# Enumeration of $CD3^{-}CD56^{+}CD16^{+}$ and $NKG2D^{+}$ Natural Killer Cells in Breast Cancer Patients

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**Article Link:** https://journals.brainetwork.org/index.php/jcihs/article/view/68

**DOI**: https://doi.org/10.69591/jcihs.2.2.3




**Volume 2, Issue 2, 2024**

**Funding**
No

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**Citation:** Majeed, N., Minhas, M., Tahir, R., Shahzad, F., Munir, B., Kashif, M., Shahid, A., & Afzal, N. (2024). Enumeration of $CD3^{-}CD56^{+}CD16^{+}$ and $NKG2D^{+}$ Natural Killer Cells in Breast Cancer Patients, *Journal of Contemporary Insights in Health Sciences, 2(2)*, 33-46.

**Conflict of Interest:** Authors declared no Conflict of Interest

**Acknowledgment:** No administrative and technical support was taken for this research

**Article History**

**Submitted:** Sep 05, 2024
**Last Revised:** Nov 10, 2024
**Accepted:** Dec 27, 2024

**An official Publication of**
**Beyond Research Advancement & Innovation Network, Islamabad, Pakistan**

Original Research Article

# Enumeration of CD3⁻CD56⁺CD16⁺ and NKG2D⁺ Natural Killer Cells in Breast Cancer Patients

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***ABSTRACT***

**Introduction:** Breast cancer is a malignant disease of breast tissue. The immune system consists of cells, molecules, and organs that play a vital role in eliminating pathogens, toxins, allergens, and tumor cells. Cytotoxic T cells ($CD8^+$) and natural killer (NK) cells are the major effector cells responsible for killing tumor cells.

**Objective:** This study was designed to determine the percentage of NK cells, NK cell subsets ($CD56^{bright}CD16^-$ and $CD56^{dim}CD16^+$), and the activating receptor NKG2D in the peripheral blood of breast cancer patients.

**Study Design:** Prospective case control

**Place and duration:** This study was conducted between August 2017 and

March 2018 at INMOL Hospital, Lahore, Pakistan.

**Methodology:** This was a comparative study that included 80 subjects (40 healthy controls and 40 breast cancer patients). The study included patients diagnosed with ductal carcinoma in situ (DCIS, n=6), invasive ductal carcinoma (IDC, n=33), and invasive lobular carcinoma (ILC, n=1). According to the American Cancer Society criteria (2017), the majority of patients were classified as grade 2 (n=25), followed by grade 3 (n=12) and grade 1 (n=3). Additionally, forty healthy controls, aged 26 to 60 years, with no history of malignancy or chronic disorders, were included.

**Results:** The mean ± SD of NK cells were higher in breast cancer patients (9.5 ± 5.2) compared to healthy controls (8.5 ± 5.3); however, the difference was not statistically significant (p = 0.39). The expression of NKG2D was higher in the control group (67 ± 16.3) compared to patients (52 ± 28.1), and this difference was statistically significant (p = 0.005). Breast cancer patients had a higher mean ± SD for the $CD56^{bright}$ subset (1.8 ± 1.1) compared to healthy controls (1.1 ± 0.5), and this difference was statistically significant (p = 0.001). Similarly, breast cancer patients had a higher mean ± SD for the $CD56^{dim}$ subset (12.4 ± 8.2) compared to healthy controls (11.2 ± 5.7); however, this difference was not statistically significant (p = 0.445).

**Conclusion:** The current study suggested that breast cancer patients modulate the abundance of NK cell subsets and alter the expression of NKG2D on the surface of peripheral blood NK cells which may decrease NK cell cytotoxicity.

**Keywords:** Breast Cancer, NK cells, NKG2D, $CD56^{bright}CD16^{-}$, $CD56^{dim}CD16^{+}$

## 1. INTRODUCTION

Cancer is defined as the uncontrolled division of abnormal cells in any part of the body [1]. These cells begin to accumulate, forming a mass of tissue known as a tumor. A tumor is composed of malignant cells and stroma, collectively referred to as the tumor microenvironment, which consists of lymphatic vessels, blood vessels, and host cells such as fibroblasts, resident, and trafficking immune cells. All of these components are embedded in the extracellular matrix [2].

The tumor microenvironment contains both innate and adaptive immune cells, including macrophages, dendritic cells, natural killer (NK) cells, neutrophils, mast cells, and T and B lymphocytes [3].

Natural killer (NK) cells constitute approximately 10% of blood lymphocytes and have a relatively short lifespan. In an adult, there are about 2 billion NK cells. These cells are large, granular, and derived from the bone marrow. They are distributed in the spleen, liver, uterus, and lymph nodes [4,5].

Phenotypically, NK cells are defined by the expression of CD56 and the absence of CD3. Based on CD56 expression, NK cells are classified into two major subsets. CD56dim NK cells, which make up about 90% of NK cells, exhibit low CD56 expression but high CD16 expression. These cells secrete low levels of cytokines, exhibit potent lymphokine-activated killer (LAK) activity, and demonstrate enhanced antibody-dependent cellular cytotoxicity (ADCC) and natural cytotoxicity. On the other hand, CD56bright NK cells, which constitute approximately 10% of NK cells, exhibit high CD56 expression but low CD16 expression. They primarily secrete immunoregulatory cytokines, exhibit potent LAK activity, but have reduced ADCC and natural cytotoxicity compared to CD56dim NK cells. Additionally, CD56bright NK cells are less granular than CD56dim NK cells [6].

NK cells possess receptors that initiate either positive or negative signaling upon interaction with their respective ligands [7]. Activating receptors include natural cytotoxicity receptors (NKp30, NKp40, and NKp46), NKG2D, 2B4 (CD244), and DNAX accessory molecule 1 (CD226). NKG2D binds to MHC class I-related chain A (MICA) and MICB, while NKp44 and NKp46 interact with viral hemagglutinins. DNAM-1 binds to the poliovirus receptor (CD155) and Nectin-2, whereas NKp30 binds to the tumor antigen B7-H6 [8].

In addition to activating receptors, NK cells express inhibitory receptors, including NKG2A and killer immunoglobulin-like receptors (KIRs) [9]. The ligands for KIRs and NKG2A are HLA class I and HLA-E, respectively [10]. Previous studies have reported that MHC class I expression is often downregulated on tumor cells [11]. According to the missing-self theory, the loss of MHC class I makes tumor cells more susceptible to NK cell-mediated lysis [11].

NK cells regulate the host immune response by secreting chemokines and cytokines. They eliminate transformed or infected cells using cytotoxic mechanisms involving perforin and granzyme release or apoptosis through death receptor pathways, such as Fas and TRAIL [12].

Increased infiltration or migration of NK cells into tumors is associated with a good prognosis [13, 14], highlighting their role in controlling tumor progression [15]. Several studies have reported a reduced expression of NK cell-activating receptors, including NKp30, NKp44, NKp46, NKG2D, and LY49I, which is linked to decreased NK cell cytotoxic activity and increased tumor metastasis [16, 17, 18].

Mamessier et al. (2011) found that patients with metastatic and locally advanced breast carcinoma had a higher proportion of immature NK cells (CD56bright, CD16$^-$) compared to those with benign and invasive localized breast carcinoma [7]. Similarly, patients with renal carcinoma exhibited decreased expression of NKG2A on peripheral blood NK cells compared to infiltrating NK cells, as NKG2A-positive infiltrating NK cells demonstrated reduced cytotoxic activity [19]. Additionally, patients with acute myeloid leukemia showed lower surface expression of NCRs compared to healthy controls, leading to diminished cytotoxic activity against malignant cells [20].

Previous reports suggest that in patients with malignant breast carcinoma, NK cells exhibit alterations in their phenotype, function, and number. Similarly, impaired NK cell maturation and function have been observed in metastatic breast carcinoma; however, the precise relationship between NK cells and breast carcinoma remains unclear [7]. Since NK cells are a crucial component of the innate immune system, specialized in detecting and eliminating "modified-self" cells such as cancer cells, this study was designed to determine the percentage of NK cells (CD56bright and CD56dim) and the expression of the activating receptor NKG2D in breast cancer patients.

## 2. MATERIAL AND METHOD

This cross-sectional study was conducted at Wah Medical College over six months, from November 22, 2021, to June 22, 2022. Using the Statulator sample size calculator, the required sample size was determined based on a prior study at the Foundation University of Dentistry, which reported fatigue and malaise as

the most common side effects (45% prevalence). With a 5% margin of error and a 95% confidence interval, the calculated sample size was 381 participants, selected through non-probability convenient sampling.

**Patients and Healthy Controls**

This study was conducted between August 2017 and March 2018. Forty female breast cancer patients, aged 24 to 71 years, who had not received any treatment, were enrolled from INMOL Hospital, Lahore, Pakistan. The study included patients diagnosed with ductal carcinoma in situ (DCIS, n=6), invasive ductal carcinoma (IDC, n=33), and invasive lobular carcinoma (ILC, n=1). According to the American Cancer Society criteria (2017), the majority of patients were classified as grade 2 (n=25), followed by grade 3 (n=12) and grade 1 (n=3). Additionally, forty healthy controls, aged 26 to 60 years, with no history of malignancy or chronic disorders, were included.

**Ethical Approval**

The study was approved by the Ethical Review Committee and the Advanced Studies & Research Board of the University of Health Sciences (UHS), Lahore, as well as INMOL Hospital, Lahore, Pakistan. Informed consent was obtained from each participant before sample collection.

**Blood Sample Collection**

Three (3) mL of venous blood was aseptically drawn from each subject using a sterile 5 mL syringe and transferred into an EDTA vacutainer. The sample was mixed thoroughly to prevent clotting. The samples were transported to the Department of Immunology, UHS, in an icebox maintained at 4˚C and were processed within two hours of collection.

**Flow Cytometry Analysis**

Peripheral blood mononuclear cells (PBMCs) were isolated using the density centrifugation method with Histopaque-1077, as described by Goreti et al. (2016) (Figure 1).

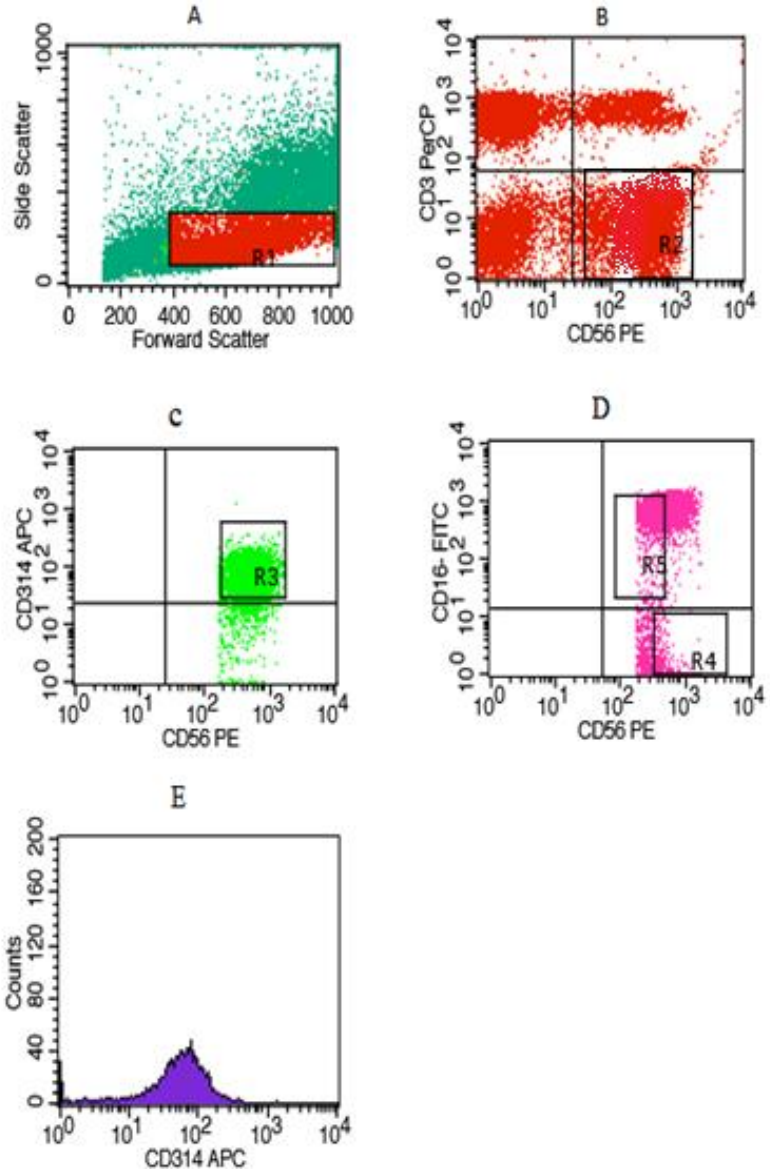



**Figure 1: Schematic Diagram for Flow Cytometric Analysis**

***Note:*** **A.** Lymphocytes were selected by placing the $R_1$ gate in the SSC-FSC dot plot. **B.** To identify NK cells, the $R_2$ gate was applied to CD3$^-$CD56$^+$ cells. **C.** The

expression of CD314 (NKG2D) and CD56 was assessed by placing the $R_3$ gate in a CD314 vs. CD56 dot plot. **D.** The $CD56^{Bright}CD16^{-}$ subset was enumerated using the $R_4$ gate, while the $CD56^{Dim}CD16^{+}$ subset was identified using the $R_5$ gate in the CD16 vs. CD56 dot plot. **E.** The mean fluorescence intensity of CD314 (NKG2D) was measured by applying the $R_2$ gate to the $CD3^{-}$ vs. CD56 dot plot.

The data was entered and analyzed using IBM SPSS 20.0 (Statistical Package for the Social Sciences). Mean ± SD was used to represent quantitative variables. For normally distributed data, an independent t-test was applied, whereas for non-normally distributed data, the Mann-Whitney U test was used. To compare different types and grades of breast cancer, a one-way ANOVA was conducted. A p-value of ≤ 0.05 was considered statistically significant.

## 3. RESULTS

Breast cancer patients between the ages of 24 to 71 years and health controls between the ages of 26 to 60 years were included in the study. The mean ± SD age of breast cancer patients was 47 ± 12.2 years, which was higher than that of the healthy controls (41 ± 11.4 years). The difference in age between the two groups was statistically significant (p=0.024) (Table 1).

**Table 1: Mean ± SD and Comparison of Age Between Breast Cancer Patients and Healthy Controls**

| Variables | Healthy Controls | Breast Cancer Patients | *p*-value |
|---|---|---|---|
| Age(years) (Mean ± SD) | 41 ± 11.4 | 47 ± 12.2 | 0.024* |

Source: Author's own Calculations.

***Note:** *p≤0.05=statistically significant*

The mean ± SD of NK cells was higher in breast cancer patients compared to healthy controls; however, the difference was not statistically significant (p=0.39) (Table 2). The expression of NKG2D was higher in the control group than in the breast cancer patients, and the difference was statistically significant (p=0.005). Although the mean fluorescence intensity (MFI) of NKG2D was also higher in the control group compared to the patients, the difference was not statistically significant (p=0.17). Breast cancer patients had a significantly higher mean ± SD for $CD56^{bright}$ NK cells compared to healthy controls (p=0.001).

Similarly, the mean ± SD for $CD56^{dim}$ NK cells was higher in breast cancer patients than in healthy controls; however, the difference was not statistically significant (p=0.445) (Table 2).

**Table 2: Mean ± SD and Comparison of NK Cells, NKG2D Expression, and NK Cell Subsets Between Breast Cancer Patients and Healthy Controls**

| Markers | Healthy Controls (mean± SD) | Breast Cancer patients (mean± SD) | p-value |
|---|---|---|---|
| NK cells | 8.5 ± 5.3 | 9.5 ± 5.2 | 0.39 |
| NKG2D | 67 ± 16.3 | 52 ± 28.1 | 0.005* |
| NKG2D MFI | 93.8 ± 41.1 | 78.6 ± 57.1 | 0.17 |
| CD 56bright CD16- | 1.1 ± 0.5 | 1.8 ± 1.1 | 0.001* |
| CD56dimCD16+ | 11.2 ± 5.7 | 12.4 ± 8.2 | 0.445 |

Source: Author's own Calculations.

***Note:*** *p≤0.05=statistically significant, NK cells=Natural Killer cells, C-type lectin receptor NKG2D= Natural Killer Group 2D, CD= cluster of differentiation, MFI= Mean fluorescence intensity.*

The mean ± SD of NK cells was highest in grade 2 breast cancer patients compared to grades 1 and 3. However, the difference was not statistically significant (p=0.14). The mean ± SD of NKG2D MFI was also highest in grade 2 breast cancer patients compared to grades 1 and 3, but the difference was not statistically significant (p=0.93) (Table 3). The mean ± SD of NKG2D was highest in grade 1 patients compared to grades 2 and 3, though this difference was not statistically significant (p=0.89). Notably, the expression of NKG2D decreased with tumor grade progression.

Grade 1 breast cancer patients had the highest mean ± SD of $CD56^{Bright}CD16^{-}$ compared to grades 2 and 3, but the difference was not statistically significant (p=0.31). Similarly, grade 1 breast cancer patients had a higher mean ± SD of $CD56^{Dim}CD16^{+}$ than grades 2 and 3, though this was also not statistically significant (p=0.29) (Table 3).

**Table 3: Mean ± SD, Frequency, and Comparison of NK Cells Across Different Breast Cancer Grades**

| Markers | Grade 1 (n = 3) | Grade 2 (n = 25) | Grade 3 (n = 12) | *p*- value |
|---|---|---|---|---|
| NK Cells | 6.6 ± 1.6 | 10.7 ± 6.0 | 7.6 ± 2.8 | 0.14 |
| NKG2D | 59.3 ± 27.9 | 51.8 ± 26.3 | 50.6 ± 33.8 | 0.89 |
| NKG2D MFI | 68.7 ± 62.4 | 80.6 ± 58.5 | 76.9 ± 57.9 | 0.93 |
| CD 56$^{BRIGHT}$ CD16$^{-}$ Subset | 2.5 ± 0.53 | 1.9 ± 1.3 | 1.5 ± 0.87 | 0.31 |
| CD56$^{Dim}$CD16$^{+}$ Subset | 17.4 ± 10.5 | 13.0 ± 9.4 | 9.7 ± 3.3 | 0.29 |

Source: Author's own Calculations.

***Note:*** *p≤0.05=statistically significant, n= number, NK cells=Natural Killer cells, MFI= Mean fluorescence intensity, C-type lectin receptor NKG2D= Natural Killer Group 2D, CD= cluster of differentiation.*

In the present study, the mean ± SD of NK cells were higher in patients with ILC compared to those with DCIS and IDC. However, the difference was not statistically significant (p=0.86) (Table 4).

The expression of NKG2D was higher in patients with DCIS compared to those with IDC and ILC, but the difference was not statistically significant (p=1.84). Furthermore, the mean ± SD of NKG2D MFI was higher in patients with IDC compared to those with DCIS and ILC, though the difference was not statistically significant (p=0.39).

Mean ± SD of CD56$^{bright}$ CD16$^{-}$ and mean ± SD of CD56$^{Dim}$ CD16$^{+}$ was the same in patients with DCIS and IDC as compared to patients with ILC and on comparison it was not statistically significant (*p*=0.93 and 0.80 respectively) (Table 4).

**Table 4: Mean ± SD, Frequency, and Comparison of NK Cells among Different Types of Breast Cancer Patients**

| Markers (%) | DCIS (n = 6) | IDC (n = 33) | ILC (n = 1) | *p*- value |
|---|---|---|---|---|
| NK cells | 8.5 ± 3.7 | 9.9 ± 5.5 | 10.8 ± 0 | 0.86 |
| NKG2D | 54.7 ± 24.0 | 53.1 ± 28.1 | 0.7 ± 0 | 1.84 |
| NKG2D MFI | 69.0 ± 45.0 | 82.5 ± 58.9 | 6.9 ± 0 | 0.39 |
| CD $56^{BRIGHT}$ $CD16^-$ Subset | 1.8 ± 0.7 | 1.8 ± 1.2 | 1.4 ± 0 | 0.93 |
| $CD56^{Dim}CD16^+$ Subset | 12.5 ± 4.3 | 12.5 ± 8.9 | 6.9 ± 0 | 0.80 |

Source: Author's own Calculations.

***Note:*** *One-way ANOVA was applied, =of breast cancer was used samplep≤0.05=statistically significant, NK cells=Natural Killer cells, MFI= Mean fluorescence intensity, DCIS= Ductal carcinoma in situ, IDC= invasive ductal carcinoma, ILC= invasive lobular carcinoma, C-type lectin receptor NKG2D= Natural Killer Group 2D, CD= cluster of differentiation*

## 4. DISCUSSION

This study examined and compared the percentage of NK cells, their subsets, and NKG2D expression between breast cancer patients and healthy controls.

The mean age of breast cancer patients (47 ± 12.2 years) was higher than that of healthy controls (41 ± 11.4 years). This finding aligns with the study by Goreti et al. (2016), in which the mean age of breast cancer patients (55.3 ± 13.5 years) was also higher than that of controls (49.6 ± 13.4) [21]. Breast cancer incidence increases with age and is relatively low in women under 40 years [22].

The percentage of NK cells was higher in breast cancer patients (9.5 ± 5.2) compared to healthy controls (8.5 ± 5.3). This result is consistent with the findings of Muzaffari et al. (2007), who also reported an increased percentage of NK cells in breast cancer patients (293 ± 47) compared to healthy controls (160 ± 32) [23]. However, our findings contrast with those of Goreti et al. (2016), who observed no significant difference in NK cell percentages between breast cancer patients and healthy controls [21].

A possible reason for this discrepancy could be the use of different CD markers to identify NK cells; in the current study, NK cells were identified using $CD3^-$ and $CD56^+$, whereas Goreti et al. (2016) used $CD3^-CD16^+$ and $CD56^+$.

Additionally, patients in the previous study were receiving adjuvant tamoxifen therapy, which has immunomodulatory effects [21].

The expression of NKG2D was higher in healthy controls (67 ± 16.3) compared to breast cancer patients (52 ± 28.1). This finding is consistent with Goreti et al. (2016), who reported a decreased expression of NKG2D on both $CD56^{dim}$ and $CD56^{bright}$ NK cell subsets in breast cancer patients compared to healthy controls [21]

Similarly, the mean fluorescence intensity (MFI) of NKG2D was higher in healthy controls (93.8 ± 41.1) compared to breast cancer patients (78.6 ± 57.1). This result aligns with the study by Mamessier et al. (2011), which also documented a reduced MFI of NKG2D in breast cancer patients compared to healthy controls [7].

NKG2D is an activating receptor for NK cells, playing a crucial role in immune surveillance. Various tumors express increased levels of NKG2D ligands, which bind to the NKG2D receptor on NK cells to trigger an immune response. A decreased expression of NKG2D may indicate reduced cytotoxic activity against tumor cells, potentially compromising the immune system's ability to target and eliminate cancerous cells [21].

In the present study, the $CD56^{bright}$ subset was higher in breast cancer patients (1.8 ± 1.1) compared to healthy controls (1.1 ± 0.5), and this difference was statistically significant ($p = 0.001$).

Mamessier et al. (2013) also reported an increased percentage of the $CD56^{bright}$ subset in patients with locally advanced (9.4 ± 2.3) and metastatic breast cancer (11.2 ± 5.2) compared to those with benign (5.8 ± 0.3) and localized breast cancer (6.1 ± 1.1). Their findings showed a statistically significant difference ($p = 0.0001$) [24].

This result is further supported by Nieto et al. (2016), who observed a higher percentage of the CD56bright subset in breast cancer patients (9.02) compared to healthy controls (8.04). However, in their study, the difference was not statistically significant ($p = 0.7277$) [21].

In the present study, the $CD56^{dim}$ subset was higher in breast carcinoma patients (12.4 ± 8.2) compared to healthy controls (11.2 ± 5.7); however, the difference was not statistically significant ($p = 0.445$).

These findings align with those of Nieto et al. (2016), who reported an increased percentage of CD56$^{dim}$ NK cells in breast cancer patients (90.98%) compared to healthy controls (91.96%), with no statistically significant difference ($p = 0.3329$) [21].

In contrast, Mamessier et al. (2013) documented a lower percentage of the CD56$^{dim}$ subset in patients with locally advanced ($76.9 \pm 5.1$) and metastatic breast cancer ($70.1 \pm 13.4$) compared to those with benign ($87.3 \pm 3.4$) and localized breast cancer ($83.5 \pm 3.8$) [24]. These discrepancies may be attributed to differences in the types of breast cancer patients included in the current study or genetic variations among individuals in these studies.

## 5. CONCLUSION

The increased percentage of NK cells and NK cell subsets (CD56$^{bright}$ and CD56$^{dim}$) in the peripheral blood of breast cancer patients suggests that breast cancer influences the distribution of NK cell subsets. Additionally, the decreased surface expression of NKG2D on peripheral blood NK cells in breast cancer patients indicates that the disease alters NK cell phenotypes. This reduction in NKG2D expression may suggest a decline in NK cell cytotoxic activity.

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