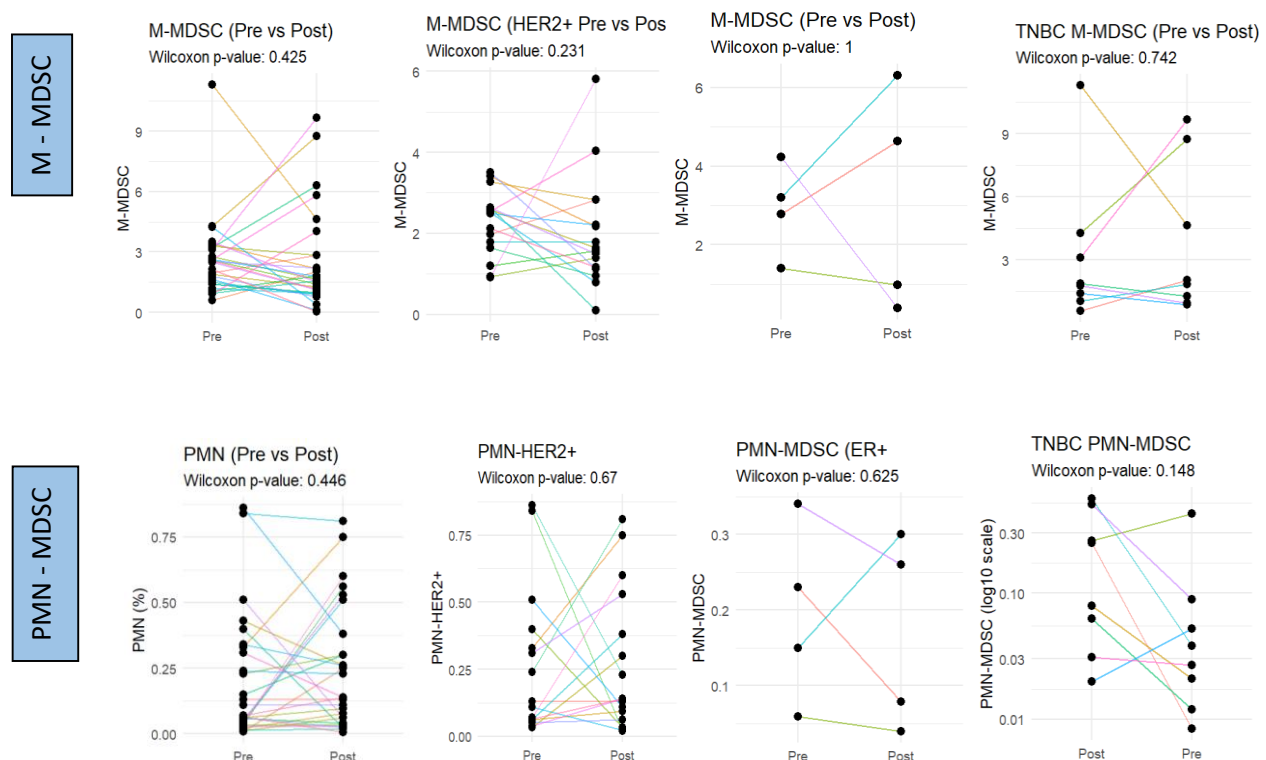


Figure 30: The plots show the relative frequencies of circulating M-MDSCs (top) and PMN-MDSCs (bottom) in breast cancer patients who achieved pathological complete response (pCR) before (Pre) and

after (Post) neoadjuvant chemotherapy. Each plot represents the total cohort as well as analyses stratified by molecular subtype ($HER2^+$, ER^+ , and TNBC). Statistical comparisons were performed using the Wilcoxon matched-pairs signed-rank test. Data are presented as mean $\pm$ SD. A p value < 0.05 was considered statistically significant.

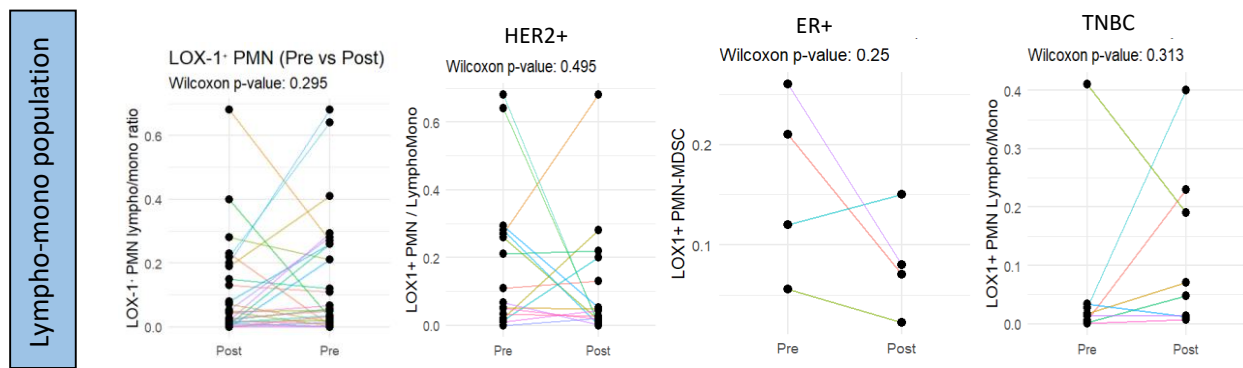
4.5.3 Modulation of LOX-1⁺ PMN-MDSCs within Lympho/Mono and PMN-MDSC Compartments in pCR Patients

To explore the modulation of LOX-1⁺ PMN-MDSCs in patients who achieved pathological complete response (pCR), we analyzed their frequencies within both the lymphocyte/monocyte gate and the PMN-MDSC population before and after neoadjuvant chemotherapy.

For the **lymphocyte/monocyte population**, no significant differences were observed between pre- and post-treatment time points in the total cohort ($n = 28$, $p = 0.2946$). Similarly, no significant changes were found in the $HER2^+$ subgroup ($n = 16$, $p = 0.4954$), ER^+ subgroup ($n = 4$, $p = 0.25$), or TNBC subgroup ($n = 8$, $p = 0.3125$).

For the **PMN-MDSC population**, in the total cohort we found a trend toward reduction in LOX-1⁺ cells following therapy, but this did not reach statistical significance ($n = 22$, $p = 0.05208$). When stratified by subtype, a significant decrease was observed in the $HER2^+$ subgroup ($n = 18$, $p = 0.0395$), whereas ER^+ ($n = 5$, $p = 0.3125$) and TNBC ($n = 6$, $p = 0.4375$) showed no significant changes.

These results suggest that neoadjuvant chemotherapy may differentially modulate LOX-1⁺ PMN-MDSCs in $HER2^+$ breast cancer, while no major effects were seen in other subtypes.



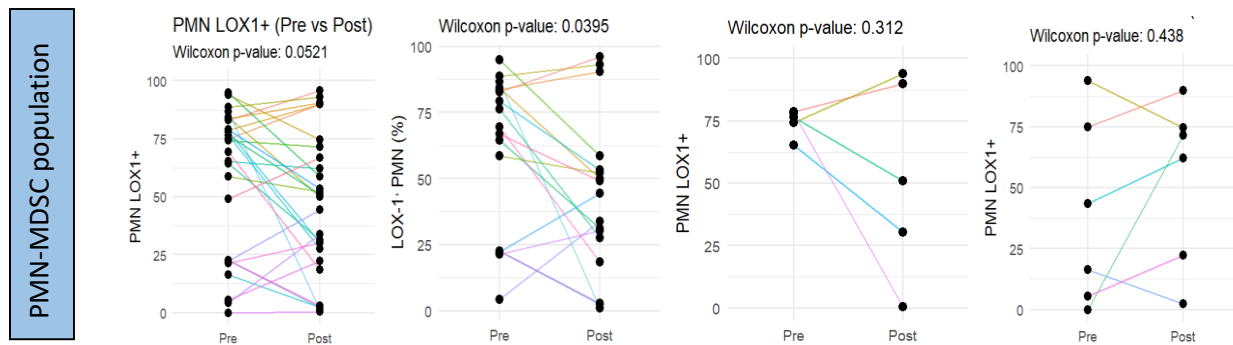


Figure 31; The plots show the relative frequencies of *LOX-1*⁺ PMN-MDSCs within the lymphocyte/monocyte compartment (top) and the PMN-MDSC population (bottom) in breast cancer patients who achieved pathological complete response (pCR) before (Pre) and after (Post) neoadjuvant chemotherapy. Statistical comparisons were performed using the Wilcoxon matched-pairs signed-rank test.

4.6 Assessment of Circulating Myeloid and Monocyte Subsets Before and After Neoadjuvant Chemotherapy in Breast Cancer Patients with Residual Disease (RD)

4.6.1 Evaluation of Total Monocytes and Subsets (Classical, Intermediate, Non-Classical) in RD Patients Before and After Neoadjuvant Chemotherapy

To investigate the effect of neoadjuvant chemotherapy on circulating monocytes and their subsets in patients with residual disease (RD), we compared their frequencies before and after treatment using the Wilcoxon matched-pairs signed-rank test.

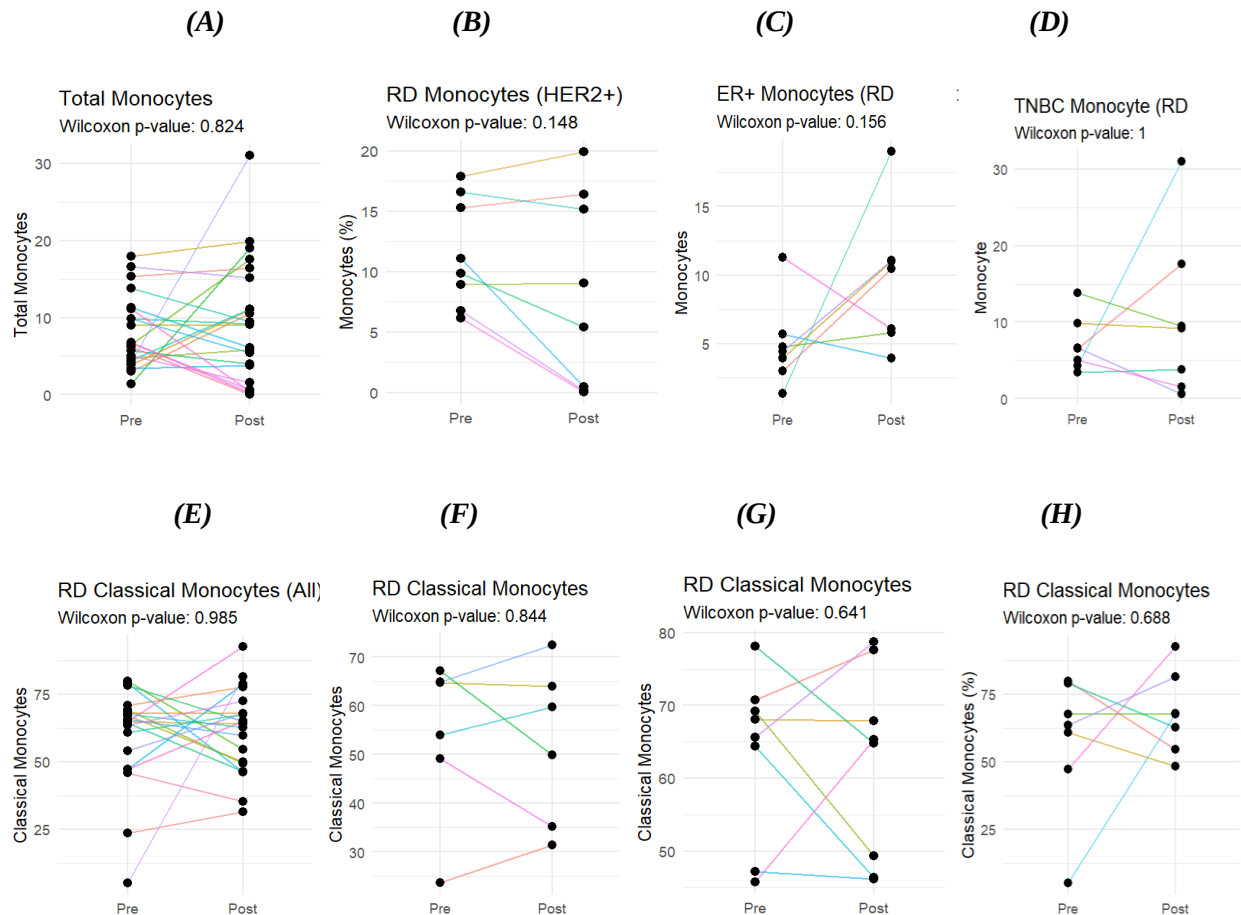
For **total monocytes**, no statistically significant differences were observed between pre- and post-treatment time points in the overall RD cohort ($n = 22$, $p = 0.824$). Similarly, no significant differences were found when stratified by breast cancer subtype: HER2^+ ($n = 8$, $p = 0.1484$), ER^+ ($n = 7$, $p = 0.1563$), and TNBC ($n = 7$, $p = 1$).

For **classical monocytes**, no significant change was detected in the total cohort ($n = 21$, $p = 0.9854$), or in the HER2^+ ($n = 6$, $p = 0.8438$), ER^+ ($n = 8$, $p = 0.6406$), and TNBC ($n = 7$, $p = 0.688$) subgroups.

For **intermediate monocytes**, no significant differences were observed in the total RD cohort ($n = 21$, $p = 0.4091$) or within subtypes (HER2⁺, $n = 6$, $p = 0.4375$; ER⁺, $n = 8$, $p = 0.383$; TNBC, $n = 7$, $p = 0.156$).

For **non-classical monocytes**, the total group also showed no significant modulation ($n = 21$, $p = 0.6742$). Subtype analyses yielded no significant changes for HER2⁺ ($n = 6$, $p = 0.0625$), ER⁺ ($n = 8$, $p = 0.383$), or TNBC ($n = 7$, $p = 0.937$).

Taken together, these results suggest that circulating total monocytes and their subsets were not significantly modulated by neoadjuvant chemotherapy in RD patients, regardless of molecular subtype.



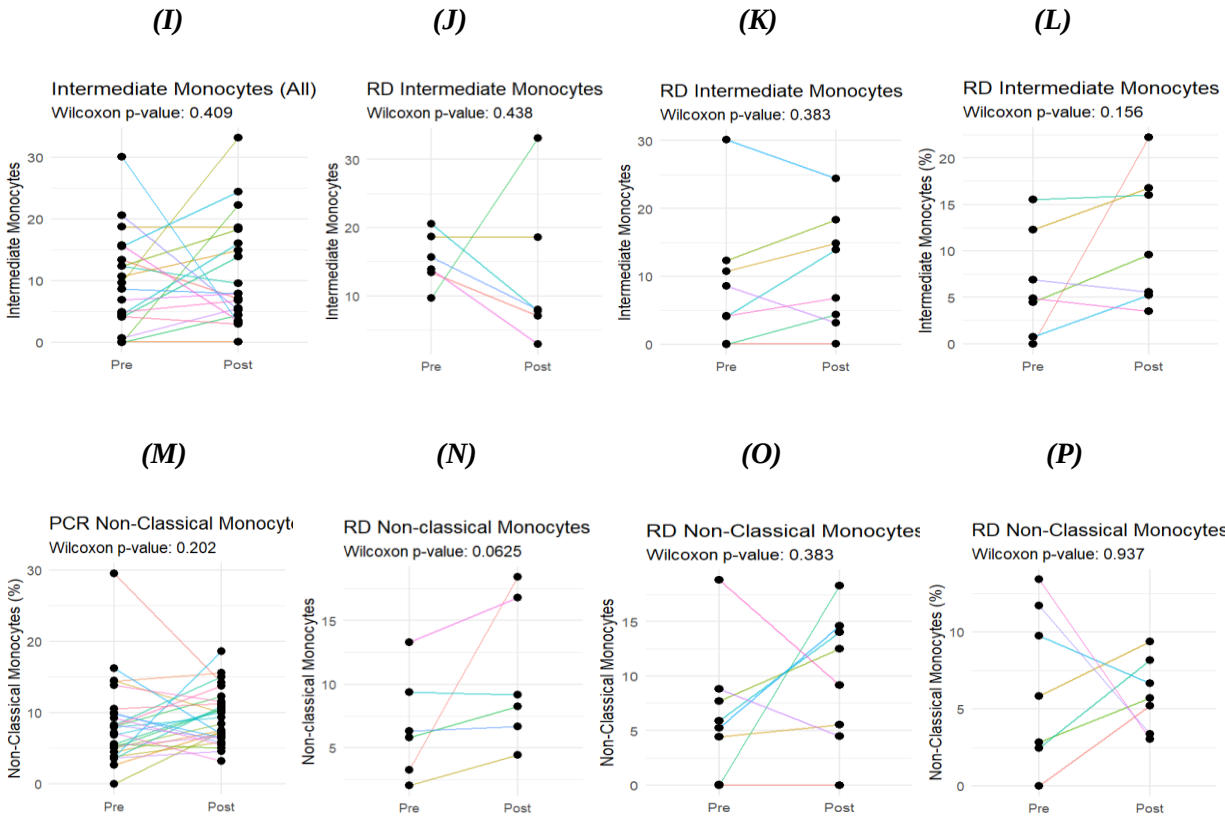


Figure 32: The plots show the relative frequencies of total monocytes and their subsets (classical, intermediate, and non-classical) in breast cancer patients with residual disease (RD) before (Pre) and after (Post) neoadjuvant chemotherapy. Panels A–D correspond to total monocytes, panels E–H to classical monocytes, panels I–L to intermediate monocytes, and panels M–P to non-classical monocytes. Statistical analysis was performed using the Wilcoxon matched-pairs signed-rank test. Data are presented as mean $\pm$ SD. A p value < 0.05 was considered statistically significant.

4.6.2. Dynamic Changes in Circulating M-MDSCs and PMN-MDSCs Before and After Neoadjuvant Chemotherapy in RD Patients

To assess the impact of neoadjuvant chemotherapy on circulating myeloid-derived suppressor cell populations in patients with residual disease (RD), the frequencies of M-MDSCs and PMN-MDSCs were analyzed before and after treatment using the Wilcoxon matched-pairs signed-rank test.

For **M-MDSCs**, no statistically significant change was observed between pre- and post-treatment in the total RD cohort ($n = 22$, $p = 0.9482$). When stratified by molecular subtype, no significant differences were observed for HER2^+ patients ($n = 8$, $p = 0.293$) or TNBC ($n = 7$, $p = 0.9375$), while ER^+ patients ($n = 7$) showed a significant increase in M-MDSCs after therapy ($p = 0.04688$).

For **PMN-MDSCs**, no significant differences were observed in the overall cohort ($n = 22$, $p = 0.156$), or when stratified by subtype (HER2^+ , $n = 8$, $p = 0.7422$; ER^+ , $n = 7$, $p = 0.2969$; TNBC, $n = 7$, $p = 0.2969$).

Taken together, these data indicate that M-MDSCs and PMN-MDSCs are largely stable following neoadjuvant therapy in RD patients, with the exception of a subgroup-specific increase in M-MDSCs among ER^+ patients.

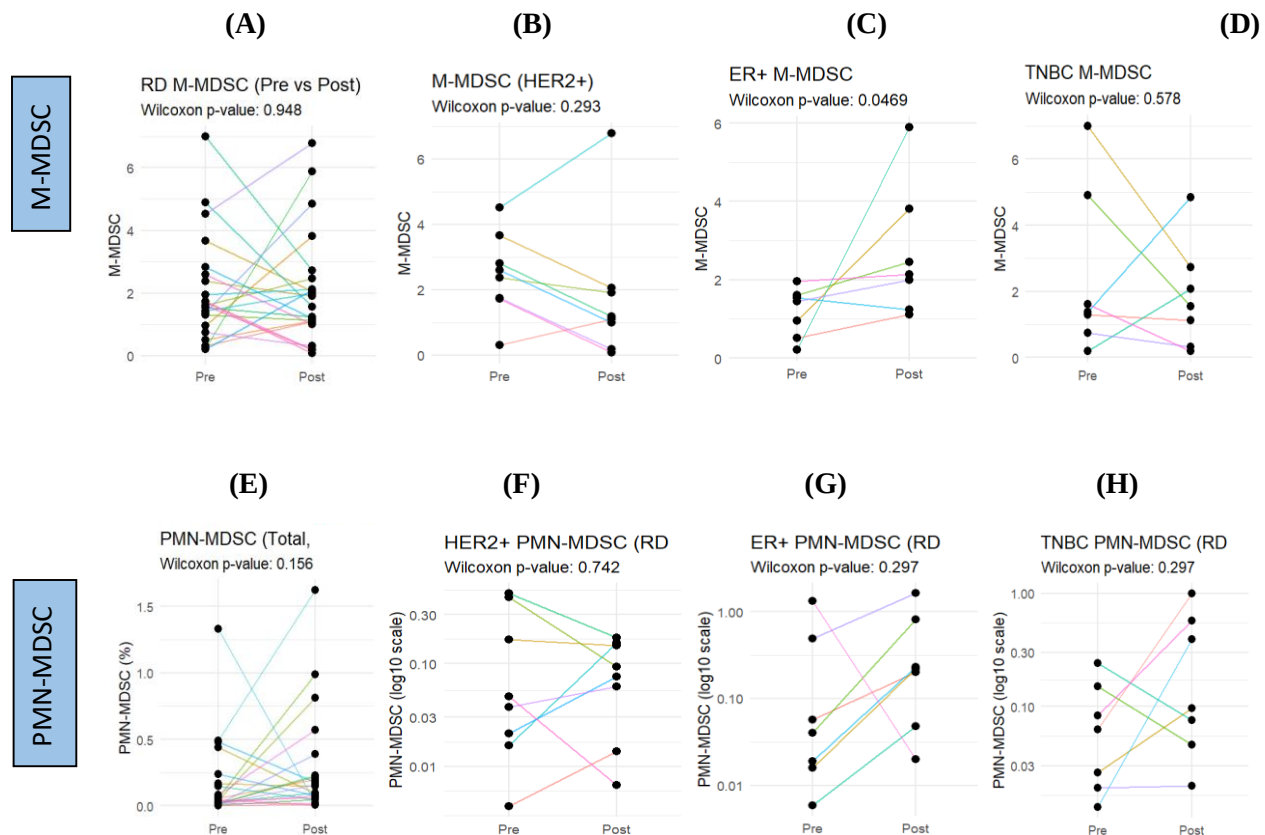


Figure 33: The plots show the relative frequencies of M-MDSCs and PMN-MDSCs in breast cancer patients with residual disease (RD) before (Pre) and after (Post) neoadjuvant chemotherapy. Panels A–D correspond to M-MDSCs: overall (A), HER2^+ (B), ER^+ (C), and TNBC (D).

Panels E–H correspond to PMN-MDSCs: overall (E), $HER2^+$ (F), ER^+ (G), and TNBC (H). Statistical analysis was performed using the Wilcoxon matched-pairs signed-rank test. Data are presented as mean $\pm$ SD. A p value < 0.05 was considered statistically significant.

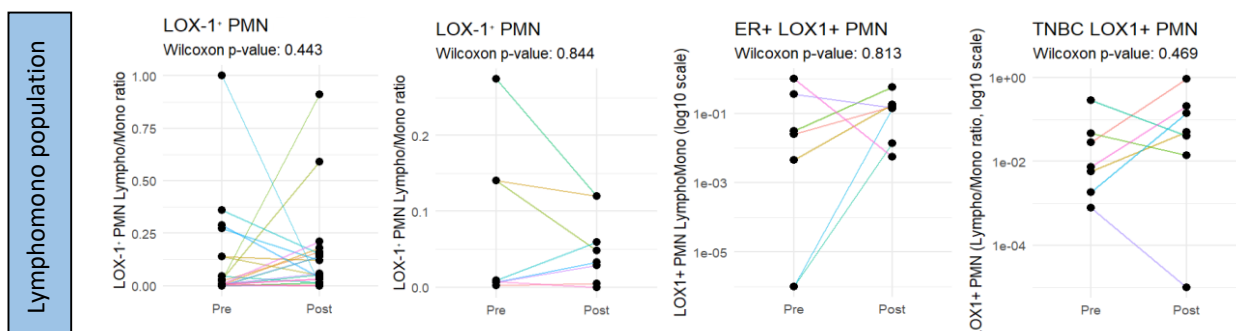
4.6.3 Dynamic Changes in LOX-1⁺ PMN-MDSCs in Lympho/Mono and PMN-MDSC Compartments in RD Patients

To investigate the impact of neoadjuvant chemotherapy on immunosuppressive neutrophil populations in patients with residual disease (RD), the frequency of LOX-1⁺ PMN-MDSCs was evaluated both within the lymphocyte/monocyte compartment and the PMN-MDSC gate before and after treatment.

Within the **lympho/mono population**, the frequency of LOX-1⁺ PMN-MDSCs remained largely unchanged after treatment in the overall RD cohort ($n = 22$, $p = 0.4434$). Consistent with this, no significant modulation was detected in any molecular subtype, including $HER2^+$ ($n = 8$, $p = 0.8434$), ER^+ ($n = 7$, $p = 0.8125$), or TNBC ($n = 7$, $p = 0.4688$) patients.

In contrast, analysis of the **PMN-MDSC population** revealed a significant post-treatment increase in LOX-1⁺ PMN-MDSC frequency in the overall RD cohort ($n = 22$, $p = 0.0296$). Although no significant differences were observed in $HER2^+$ ($n = 4$, $p = 0.375$) or ER^+ ($n = 7$, $p = 0.8125$) patients, a significant enrichment of LOX-1⁺ PMN-MDSCs was detected in TNBC patients ($n = 8$, $p = 0.01563$) following therapy.

These results indicate that LOX-1⁺ PMN-MDSCs are preferentially expanded after neoadjuvant treatment in TNBC patients with residual disease, reflecting a potential mechanism of persistent systemic immunosuppression in this specific cohort.



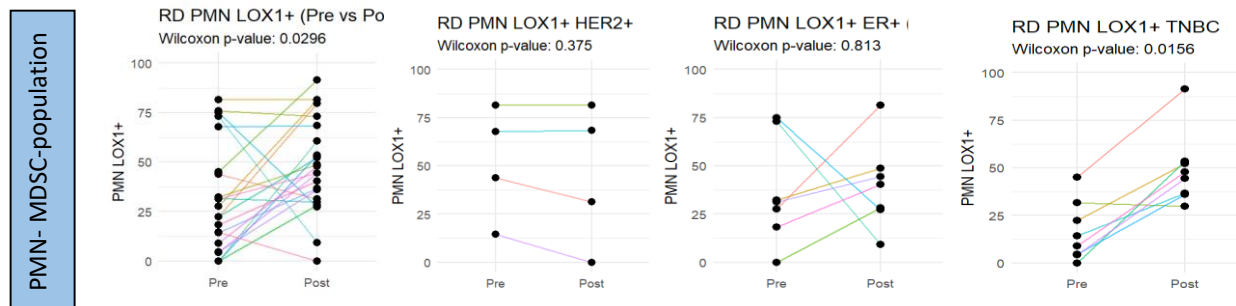


Figure 34: The plots show the relative frequencies of LOX-1⁺ PMN-MDSCs in breast cancer patients with residual disease (RD) before (Pre) and after (Post) neoadjuvant chemotherapy. Panels A–D correspond to LOX-1⁺ PMN-MDSCs measured in the lympho/mono population: total (A), HER2⁺ (B), ER⁺ (C), and TNBC (D). Panels E–H correspond to LOX-1⁺ PMN-MDSCs measured in the PMN-MDSC population: total (E), HER2⁺ (F), ER⁺ (G), and TNBC (H). Statistical analysis was performed using the Wilcoxon matched-pairs signed-rank test. Data are presented as mean $\pm$ SD. A p value < 0.05 was considered statistically significant.

4.7 PD-L1 Expression Profile and Dynamics of Circulating MDSC Subsets in Breast Cancer Patients Undergoing Neoadjuvant Treatment

4.7.1 PDL-1 expression on MDSCs subset at baseline

At baseline ($n = 18$), the mean fluorescence intensity (MFI) of PD-L1 was found to be higher on PMN-MDSCs compared to M-MDSCs. Although the difference did not reach statistical significance ($p = 0.23$), this trend suggests that PMN-MDSCs may express relatively higher levels of PD-L1 at diagnosis. This observation could reflect an early immunoregulatory activity of this subset within the tumor microenvironment, potentially contributing to immune escape mechanisms. While further studies in larger cohorts are needed to confirm this finding, the pattern observed here provides an indication of the differential immune checkpoint profile between MDSC subsets.

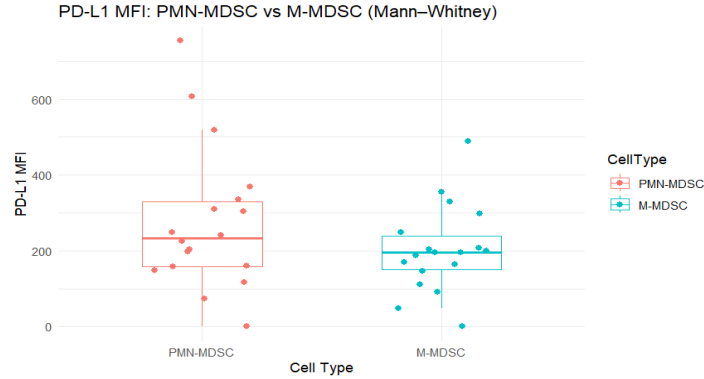


Figure 35: The plots show the PD-L1 mean fluorescence intensity (MFI) on PMN-MDSCs and M-MDSCs at baseline. No significant difference was observed between the two subsets (Mann–Whitney test, $p = 0.23$).

4.7.2 PD-L1 MFI on MDSC Subsets After Neoadjuvant Treatment

At the post-treatment time point ($n = 7$), the mean fluorescence intensity (MFI) of PD-L1 was assessed on PMN-MDSCs and M-MDSCs. The analysis showed no significant difference between the two subsets (Mann–Whitney test, $p = 0.52$). This indicates that, unlike the baseline measurement, PD-L1 expression levels remained comparable across both MDSC populations after therapy, suggesting no preferential upregulation of PD-L1 in either subset following treatment.

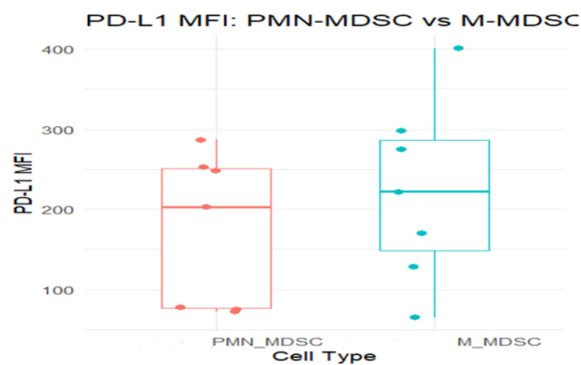


Figure 36: The plots show the PD-L1 mean fluorescence intensity (MFI) on PMN-MDSCs and M-MDSCs after neoadjuvant treatment. No significant difference was observed between the two subsets (Mann–Whitney test, $p = 0.52$).

4.7.3 PD-L1 MFI on MDSC Subsets Before and After Neoadjuvant Treatment

For the **M-MDSC subset**, PD-L1 mean fluorescence intensity (MFI) was compared between pre- and post-treatment time points. The analysis showed no significant difference in PD-L1 expression ($p = 0.933$, Wilcoxon signed-rank test, $n = 7$). This indicates that neoadjuvant therapy did not significantly alter PD-L1 expression levels on M-MDSCs.

For the **PMN-MDSC subset**, PD-L1 MFI was also assessed at pre- and post-treatment time points. Similar to M-MDSCs, no significant difference was observed between the two time points ($p = 0.27$, Wilcoxon signed-rank test, $n = 7$). Although the median MFI appeared lower after treatment, this change was not statistically significant.

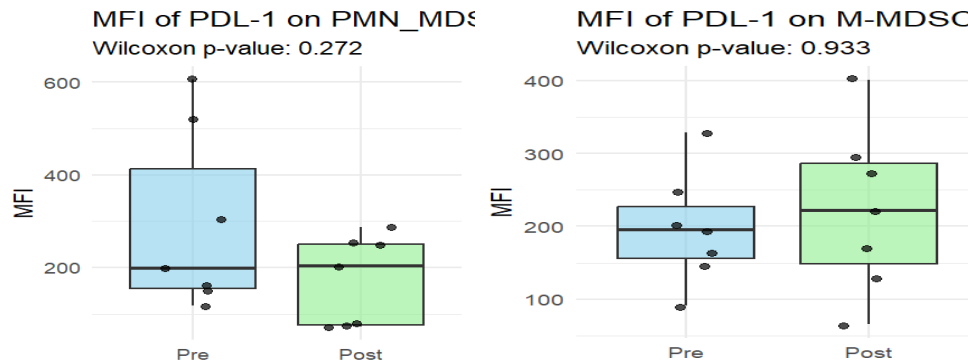


Figure 37: The plots show PD-L1 mean fluorescence intensity (MFI) on M-MDSCs and PMN-MDSCs before (Pre) and after (Post) neoadjuvant treatment ($n = 7$). No significant differences were observed between pre- and post-treatment time points for either subset (Wilcoxon signed-rank test, $p = 0.933$ for M-MDSCs; $p = 0.27$ for PMN-MDSCs).

4.7.4 PD-L1 frequency on M-MDSCs and PMN-MDSCs in Breast Cancer Patients

To evaluate the expression of PD-L1 on different MDSC subsets, we compared PD-L1 frequency on monocytic (M-MDSCs) and polymorphonuclear (PMN-MDSCs) myeloid-derived suppressor cells in breast cancer patients ($n = 19$). The analysis revealed a significantly higher frequency of PD-L1 on PMN-MDSCs compared to M-MDSCs ($p = 0.0169$), suggesting that PMN-MDSCs may contribute more strongly to PD-L1-mediated immunosuppressive mechanisms within the tumor microenvironment (Figure 32).

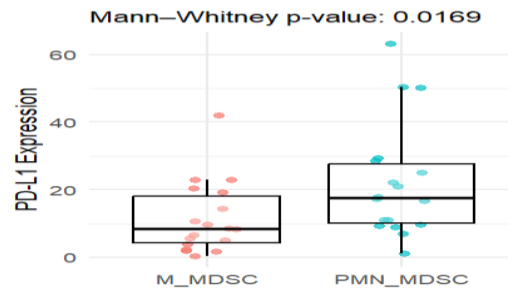


Figure 38. PD-L1 expression on M-MDSCs and PMN-MDSCs in breast cancer patients. The plot shows the relative expression levels of PD-L1 on circulating M-MDSCs and PMN-MDSCs ($n = 19$). Statistical analysis was performed using the Mann–Whitney U test. Data are presented as median with interquartile range. A significant increase in PD-L1 expression was observed on PMN-MDSCs compared with M-MDSCs ($p = 0.0169$).

4.7.5 PD-L1 Expression in M-MDSC and PMN-MDSC Subsets After Neoadjuvant Therapy

The expression of PD-L1 was evaluated in monocytic (M-MDSC) and polymorphonuclear (PMN-MDSC) myeloid-derived suppressor cell subsets in post-treatment samples. Statistical analysis was performed using the Mann–Whitney U test on paired patient data ($n = 7$). No significant difference was observed in PD-L1 expression between the two subsets ($p = 0.6093$). These results indicate that PD-L1 expression is distributed similarly across M-MDSCs and PMN-MDSCs in the post-treatment setting, suggesting a comparable contribution of both subsets to PD-L1-mediated immunoregulatory mechanisms.

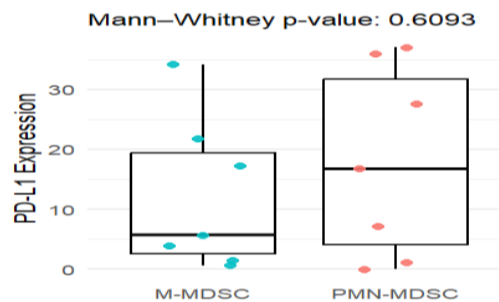


Figure 39: PD-L1 expression on M-MDSCs and PMN-MDSCs in breast cancer patients after neoadjuvant therapy. Statistical comparison between the two subsets was performed using the Mann–Whitney U test. Data are presented as median with interquartile range. No statistically significant difference in PD-L1 expression was observed between M-MDSCs and PMN-MDSCs ($p = 0.6093$).

5. Discussion

Early breast cancer refers to stages of breast cancer where the tumor is confined to the breast or nearby lymph nodes and has not metastasized to distant organs. Treatment for early breast cancer typically involves surgery, often combined with neoadjuvant treatment (NAT) such as chemotherapy or targeted therapies. Neoadjuvant treatment (NAT) is particularly beneficial in patients with locally advanced tumors or aggressive subtypes like HER2+ and triple-negative breast cancer (TNBC), as it can reduce tumors before surgery, making them operable and allowing for breast-conserving surgery. Additionally, NAT provides critical insights into the tumor's response to treatment, with a pathological complete response (pCR) often linked to better long-term outcomes like overall survival (OS) and disease-free survival (DFS). In this study, we comprehensively examined circulating myeloid and monocyte subpopulations in early breast cancer patients, focusing on their baseline levels, dynamic changes in response to NAT, and their correlation with pCR and RD. The goal was to delineate the immunological landscape that may influence therapeutic outcomes and uncover potential predictive biomarkers.

At baseline, the proportions of classical, intermediate, and non-classical monocytes did not differ significantly among TNBC, HER2+, and ER+ subtypes. Total monocyte levels were also not directly associated with response to NAT, indicating that their overall frequency alone may not be sufficient to predict therapeutic outcome.

Patients with triple-negative disease who achieved a pathological complete response (pCR) displayed higher baseline frequencies of intermediate monocytes compared with those with residual disease. Interestingly, this trend contrasts with previous reports associating elevated intermediate monocytes with poorer prognosis [51]. A plausible explanation for this divergence lies in the unique immune microenvironment of TNBC. Owing to its highly inflammatory and immunogenic nature, TNBC may convert intermediate monocytes from a typically tumor-supporting phenotype into one that amplifies antitumor responses. This context-dependent plasticity highlights how the same immune subset can exert different functional effects depending on the tumor microenvironment.

Logistic regression analysis further revealed a significant association between non-classical monocytes and pCR, supporting earlier observations that these cells can serve as independent predictors of treatment outcome [52]. Non-classical monocytes are characterized by their patrolling behavior and immune-activating functions, which may promote enhanced immune surveillance and cytotoxic activity during neoadjuvant therapy. In line with the findings of Hao et al, early-stage breast cancer can induce functional reprogramming of monocyte subsets, with non-classical monocytes displaying a transcriptional profile enriched for genes involved in antigen presentation, cytokine production, and interferon signaling. This suggests that these cells may act as early immune sensors, bridging innate and adaptive responses and contributing to more effective tumor control.

Analysis of the immunosuppressive compartment showed a clear predominance of monocytic MDSCs over PMN-MDSCs across breast cancer subtypes and tumor grades, particularly in G2 and G3 tumors. This is consistent with previous findings, which demonstrated early enrichment of Mo-MDSCs in breast cancer patients and their further increase with disease progression [53]. The predominance of M-MDSCs likely reflects early tumor-driven reprogramming of circulating monocytes toward an immunosuppressive phenotype. This pattern likely reflects the fact that M-MDSCs can expand rapidly in response to chronic inflammatory and tumor-derived signals, such as IL-6 and GM-CSF, which are commonly elevated in more advanced tumors. However, despite this numerical predominance, baseline M-MDSC levels were not associated with pCR, indicating that their clinical impact may be more dependent on therapy-induced modulation rather than static pre-treatment abundance.

Following neoadjuvant therapy, in TNBC patients, a marked post-treatment expansion of LOX-1⁺ PMN-MDSCs was observed, particularly in those with residual disease. This pattern reflects an adaptive immunosuppressive response, consistent with the established role of LOX-1⁺ PMN-MDSCs in suppressing T and NK cell activity [56]. In contrast, HER2⁺ patients exhibited a significant decline in LOX-1⁺ PMN-MDSCs, especially among responders, suggesting a therapy-driven alleviation of myeloid suppression and the establishment of a more permissive immune

environment. These opposite trajectories in TNBC and HER2⁺ disease underscore the context-dependent nature of immune adaptation during treatment.

In ER⁺ disease, overall M-MDSC frequencies remained stable; however, patients with residual disease exhibited a clear post-treatment increase. This pattern may reflect the effect of early tumor-driven monocyte reprogramming, as previously described in early-stage breast cancer [52], which could create a myeloid environment more prone to immunosuppressive expansion after therapy. Consistent with previous studies linking elevated M-MDSC levels to poor prognosis [55–56], our findings indicate that this increase is particularly relevant in non-responders, highlighting its potential contribution to therapy resistance in this subgroup.

Baseline analysis also revealed that HER2⁺ patients had significantly higher frequencies of LOX-1⁺ PMN-MDSCs compared with TNBC. Given that LOX-1 is a well-established marker of functionally suppressive PMN-MDSCs [56], this finding suggests that HER2⁺ tumors may possess a more immunosuppressive myeloid profile from the outset, potentially influencing therapeutic responses.

Evaluation of PD-L1 expression across MDSC subsets showed similar mean fluorescence intensity between PMN-MDSCs and M-MDSCs; however, a significantly higher proportion of PMN-MDSCs expressed PD-L1. This aligns with previous reports demonstrating preferential PD-L1 expression on PMN-MDSCs [57], as well as studies in breast cancer linking this expression to T and NK cell suppression [58]. Such checkpoint-mediated inhibition may synergize with other suppressive mechanisms, further contributing to therapy resistance.

In conclusion, our results show that circulating myeloid populations undergo distinct subtype-specific changes during neoadjuvant therapy in breast cancer. Intermediate and non-classical monocytes are linked to better treatment response, whereas therapy resistance is associated with the expansion of suppressive MDSC subsets—particularly LOX-1⁺ PMN-MDSCs in TNBC and M-MDSCs in ER⁺ disease. The higher PD-L1 expression on PMN-MDSCs further highlights their central role in immune escape. These findings support the potential use of myeloid signatures as predictive biomarkers and therapeutic targets to enhance treatment efficacy.

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