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Low-Density Neutrophils dynamics as a potential biomarker for immunotherapy response in Breast Cancer patients: A Case Report

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Case Report

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Abstract

Triple-negative breast cancer (TNBC) is an aggressive subtype with limited treatment options. Although immunotherapy has improved outcomes in selected patients, predictive biomarkers remain scarce. Low-density neutrophils (LDNs), a subpopulation of immunosuppressive neutrophils, have been implicated in resistance to immune checkpoint inhibitors (ICIs), but their longitudinal dynamics and correlation with clinical parameters and immune markers have not been investigated to date, especially in TNBC.

We report a case of a 48-year-old woman with early-stage TNBC treated with neoadjuvant chemoimmunotherapy per the KEYNOTE-522 protocol. Circulating LDNs, following density gradient centrifugation, as well as activation status of systemic T lymphocytes, were monitored longitudinally by flow cytometry. Parallel assessments included lactate dehydrogenase (LDH), carcinoembryonic antigen (CEA), cancer antigen 15.3 (CA 15.3), platelet-to-lymphocyte ratio (PLR), neutrophil-to-lymphocyte ratio (NLR). Tumor-infiltrating lymphocytes (TILs) were characterized by immunohistochemistry, in the surgical specimen.

LDNs were absent at baseline but increased during neoadjuvant therapy, peaking before radiologic progression and remaining elevated during palliative treatment. Notably, systemic T lymphocytes markers, such as CD69 and HLA-DR, declined as LDNs rose. This trajectory preceded disease evolution while conventional markers, such as LDH, CA 15.3, PLR and NLR rose later. The patient progressed during adjuvant immunotherapy and partially responded to first-line antibody-drug conjugate therapy. In line with the systemic immunosuppression profile, and resistance to immunotherapy, TIL analysis revealed low CD8 + infiltration and sparse CD20 + B cells.

This case illustrates the potential of LDNs as minimally invasive biomarkers for monitoring treatment response and detecting therapy resistance in breast cancer (BC). Their longitudinal profile captured disease progression more precisely than conventional markers, underscoring their translational relevance for patient stratification and therapeutic guidance.

Although limited by the single-patient design, these findings warrant investigation of LDNs dynamics in larger cohorts to validate their predictive value and clinical utility in optimizing immunotherapy outcomes in BC.

Introduction

Breast cancer (BC) remains the most common malignancy among women worldwide, accounting for over 2.3 million new diagnoses and nearly 700,000 deaths annually [1]. Although the majority of cases occur in postmenopausal women, approximately 7–10% are diagnosed before the age of 40, a population in which the disease often exhibits more aggressive biological behavior and poorer clinical outcomes [2].

In addition to histological classification, BC is subdivided into molecular subtypes based on hormone receptor and human epidermal growth factor receptor 2 (HER2) expression. These include hormone receptor-positive (estrogen and/or progesterone receptor-positive), HER2-positive, and triple-negative breast cancer (TNBC). Each subtype exhibits distinct biological behavior, therapeutic targets, and clinical outcomes. Among them, TNBC, defined by the absence of estrogen receptor (ER), progesterone receptor (PR), and HER2 expression, represents 10–15% of all BCs [3]. TNBC disproportionately affects younger women and is associated with higher histological grade, early recurrence, and limited therapeutic options compared with other subtypes. Despite recent advances, TNBC continues to pose major clinical challenges, particularly in patients with residual disease following neoadjuvant therapy [4].

Immunotherapy, particularly immune checkpoint inhibitors (ICIs) targeting the PD-1/PD-L1 axis, and, more recently, antibody-drug conjugates (ADCs), have transformed the therapeutic landscape for TNBC. Clinical trials have demonstrated improved outcomes in subsets of patients receiving these agents, either as monotherapy or in combination with chemotherapy [5, 6]. However, durable responses remain limited to a fraction of patients, highlighting the urgent need for predictive biomarkers that can identify those most likely to benefit. As immunotherapy efficacy depends on the complex interplay between tumor cells and the host immune system, immune-related biomarkers have gained particular interest. In this context, both tumor-intrinsic and systemic immune features are being explored as potential predictors of therapeutic response [7, 8].

Beyond classical clinicopathological parameters, increasing attention has been directed toward the tumor microenvironment (TME) and systemic immune alterations as key modulators of response to therapy. Tumor-infiltrating lymphocytes (TILs), for example, have been correlated with a higher probability of achieving pathological complete response (pCR) after neoadjuvant chemotherapy in patients with BC [9, 10]. At the same time, myeloid-derived populations, particularly neutrophils, are emerging as important regulators of cancer progression and therapeutic resistance.

Tumor-associated neutrophils (TANs) display remarkable plasticity and can be broadly classified into two functional phenotypes: N1 neutrophils, which exert pro-inflammatory and anti-tumor functions, including the activation of cytotoxic T lymphocytes; and N2 neutrophils, which promote tumor growth, angiogenesis, and immune suppression. The tumor milieu, particularly transforming growth factor-β (TGF-β), drives neutrophil polarization toward the N2 phenotype [11, 12].

Analogous to TAN heterogeneity, circulating neutrophils can be subdivided into high-density neutrophils (HDNs) and low-density neutrophils (LDNs), which are distinguishable by density gradient separation. These subpopulations mirror the phenotypes of N1 and N2 neutrophils, respectively. LDNs are typically absent in healthy individuals and emerge under pathological or inflammatory conditions. Comprising both mature and immature neutrophils, LDNs exhibit altered functionalities and pronounced immunosuppressive properties. Recent evidence, mostly from preclinical and translational studies, including our findings [13], has implicated LDNs in tumor progression, metastatic spread, and modulation of anti-tumor immunity. In cancer patients, elevated circulating LDN levels have also been

associated with reduced efficacy of ICIs, suggesting their potential role as negative regulators of immunotherapy response [14].

Monitoring the dynamics of circulating LDNs during treatment may provide valuable insight into the evolving immune landscape of cancer and serve as an early indicator of therapeutic efficacy or emerging resistance. However, longitudinal data describing LDNs variations in patients receiving immunotherapy are still lacking. Considering that systemic immune regulation critically governs tumor progression and therapeutic response, elucidating these dynamics could advance precision immune-oncology strategies.

Here, we present, for the first time to our knowledge, a case of a 48-year-old woman with TNBC in whom circulating LDNs were longitudinally monitored throughout systemic therapy, including ICIs.

It was observed that LDNs were initially undetectable but increased during neoadjuvant therapy, peaking prior to radiologic progression and remaining elevated throughout palliative treatment. This rise was accompanied by a decline in systemic T-cell activation and preceded changes in conventional indicators such as LDH, CA15-3, PLR, and NLR. Clinically, the patient progressed on adjuvant immunotherapy but showed a partial response to first-line ADC therapy. Consistent with blood immunosuppression and immunotherapy resistance, low $CD8^+$ T-cell and $CD20^+$ B cells were infiltrating the tumor tissue.

This report offers translational insight into the relationship between LDN dynamics and treatment response in TNBC and highlights the potential of LDN monitoring as a minimally invasive biomarker to support patient stratification, guide therapeutic adjustments, and ultimately optimize immunotherapy outcomes.

Case Report

A 48-year-old perimenopausal nulliparous woman (G0P0A2) was referred in September 2023 for evaluation of a palpable mass in the left breast. Her past medical and surgical history was unremarkable. Family history was notable for multiple malignancies: on her paternal side, her grandmother had uterine cancer at age 60 and her aunt had BC at 49; on her maternal side, one uncle had prostate cancer at 50, another uncle had pancreatic cancer at 50, and an aunt had BC at 80.

Breast ultrasound and core needle biopsy revealed a 21 × 13 mm solid mass in the upper outer quadrant of the left breast, with no suspicious axillary lymph nodes. Histopathological examination confirmed invasive breast carcinoma of no special type (NST), grade 2, triple-negative (ER-negative, PR-negative, and HER2-negative by immunohistochemistry, score 0), with a Ki-67 proliferation index of 15%. Breast magnetic resonance imaging (MRI) demonstrated a 30 × 22 mm lesion with subcutaneous tissue infiltration and a 6 mm satellite nodule. Staging with bone scan and computed tomography of the chest, abdomen, and pelvis (CT CAP) showed no evidence of distant metastasis, consistent with clinical stage T2N0M0 TNBC. Multigene next-generation sequencing did not identify any pathogenic germline mutations.

Based on the patient's clinical and pathological findings, the multidisciplinary team (MDT) recommended neoadjuvant systemic therapy guided by the KEYNOTE-522 trial protocol [15]. The initial phase consisted of four cycles of intravenous pembrolizumab (200 mg every 3 weeks) combined with weekly carboplatin (dosed based on an area under the concentration-time curve of 1.5 mg/mL per minute), and weekly paclitaxel (80 mg/m^2). These treatments were administered over a total duration of 12 weeks. This was followed by four cycles of intravenous pembrolizumab (200 mg) with doxorubicin (60 mg/m^2) and cyclophosphamide (600 mg/m^2), administered every 3 weeks. The treatment took place from December 2023 to June 2024. Reported toxicities included a grade 2 carboplatin infusion reaction (cycle 4, day 8), grade 2 hypothyroidism requiring levothyroxine replacement, and grade 2 alopecia. Post-treatment MRI demonstrated a partial but incomplete radiologic response.

In August 2024, the patient underwent breast-conserving surgery with sentinel lymph node biopsy. Pathological examination revealed residual invasive carcinoma, staged as ypT2N0, indicating lack of response to neoadjuvant chemoimmunotherapy. Adjuvant three-dimensional conformal radiotherapy (3D-CRT) was delivered to the left breast (40.05 Gy in 15 fractions, followed by a tumor bed boost of 13.35 Gy in 5 fractions). The patient subsequently initiated adjuvant therapy with capecitabine (1250 mg orally, twice daily) combined with pembrolizumab (200 mg, intravenously) in November 2024.

In January 2025, during adjuvant treatment, serial blood analyses showed gradual increases in lactate dehydrogenase (LDH) and tumor markers (carcinoembryonic antigen [CEA] and cancer antigen 15.3 [CA 15.3]). A restaging CT CAP scan revealed new mediastinal lymphadenopathy and lung metastases, confirming disease progression. In February 2025, first-line therapy with sacituzumab govitecan (at a dose of 10 mg/m^2, intravenously), an ADC, was initiated, resulting in a partial response on follow-up imaging. Reported toxicities included grade 2 fatigue and grade 2 alopecia.

Systemic LDN profiling and assessment T lymphocytes activation status were performed longitudinally throughout treatment, using flow cytometry. In parallel, serial peripheral blood analyses were conducted to monitor laboratory parameters, including CEA, CA 15.3, LDH, general neutrophils and lymphocytes, total leukocytes and platelets. From these measurements, neutrophil-to-lymphocyte ratio (NLR) and platelet-to-lymphocyte ratio (PLR) were calculated over time. Additionally, TILs were characterized in the surgical specimen using immunohistochemistry for CD3 (pan-T cells), CD4 (helper T cells), CD8 (cytotoxic T cells), and CD20 (B cells).

As of September 2025, the last assessment before this submission, the patient remains on sacituzumab govitecan therapy, with continued longitudinal monitoring of LDNs and other laboratory parameters. The most recent CT CAP scan confirmed stable disease.

Methods

1. Patient Samples

For this case study, blood samples were obtained from the BC patient at Hospital Prof. Doutor Fernando Fonseca (HFF). Approximately 9 mL of whole blood were collected into Vacutainer tubes containing EDTA (BD Biosciences). The first collection was performed before treatment initiation, followed by subsequent collections throughout the course of therapy. Blood samples were processed at NOVA Medical School, within 24 hours of collection.

In addition, hematologic and biochemical parameters were assessed in whole blood collected as part of routine clinical monitoring. These analyses, including LDH, CEA, CA 15.3, and hematologic indices such as PLR and NLR, were performed at the laboratory of HFF using standard clinical procedures.

Formalin-fixed, paraffin-embedded tumor tissue sections from the surgical specimen were also obtained for analysis of TILs. Sections of 3µm thickness were mounted on X-tra^{TM} adhesive slides and processed for immunohistochemistry at the Anatomical Pathology department, HFF.

This study was approved by the Ethics Committees of HFF and NOVA Medical School. The participant was recruited voluntarily and provided written informed consent before sample and data collection. Blood and tissue collection were conducted as part of routine clinical procedures and did not interfere with patient diagnosis or treatment.

Sample processing and analyses were performed in accordance with the principles of the Declaration of Helsinki. The study complied with Portuguese national legislation regulating the scientific use of human biological samples (Law No. 12/2005 and Decree-Law No. 131/2014). Clinical data were managed in compliance with the General Data Protection Regulation (GDPR; Regulation [EU] 2016/679) and Portuguese Law No. 58/2019, which implements GDPR nationally. Patient data were anonymized and securely stored according to these regulations.

2. Neutrophil Isolation

To isolate neutrophils, peripheral blood mononuclear cells (PBMCs), and plasma, density gradient centrifugation was performed. Whole blood was layered on top of equal volumes of Histopaque-1119 and Histopaque-1077 (Sigma-Aldrich) mixed in a 1:1 ratio and centrifuged at 2000 rpm for 20 min without brake. After centrifugation, distinct layers were obtained according to density: plasma, PBMCs (comprising lymphocytes, monocytes, and, in some cases, LDNs), Histopaque solution, granulocytes (containing HDNs), and red blood cells (Figure 1).

Plasma was collected and stored at −80°C. The PBMC and granulocyte fractions were carefully recovered with a Pasteur pipette, transferred to 15 mL Falcon tubes, and aliquoted for subsequent flow cytometry characterization.

3. Immunophenotyping by Flow Cytometry

Immunophenotyping was performed on PBMCs and granulocyte fractions to evaluate LDNs, HDNs, and T lymphocyte subsets. A panel of fluorescently conjugated mouse anti-human monoclonal antibodies

(mAbs) targeting cell surface markers was used. For each sample, 100 µL of cell suspension were incubated with 1 µL of each selected antibody for 15 min at RT in the dark.

Staining was followed by red blood cell lysis using 2 mL RBC lysis buffer (BioLegend) for 20 min at 4°C. All samples were then washed with 1 mL phosphate-buffered saline (PBS) and centrifuged at 300 × g for 5 min. At the end of staining, cells were fixed in 200 µL FlowFix (Polysciences) and resuspended in 200 µL PBS for acquisition.

Samples were acquired on a BD FACS Canto II flow cytometer using FACSDiva software v8.0.1 (BD Biosciences). Data analysis was performed with FlowJo v10. Compensation was applied using single-stained controls for each fluorophore to correct for spectral overlap, and unstained controls were included to define negative populations.

3.1. Gating Strategy and Data Analysis

The following monoclonal antibodies were used for surface marker staining: anti-CD4-FITC (clone OKT4), anti-CD8-PE (clone HIT8a), anti-CD15-PE (clone HI98), anti-CD69-PerCP (clone FN50), and anti-HLA-DR (clone L243), all from BioLegend.

Immune cell populations were defined based on the following gating strategy: neutrophils were identified as $CD15^+$ cells, helper T lymphocytes as CD4+ cells, and cytotoxic T lymphocytes as $CD8^+$ cells. For each population, the frequency of positive cells was determined. Lymphocyte activation markers, CD69 and HLA-DR, were analyzed as the percentage of positive cells within the $CD4^+$ and $CD8^+$ subsets.

4. Hematologic and Biochemical Parameters

Blood biomarkers, including LDH, CEA, CA 15.3, as well as hematologic indices such as PLR and NLR, were obtained from routine blood samples collected during patient monitoring. Blood samples were collected in EDTA-containing Vacutainer tubes (BD Biosciences) for neutrophil, lymphocyte, and platelet counts, and in dry vacuum tubes (BD Biosciences) for serum-based analyses of LDH, CEA, and CA 15.3, as part of standard clinical procedures. PLR and NLR were calculated from complete blood counts obtained in routine laboratory analyses, using the formulas: PLR=platelets/lymphocytes and NLR=neutrophils/lymphocytes. These parameters were measured as part of standard hospital laboratory assessments, without any intervention from the study protocol.

5. Immunohistochemistry for TILs

TILs were evaluated in formalin-fixed, paraffin-embedded tumor tissue sections obtained from surgical tumor specimens. Sections of 3µm thickness were mounted on X-tra™ adhesive slides. Immunohistochemistry was performed using the automated Leica BOND-Prime system following standardized hospital protocols. Tissue sections were stained with antibodies against CD3 (T cells, clone LN10 from Leica), CD4 (helper T cells, clone EP204 from Vitro Master Diagnóstics), CD8 (cytotoxic T cells, clone SP16 from NeoMarkers), and CD20 (B cells, clone L26 from Dako), with tonsil tissue

serving as a positive control. Following staining, slides were examined under a light microscope (Olympus), and TIL density was assessed qualitatively by visual inspection, considering both intratumoral and stromal regions. The automated Leica BOND-Prime system ensured standardized deparaffinization, antigen retrieval, and staining across all slides.

Results

Systemic immune monitoring was performed over 22 months to evaluate the relationship between circulating biomarkers and disease evolution. As shown in Figure 2, the classical clinical biomarkers used in BC follow-up - CA 15.3, CEA, LDH, NLR, and PLR - exhibited marked fluctuations during the initial treatment phase. Although some transient decreases were observed following therapy initiation (four cycles of pembrolizumab combined with carboplatin and paclitaxel, followed by four cycles of pembrolizumab with doxorubicin and cyclophosphamide), these markers remained above the normal range and increased steadily after approximately 14 months, coinciding with the clinical detection of metastatic disease.

In contrast, the frequency of LDNs within the PBMC fraction showed a distinct trajectory. LDNs progressively increased during therapy, reaching their peak shortly before the clinical confirmation of metastasis, and remained elevated during subsequent palliative treatment, even when the disease appeared clinically stable. This trend paralleled disease evolution more closely than the conventional serum markers, supporting the potential of LDNs frequency as a dynamic indicator of therapeutic response.

Consistent with our previous observations showing that LDNs can suppress T cell activation [13], the expression of activation markers (CD69 and HLA-DR) on circulating $CD8^+$ T cells showed an early transient increase during neoadjuvant immunotherapy, followed by a sustained decline coinciding with the rise in LDNs frequency. Both activation markers on $CD8^+$ T cells remained low during disease progression, suggesting persistent impairment of cytotoxic T cell activation, likely associated with elevated circulating LDNs (Figure 2). Moreover, circulating $CD4^+$ T cells exhibited generally low levels of activation markers throughout monitoring, with HLA-DR and CD69 both remaining below 10% for most of the period (data not shown). HDN frequency was also assessed. As anticipated, it showed a slight decrease with higher LDN levels, however, this change was not significant over the follow-up period and is therefore not shown.

To complement the systemic analysis, immunophenotyping of TILs was performed on the surgical specimen. Staining for $CD3^+$, $CD4^+$, $CD8^+$, and $CD20^+$ cells revealed an overall scarce immune infiltrate, with $CD4^+$ T cells being relatively more frequent than other subsets, although still present at low levels. $CD8^+$ and $CD3^+$ T cells were even less abundant, and B cells ($CD20^+$) were rarely detected (Figure 3). The overall low infiltration of T cells within the tumor microenvironment, together with the persistently reduced activation of circulating $CD8^+$ (and low-activation $CD4^+$) T cells, supports an

immunosuppressive milieu consistent with elevated levels of LDNs and reflective of an unfavorable therapeutic response.

Collectively, these findings link elevated circulating LDNs with impaired T cell activation and a weak intratumoral immune infiltrate, supporting their role in systemic and local immune dysfunction and their potential as an early biomarker of poor therapeutic response in BC.

Discussion

This case provides real-world evidence of the clinical challenges associated with TNBC, a subtype characterized by poor prognosis and intrinsic resistance to therapy [16]. Despite neoadjuvant chemoimmunotherapy followed by adjuvant treatment, the patient experienced early relapse and metastatic progression, underscoring the aggressive nature of TNBC and the limitations of current therapeutic strategies. pCR is a well-established surrogate marker of long-term survival in TNBC, particularly among patients receiving neoadjuvant immunotherapy [16]. In this patient, the absence of pCR (ypT2N0), despite a partial radiologic response, reflected suboptimal treatment efficacy and correlated with early disease recurrence, highlighting the persistent challenge of TNBC resistance.

Emerging evidence suggests that myeloid-derived cells, particularly LDNs, play a critical role in shaping systemic immune responses and influencing therapy outcomes [13, 17]. LDNs are immunosuppressive subsets typically absent or present at low frequencies in healthy individuals [13], emphasizing that their elevation reflects a disease-associated immunosuppressive state rather than normal physiological variation. While previous studies linked LDN prevalence to worse prognosis in breast and lung cancer [13, 18, 19], our longitudinal data not only reinforces this, but also provide compelling evidence that routine LDN assessment could support real-time disease monitoring. Tracking LDNs may complement existing biomarkers, aiding in early detection of progression and timely treatment adaptations in high-risk patients.

The concept of using circulating immune subsets as dynamic biomarkers is increasingly supported by data from other malignancies [7, 20, 21]. In non-small-cell lung cancer (NSCLC), immunophenotyping studies revealed that elevated monocytes with low HLA-DR expression correlate with poor survival [20]. These findings support the concept that specific myeloid subsets can serve as dynamic biomarkers, with our data highlighting LDNs as particularly informative in TNBC. Similarly, multicellular analyses of the “cellular immunome” in blood and tumor samples from cancer patients, including BC, demonstrated that peripheral immune cell subsets change dynamically during therapy, reinforcing the idea that longitudinal monitoring of immune cells can provide actionable insights into treatment response [21].

Notably, longitudinal monitoring in this patient revealed an early and sustained rise in LDNs during neoadjuvant therapy with immunotherapy in addition to chemotherapy, peaking prior to radiologic progression and remaining elevated throughout palliative treatment. This trajectory paralleled clinical deterioration more closely than conventional biomarkers such as CEA and CA 15.3, which have limited sensitivity for minimal residual disease [22]. Similarly, systemic inflammatory indices like NLR and PLR

demonstrated modest predictive value [23, 24], highlighting their limited utility as standalone monitoring tools. Importantly, the rise in LDNs coincided with a decline in T cell activation markers (CD69, HLA-DR), supporting the hypothesis that LDNs contribute to systemic immunosuppression by impairing T cell [25]. This observation aligns with preclinical evidence demonstrating LDN-mediated inhibition of T cell proliferation and cytokine production, which may facilitate tumor immune evasion [13].

Notably, although anti-PD-1 therapy is intended to enhance T cell activation, in this patient, we observed that the initial transient increase in activation markers at the start of the treatment was not sustained. Instead, T cell activation declined and remained low throughout therapy, mirroring the rise and persistence of elevated LDN frequencies. This pattern suggests that increasing LDN levels may attenuate the expected immunostimulatory effect of checkpoint blockade and contribute to therapeutic resistance. This observation is also supported by other studies reporting that elevated LDNs or immunosuppressive neutrophils/myeloid-derived suppressor cells were associated with primary resistance to PD-1/PD-L1 blockade and with suppression or exhaustion of T cell responses [14, 26, 27].

LDNs possess both pro-tumorigenic and immunosuppressive properties, and their increased prevalence in metastatic disease underscores their potential as biomarkers and therapeutic targets [13]. Nonetheless, LDNs represent a heterogeneous population, and not all subsets contribute equally to cancer progression. In line with this, distinct neutrophil subpopulations have been associated with tumor progression and immunosuppression, although standardized markers for their identification are still scarce [28]. This complexity highlights the limitations of current knowledge and the need for precise markers to distinguish pathological LDN subsets from homeostatic neutrophils, enabling selective modulation of pro-tumor populations while preserving protective immunity.

Moreover, our analysis of the surgical specimen revealed low TILs infiltration. The paucity of intratumoral lymphocytes, particularly of cytotoxic $CD8^+$ T cells, aligns with previous reports describing “immune-desert” or “cold” tumor phenotypes, which are often associated with poor T-cell recruitment and ineffective antitumor immunity [29, 30]. Low baseline TILs have indeed been associated with poor response to immunotherapy, although functional status and activation markers are increasingly recognized as more informative than absolute counts [31, 32]. In this case, the inverse relationship between increasing LDNs and declining TILs infiltration suggests that systemic immunosuppression may also compromise local immune responses [33, 34].

In line with this interpretation, Maynard *et al*. (2025) emphasized the need for novel biomarkers beyond PD-L1 and TILs to better predict immunotherapy response in BC [35], highlighting the potential of integrating systemic immune markers into clinical practice. Our findings align with this perspective, suggesting that LDN dynamics, may serve as promising candidates for real-time monitoring of immune status and therapeutic response. Validating LDN kinetics in larger, independent patient cohorts will be critical to assess robustness, clinical applicability, and integration into precision medicine strategies. Ultimately, understanding neutrophil heterogeneity may not only improve patient stratification but also

lay the groundwork for personalized interventions aimed at enhancing the efficacy of current BC therapies.

Although limited by its single-patient nature, this case provides preliminary yet compelling evidence that dynamic monitoring of LDNs may outperform conventional biomarkers in predicting immunotherapy response. These findings underscore the broader relevance of the myeloid-driven immunosuppressive cells, which likely contributes to resistance to ICIs in TNBC. Although LDN, have been shown to impair T-cell proliferation and effector function through multiple mechanisms, including the release of reactive oxygen species, arginase-1, and immunosuppressive cytokines [13, 36–38], further elucidating the mechanisms through which these cells suppress T cell anti-tumor function and remodel the tumor immune landscape would fully uncover actionable targets to counteract immunotherapy resistance, optimizing therapeutic responsiveness, and ultimately improving outcomes for patients with this aggressive disease.

Declarations

Ethics approval and consent to participate:

Written informed consent was obtained from the patient who participated in this study. The study was approved by the Ethics Committees of Hospital Prof. Doutor Fernando Fonseca and NOVA Medical School.

Consent for publication:

Consent for publication was obtained from the participant and from all authors involved in the study.

Conflict of interest:

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author contributions:

TM drafted the manuscript, performed data analysis, and contributed to the clinical follow-up and discussion of the patient. MV revised the manuscript and participated in the clinical follow-up and discussion. RS, BFC, and DG processed blood samples, carried out all flow cytometry analyses, and contributed to manuscript writing. ADM, IE and MVB contributed to the clinical discussion. GG analyzed the tumor immune infiltrate. SB had a role in the interpretation of clinical data and overall discussion. MGC designed and supervised the study and contributed to data interpretation and manuscript writing. The final manuscript received approval from all authors.

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Figures

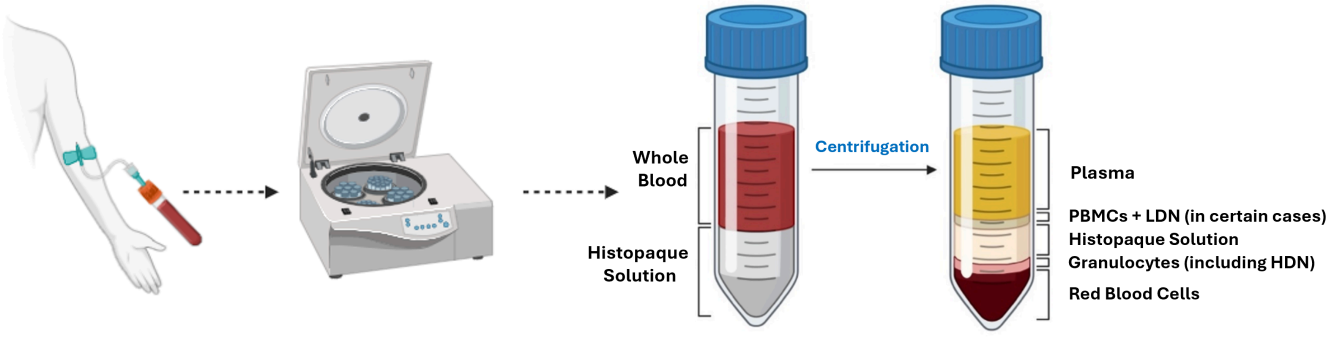


Figure 1

Diagram illustrating the distinct whole blood fractions after density gradient centrifugation. This process separates whole blood into different layers: the top layer consists of plasma; the upper-middle layer contains PBMCs and, in some cases, LDNs; below this lies the histopaque layer followed by a layer enriched in granulocytes, including HDNs; and the bottom layer consists of red blood cells.

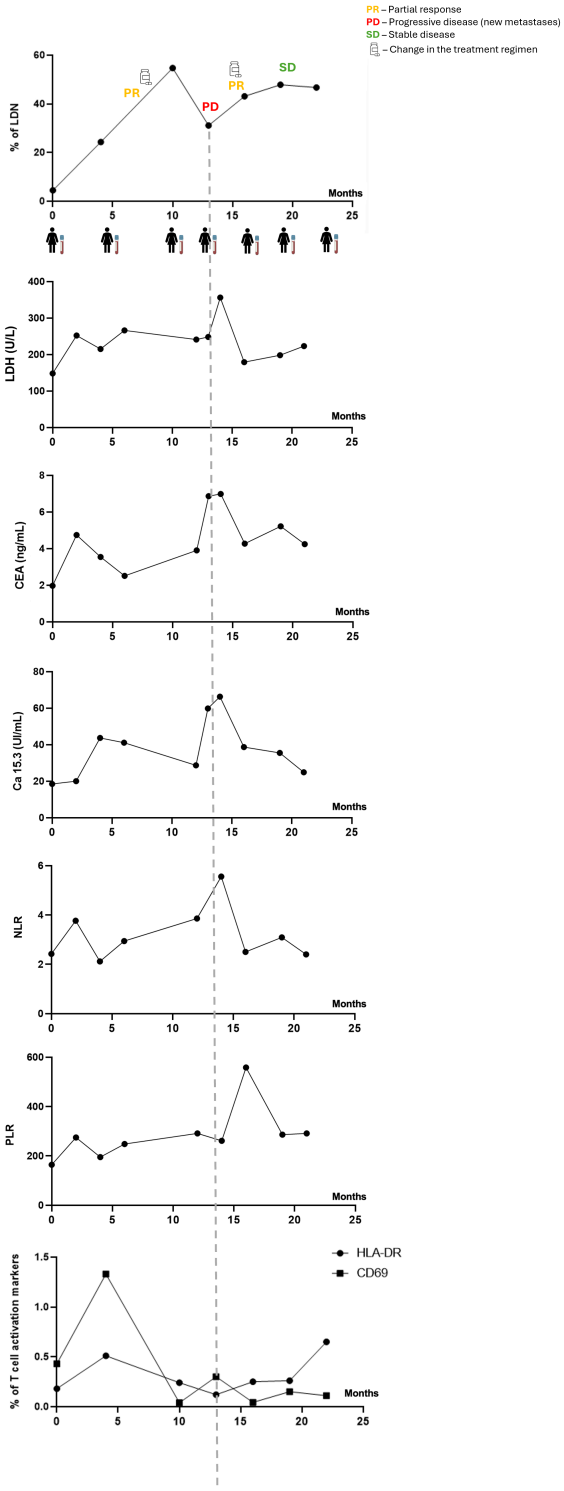


Figure 2

Longitudinal monitoring of systemic and immune biomarkers in a BC patient across neoadjuvant and palliative therapy. Dynamic changes in the frequency of LDNs (% of LDN), classical clinical biomarkers (LDH, CEA, CA 15.3, NLR, and PLR), and T cell activation markers (CD69 and HLA-DR) were monitored for 22 months. The timeline indicates the corresponding clinical outcomes (PR - partial response; SD - stable disease; PD - progressive disease).

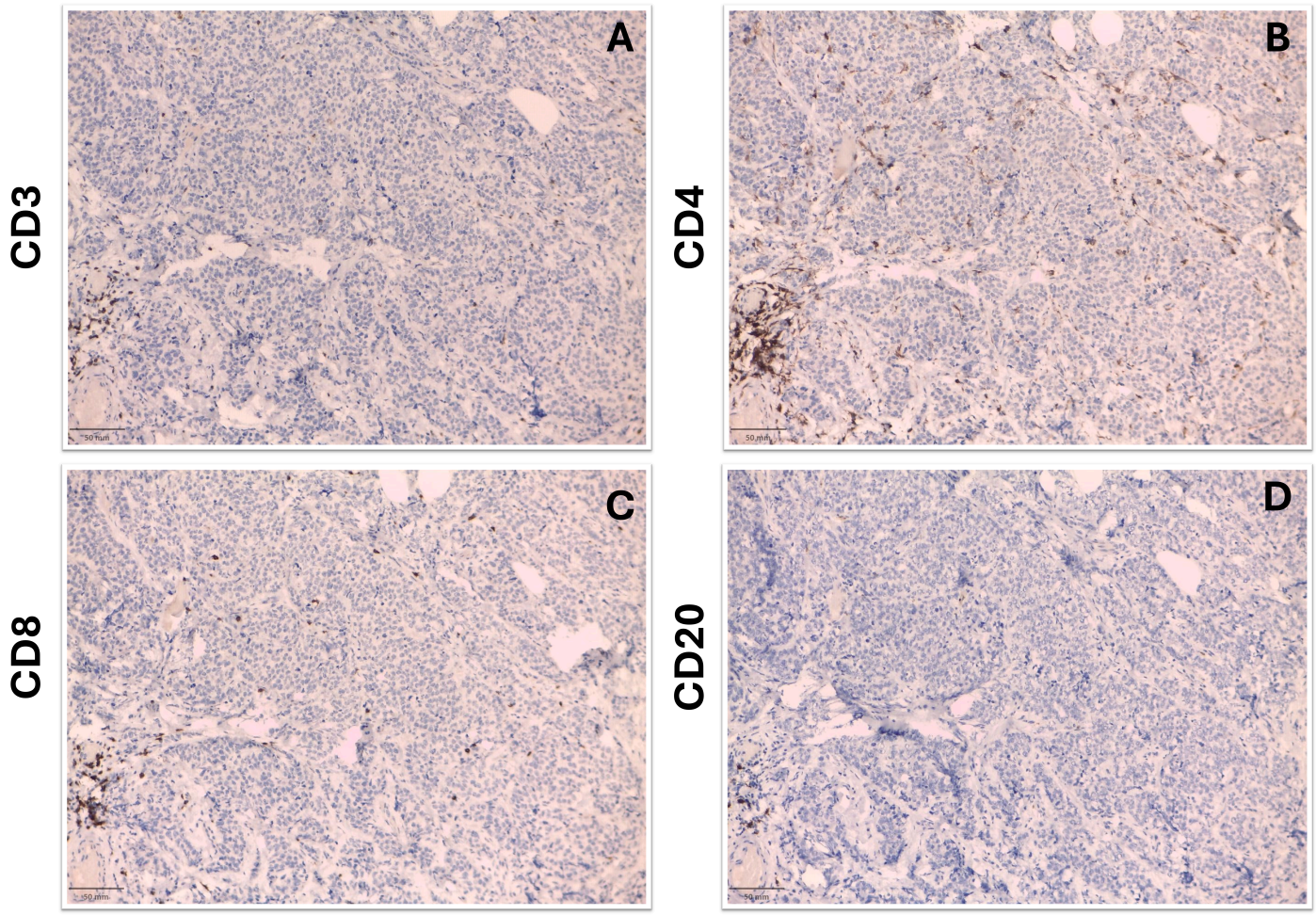


Figure 3

Immunophenotyping of TILs in the surgical specimen. Representative images of immunohistochemical staining for **(A)** $CD3^+$, **(B)** $CD4^+$, **(C)** $CD8^+$, and **(D)** $CD20^+$ cells in the tumor tissue (200× magnification). Scale bar = 50 mm.