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Differences in tolerogenic status of NK cells between luminal A type, luminal B type, and triple-negative breast cancer

Alma STARČEVIĆ[1,#], Damir GREBIĆ[2,#], Manuela AVIROVIĆ[3], Martina MAVRINAC[4], Petra Valković ZUJIĆ[5], Danijela Veljković VUJAKLIJA[5], Tamara GULIĆ[6,*]

[1]*Clinical Institute for Transfusion Medicine, Clinical Hospital Center Rijeka, Rijeka, Croatia;* [2]*Department of General and Oncological Surgery, Clinical Hospital Center Rijeka, Faculty of Medicine, University of Rijeka, Rijeka, Croatia;* [3]*Department of Pathology, Faculty of Medicine, University of Rijeka, Rijeka, Croatia;* [4]*Department of Medical Informatics, Faculty of Medicine, University of Rijeka, Rijeka, Croatia;* [5]*Department of Radiology, Clinical Hospital Center Rijeka, Faculty of Medicine, University of Rijeka, Rijeka, Croatia;* [6]*Department of Physiology and Immunology, Faculty of Medicine, University of Rijeka, Rijeka, Croatia*

**Correspondence: tamara.gulic@uniri.hr*
[#]Contributed equally to this work.

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Globally, breast cancer is the main cause of death among female cancer patients. The tumor-infiltrating lymphocytes (TILs) in breast cancer are associated with a more favorable outcome of a disease. Natural killer (NK) cells are important cytotoxic cells involved in tumor immunosurveillance, causing the direct killing of tumor cells. In solid tumors, peripheral NK cells and tumor-infiltrating NK cells display an altered phenotype characterized by reduced cytotoxicity or anergy. The goal of this study was to investigate the NK cells' phenotype and activation status in order to get into the pathological process of breast cancer subtypes. In our study, the normal tissues and tumoral breast tissue were fixed in formalin, embedded in paraffin, and the phenotypic marker CD56 and proinflammatory cytokine IL-15 were identified by immunohistology. The distribution and expression of receptors repertoire (NKG2A, NKG2C, NKp46, CD94, CD69, and CD107a) were investigated in peripheral NK cells of mononuclear cells by flow cytometry. mRNA of cytolytic mediators was determined by real-time PCR. The frequency of CD56+ and IL-15+ cells were significantly higher in triple-negative breast cancer tissue. The frequency of NK cell activating receptors was decreased in investigated breast cancer subtypes while the inhibitory NKG2A receptor was increased. Decreased percentage of CD69+/CD107a+ in NK cells could indicate lower cellular activation and cytotoxicity. In luminal B breast cancer, the mRNA of cytolytic mediators was upregulated. In conclusion, modulation of activation status in tumor-infiltration NK cells could be involved in the pathogenesis of molecular breast cancer subtypes. This highlights the importance of NK cells as an appropriate target for potent anti-tumor response in the immunosuppressive tumor microenvironment of breast cancer.

Key words: NK cells, tumor microenvironment, NKG2A, IL-15, breast cancer

Globally, breast cancer is the main cause of death among female cancer patients. High incidence and still high mortality of breast cancer assign as a health and sociology problem [1–3]. The classification of breast cancer subtypes is based on histological and molecular characteristics [4]. Estrogen (ER) and progesterone (PR) receptors, human epidermal growth factor receptor 2, and the proliferation-associated nuclear protein Ki-67 are the most common immunohistochemical biomarkers used to classify breast cancer into four subtypes a) luminal A, b) luminal B, c) HER2-positive cancer, and triple-negative cancers [5–7]. Of all carcinomas listed above, the luminal A (ER+, PR+, and HER2–) has the best prognosis, while triple-negative carcinoma (ER–, PR–, HER2–, Ki-67 highly expressed) is more aggressive and has poorer outcomes with a quick relapse [6, 7]. One of the hallmarks of tumorigenesis is chronic inflammation, where the immunosuppressive microenvironment prevails in cancer and autoimmune/neurodegenerative disease [7–9]. Mediators of inflammation modulate normal cell activity by upregulation of oncogenes, promoting resistance to

apoptosis, progression of preneoplastic lesions, and dissemination [9, 10]. In addition, tumor-associated macrophages (TAMs) [11], tolerogenic NK cells [12], myeloid-derived suppressor cells (MDSCs) [13], regulatory T cells [14], and other immune cells are notable contributors to the immunosuppressive tumor microenvironment (TME). TAMs have an important influence on tumor development, invasion, and metastasis as well as vascularization [12, 15, 16]. Such a microenvironment is capable of dampening NK cell activation and cytotoxicity, promoting NK cells' dysfunctionality via the production of soluble modulators, low nutrient levels, and hypoxic conditions [15–17].

NK cells as a constituent part of innate immunity play an important anti-tumor response [18, 19]. Peripheral blood NK cells can be distinguished into two subsets: the most prevalent $CD16^{+}CD56^{dim}$ subset (85–90%, cytotoxic) and the $CD16^{-}CD56^{bright}$ subset (5–10%) [10, 17]. However, usually NK cells' ability to recognize and kill tumor cells is significantly weakened in cancer patients [20]. Konjevic *et al.* also demonstrated a significantly decreased NK cell activity in cancer patients with the progression of the disease [21]. A recent study has reported that decreased expression of IFN-γ and pSTAT could contribute to the dysregulation of NK cells in women with breast cancer [22, 23]. The tumor secretes soluble factors that induce suppression of NK cell activity allowing the tumor to bystander cytotoxicity of NK cells [17, 20]. Furthermore, NK cell activation depends on the engagement of activating and inhibitory receptors with their interaction [23, 24]. Moreover, NK cell receptors are classified based on their functions: activating, inhibitory, and mixed receptors. The main activating receptors expressed on human NK cells include the natural cytotoxicity receptors (NCRs: NKp30, NKp44, NKp46, NKp80), NKG2D, activating forms of killer cell Ig-like receptors (KIR), C-type lectin receptors (CD94/NKG2C) [25–28]. The activating receptors NKG2D and NKp46 are found to be reduced in many tumors such as gastric cancer, colorectal carcinoma, and metastatic melanoma [29–31]. Inhibitory receptors (killer cell immunoglobulin-like receptors; KIRs, CD94/NKG2A) prevent NK cells from killing healthy cells [17, 31]. However, several research studies have confirmed that the immune profile of NK cells is characterized by an increased expression of the inhibitory receptor (CD94/NKG2A) in solid tumors [26, 27, 31]. Mamesier *et al.* [32] observed the importance of tolerogenic NK cells in breast cancer, which results in a weakened cellular immunity that involved a decrease of activating receptors (such as NKp30, NKG2D, DNAM-1, and CD16) and an increase of the inhibitory receptor NKG2A. NKG2A is one of the recently discovered checkpoints that decrease NK cell cytotoxicity [33]. The killing of tumor cells is mediated by cytotoxic granules (perforins and granzymes), death receptor-induced target cell apoptosis TRAIL (tumor necrosis factor-related apoptosis-inducing ligand), and Fas-L (Fas ligand) [34–36].

IL-15 is a novel cytokine familiar with the structure of IL-2 and the essential chemo-attractant and the key regulator of NK cell proliferation [37–39]. IL-15 increased the expression of perforin, granzyme B, natural cytotoxicity receptors NKp30 and NKp44 [17], and the activating receptor NKG2D [37]. In several tumor models, the presence of IL-15 is critical for delaying primary tumor formation and preventing metastasis [37–39]. The absence of IL-15 drastically alters breast cancer dissemination due to the alteration of NK cell function, and the effects on other immune cells such as macrophages and CD4 T cells [38]. Santana Carrero *et al.* [38] showed that IL-15 loss in colorectal tumors correlated with decreased density and proliferation of T cells, and NK cells, leading to a higher risk of relapse, and decreased survival. Nonetheless, the mechanism of IL-15 expression by the TME is still unclear [40].

In breast cancer, NK cells could act as a double edge sword, on one hand, may control the development of cancer, and on the other, favor tumor progression [41, 42]. Moreover, in advanced stages of the disease in patients with breast cancer has been reported an increased amount of immature NK cells, leading to a decrease in cytotoxic function consequently promoting tumor progression [8, 43]. Muraro *et al.* [44] have reported an increase of NK cells and regulatory T cells in patients with locally advanced HER2+ breast cancer at assessing the possible predictive and/or prognostic role of CD56 immune markers. Furthermore, increased expression of activating receptors within tumor progression correlated with the predicted survival in breast cancer [42–44]. NK cell therapy has the potential for treating cancer and preventing relapse of the disease [45]. Despite, significant progress in understanding the interrelation and complexity of causal events between a tumor cell and NK cells over the last two decades there are still many questions yet to be answered. We decide to focus our attention on elucidating the immunophenotype and activation status of NK cells in molecular breast cancer subtypes.

The aim of our study was to determine the expression pattern and distribution of TILs, NK cells, IL-15, and APAF-1 in molecular breast cancer subtypes (luminal A, luminal B, and triple-negative breast cancer), as well as to assess the activation status and cytotoxic mediators (perforin, TRAIL, and FAS-L) expression in NK cells on protein and gene level.

Materials and methods

Human tissue samples. The study included 141 females, 111 with breast cancer and 30 healthy in the control group. Median age for all was 60 years (minimum 30 to maximum 92). Breast cancer diagnosis were luminal A (N = 38, median age 61.5; 35–92), luminal B (N = 41, median age 65; 37–87), and triple-negative breast cancer (N = 32, median age 56.5; 32–83), cT2–cT3 primary tumor stage, pN0–pN3 regional lymph node stage, without distant metastases (M0). A core needle biopsy samples (80) from patients with different

subtypes of breast cancer (luminal A (20 samples), luminal B (20 samples), and triple-negative cancer (20 samples)) were obtained in order to collect 3–5 cylinders of breast tumor tissue samples 22 mm long and 2 mm wide, which were further processed for pathohistological analysis. Briefly, they were stained with hematoxylin-eosin (HE) as well as immunohistochemical markers (ER, PR, Ki-67, and HER-2), based on which breast cancer is diagnosed and categorized by its immunophenotype. One of the cylinders was used for gene analysis.

Control group (N = 30, median age 56.5; 30–76) samples were obtained retrospectively from breast tissue samples due to the microcalcifications present on the mammogram, for which the Department of pathology and cytology of CHC Rijeka has set the diagnosis of nonproliferative changes involving microcysts, cysts, apocrine metaplasia, and fibrosis. Peripheral blood samples (N = 20) were derived from healthy women without any sign of systemic disease or any infections, as observed in medical history. All the women involved in the study signed informed consent. The Ethics Committee of the Medical Faculty, University of Rijeka, Croatia approved the study.

Determination of TILs. HE-stained slides were used to determine the percentage of tumor-infiltrating lymphocytes (TILs) within the control healthy group and breast cancer subtypes (luminal A, luminal B, and triple-negative breast cancer) group. TILs were determined as a continuous variable by estimating the percentage of TILs in the stromal portion of tumor tissue, in HE-stained sections, at low magnification of a light microscope ×20 [16]. The determination of TILs was done manually, by three independent researchers including one transfusiologist, one immunologist, and one pathologist (A.S., T.G, and M.A.) without insight into the clinical and pathological characteristics of the patient and the histological characteristics of the tumor.

Tissue labeling of CD56, IL-15, and APAF-1 by immunohistology. Paraffin-embedded breast tissue sections from the healthy control group and breast cancer subtypes (luminal A, luminal B, and triple-negative cancer) group (thick 6 μm) were single labeled for CD56 (N=20 for each tissue), IL-15 (N=10 for each tissue), and APAF-1 (N=10 for each tissue) detection as described earlier [46, 47].

Briefly, the tissue sections were deparaffinized in Tissue Clear (Paraffin Cleaning Agent, Sakura Fintek Europe, Zoeterwoude, Netherland), hydrated in decreasing concentrations of ethanol (from 100% to 75%), and washed in phosphate-buffered saline (PBS; 0.05 M containing 0.3 M NaCl; pH 7.4) (Kemika, Zagreb, Croatia). 10 mM sodium citrate (pH 6.0) was used as an antigen retrieval following the washing step in PBS and blocking of endogenous peroxidase activity with 3% H_2O_2 (Kemika) for 10 min and non-specific antibody binding was prevented with Background Sniper (Biocare Medical) for 15 min. The sections were then incubated for 1 h with anti-CD56, anti-IL-15, and anti-APAF-1 mAb (Table 1), or without a primary antibody as a negative control. Subsequently, the slides were incubated for 20 min with MACH 1 Universal HRP-Polymer and then were washed in PBS between steps. The positive reaction was seen by using 3,3-diaminobenzidine (DAB; Biocare Medical). The nuclei of the cells were stained with hematoxylin solution Gill No. 3 (Sigma Aldrich Chemie, Steinheim, Germany). In the end, tissue sections were dehydrated by immersion in increasing concentrations of ethanol from 75% to 100% and dipped in Tissue Clear. The slides were mounted using Entellan (MerckKgaA, Darmstadt, Germany). Tissue sections were analyzed with a light microscope Olympus BX51 and a camera Olympus DP71 (Olympus, Tokyo, Japan). Cell^A imaging software, version 3.0 (Olympus, Tokyo, Japan) was used to acquire the microphotographs.

Semiquantitative analysis of immunohistological staining. The Alphelys Spot Browser 2 integrated system (Alphelys, Plaisir, France) was used to quantify the CD56 and IL-15 immunohistological staining as described previously [46, 47]. Nikon Eclipse 50i microscope (Nikon, Tokyo, Japan) mounted on a 1360 9 1024 resolution Microvision CFW-1310C digital camera (Microvision Instruments, Evry, France) under the control of Alphelys Spot Browser 2.4.4 (Alphelys) was involved in the quantification. The Nazca program (Micro Vision Instruments, France), which determines the size of a point for each lens (0.3311 lm for 209 lens and 3.320 lm for 29 lens) is used to calibrate the system. With a constant camera, microscope settings, and light intensity, slides were scanned at 209 magnifications to identify the section area of the slide and then scanned at 2009 magnification to create images for quantification in the program. The software was arranged to distinguish between the empty background space and the section area to determine the homogenous area, representing tumoral tissue. During digital image analysis, the software detected objects of interest on the basis of pixel color properties (wavelength, intensity, and saturation), grouping, and morphological attributes such as size and shape. The algorithms were designed to determine the count of unstained and stained objects (cells). Output was expressed as the arithmetic mean of the percentage of positive cells analyzed in 12 fields. The final result is expressed as "percentage of positive cells of total cells".

Isolation of human peripheral blood mononuclear cells by density gradient centrifugation. Human peripheral blood mononuclear cells (PBMCs) are routinely isolated from blood samples from the healthy control group and breast cancer subtypes (luminal A, luminal B, and triple-negative carcinoma) group, *in vitro*, according to the method used in our laboratory [47]. The peripheral blood samples were resuspended in RPMI 1640 and layered on top of Lymphoprep™ solution (Axis-Shield PoC AS, Oslo, Norway) following the centrifugation at 600×g for 20 min without brake. The mononuclear cell fraction was carefully layered up by pipet and washed in RPMI 1640. The mononuclear cells were resuspended in RPMI 1640 containing 3% fetal calf serum (FSC; Gibco, Gaithersburg, MD, USA), 0.3 mg/ml L-gluta-

mine and 100 U/ml penicillin/streptomycin, and adjusted to a concentration of 10^6 cells/ml. Trypan blue (Serv, Heidelberg, Germany) was used to assess cellular viability that was checked by a light microscope (Carl Zeiss, Jena, Germany). Cell viability was always more than 95%.

Antigen detection in PBMCs using flow cytometry. Briefly, freshly isolated PBMCs (3×10^5 cells/sample) were washed in florescence-activated cells sorter (FACS) buffer (NaCl [8.12 g/l], KH_2PO_4 [0.26 g/l], Na_2HPO_4 [2.35 g/l], KCl [0.28 g/l], all from Kemika (Zagreb, Croatia); Na_2EDTA [0.36 g/l] (Fluka, Buchs, Switzerland); NaN_3 [0.1 g/l] and fixed using 4% paraformaldehyde (Kemika) for 10 min at room temperature. After 2 washes in cold FACS buffer, we used human AB serum to block unspecific binding for 20 min at room temperature. The PBMCs were triple stained with FITC-conjugated anti-CD107a mAb or with PE-conjugated anti-NKp46 mAb or with PE-conjugated anti-NKG2A mAb or with PE-conjugated anti-NKG2A mAb or with PE-conjugated anti-NK2C mAb, and for the lineage-specific markers of $CD3^-CD56^+$ NK cells, $CD69^+CD56^+$ NK cells and incubated for 30 min at 4°C. The following mAbs were used: PE-Cy5-conjugated mouse anti-human CD56 (IgG1), APC-conjugated mouse anti-human CD3 (IgG1), PE-conjugated mouse anti-human CD69 (IgG1), FITC-conjugated mouse anti-human CD56 (IgG1), all from BD Pharmingen (San Diego, CA) and described in Table 1. Directly conjugated (FITC, PE, PE-Cy5, and APC) mouse isotype-matched IgG1s were used as isotype controls (all from BD Pharmingen). Samples were washed twice in cold FACS buffer. Labeled cell samples were fixed in 200 µl 2% paraformaldehyde and assessed by flow cytometry (Becton Dickinson FACSCalibur) using CellQuestPro software (BD Biosciences, San Jose, CA, USA).

RNA extraction and real time quantitative-polymerase chain reaction (RT-qPCR) analysis. Total RNA was purified from the core tissue of luminal A, luminal B, and triple-negative breast cancer with the TRIzol method (Invitrogen, Carlsbad, CA, USA). The RNA concentration and the quality were measured and examined by a NanoDrop spectrophotometer (Thermo Fisher Scientific) (high OD260/OD280 ratio) and by agarose gel electrophoresis (sharp 18S and 28S ribosomal RNA bands). The Turbo DNA-free kit (Ambion Inc, The RNA Company, Austin, TX, USA) was used to eliminate any traces of DNA contamination in the samples. Subsequently, 2 µg of total RNA were reverse transcribed using the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer protocol. RT-qPCR analysis was performed with the ABI PRISM 7300 SDS (Applied Biosystems) for the following genes: perforin, Fas-L, TRAIL, and human eukaryotic 18S rRNA (housekeeping gene) using the commercially available TaqMan assay for perforin (Hs00169473 m1), Fas ligand (Hs00181225 m1), TNFSF10 (TRAIL) (Hs00234356 m1), and 4352930E (18S rRNA) (Applied Biosystems). Quantitative RT-PCR reactions were performed in duplicate for each sample (50 ng of cDNA as template) using 7500 Real-Time PCR System thermocycler (Applied Biosystems, USA) and TaqMan Universal PCR master mix (Applied Biosystems). The reactions were carried out in 25 µl following the manufacturer's protocols. Normalization of the target genes with housekeeping gene (18S rRNA) was performed in each experiment to compensate for a possible inter-PCR variation. Quantification was performed using the 2–ΔCt method calculated as the difference between the ΔCt values of the target genes and an endogenous control gene and 18S was used as the housekeeping gene control. The results were presented as relative expression of the target gene to the endogenous control gene.

Statistical analysis. Numerical data were expressed as median and 25^{th} and 75^{th} percentiles and categorical data as the percentages. Age was expressed as median and range. The significance of the differences between multiple groups of interest was analyzed using the Kruskal-Wallis non-parametric and as a post hoc was used Mann-Whitney U-test. For the correlations between values, Spearman's correlation coefficient (rho) was used. We performed the AUC (Area Under the Curve) and ROC (Receiver Operating Characteristics) curve to distinguish between different diagnoses. All the p-values <0.05 were considered statistically significant. Statistical analyses were performed with the data analysis software system Statistica 14.0.0.15.0 (TIBCO Software, Inc., Palo Alto, CA, USA) and MedCalc 19.1.7 (MedCalc Software, MariaKerke, Belgium).

Results

Association between TILs and molecular subtypes of breast cancer. In clinical practice breast cancer is divided into four molecular subtypes and the TILs percentage was analyzed in breast cancer subtypes. Several studies have attempted to determine the prognostic value of TILs in breast cancer subtypes (luminal A, luminal B, and triple-negative breast cancer) [48, 49]. In detail, increased TILs levels have been linked to better response to chemotherapy, and patients with triple-negative cancer have better survival [50, 51]. On the other hand, the prognostic and predictive value of TILs in luminal breast cancer remains poorly understood [48]. Hematoxylin and eosin (H&E, upper row) stained sections illustrating different TILs distribution in our series (Figure 1A). Higher magnification (H&E, ×400, lower row) of the same case illustrating the TILs distribution in tumor stroma (Figure 1A). Higher magnification of the same case (H&E, ×100) demonstrates the increased TILs distribution in tumor stroma especially in triple-negative breast cancer compared to another investigated group. Our histopathological evaluation of TILs in triple-negative cancer was significantly increased compared to the healthy control group and to less malignant subtypes of breast cancer (luminal A and luminal B) (Figure 1B). In addition, a similar trend was found in hormone-receptor positive

breast cancers (luminal A and luminal B) in comparison to the healthy control group (Figure 1B).

Increased expression of stromal and intratumoral NK cells in triple-negative breast cancer. NK cells play a role in tumor immune surveillance and are an important arm of the innate immune system in the prevention of tumor growth and of deleterious metastatic potential [34–36]. Herein, we investigated the distribution and expression of NK cells in molecular subtypes of breast cancer by immunohistology. Compared to negative controls, in the healthy control group and molecular subtypes of breast cancer group mammary gland tissue, $CD56^+$ cells, stained brown due to DAB, were expressed in the cytoplasm and membrane of cells lining the glands and in cells diffuse disperse in the normal and tumoral stroma as well as intratumoral, indicated with black arrows (Figure 2A). A statistically increased percentage of $CD56^+$ cells was observed in triple-negative cancer when compared to normal and hormone-receptor positive breast cancers (luminal A and luminal B) tissue sections (Figure 2B). By contrast, the percentage of $CD56^+$ cells was statistically higher in normal tissue compared to less proliferative breast cancers (luminal A and luminal B) (Figure 2B).

Assessment of IL-15 in the mammary gland of normal and breast cancer tissue. The cytokine IL-15 is can affect many immune cells, including CD8 T cells and NK cells [38]. The involvement of these cell types in tumor immunosurveillance and eradication has led our interest on this cytokine [37, 38].

In paraffin-embedded tissue sections of the normal and breast cancer subtypes (luminal A, luminal B, and triple-negative cancer) were IL-15 positive and were compared with the isotype control (Figure 3A). Specifically labeled $IL\text{-}15^+$ cells (brown) were abundant in all analyzed molecular subtypes of breast cancer tissue sections (Figure 3A). Moreover, infiltrating tumor cells and cells diffuse disperse in the normal and tumoral stroma as well as an intratumoral part in all investigated tissue sections were determined as $IL\text{-}15^+$ cells (Figure 3A). Further, we observed cytoplasmatic and nuclear IL-15 labeling in all investigated groups (Figure 3A). The number of $IL\text{-}15^+$ cells/400x HPF of luminal A, luminal B, and triple-negative breast cancer tissue sections was significantly higher as compared to the mammary tissue samples of healthy donors (Figure 3B).

CD56 biomarker is a very good measure for distinguishing diagnosis between triple-negative and hormone-positive receptors subtypes of breast cancer. There was no association between the expression of CD56 and different diagnoses including the healthy control group and hormone-receptor positive breast cancer group (luminal A and luminal B) (Figure 4A, rs=0.16; p=0.059). However, a high

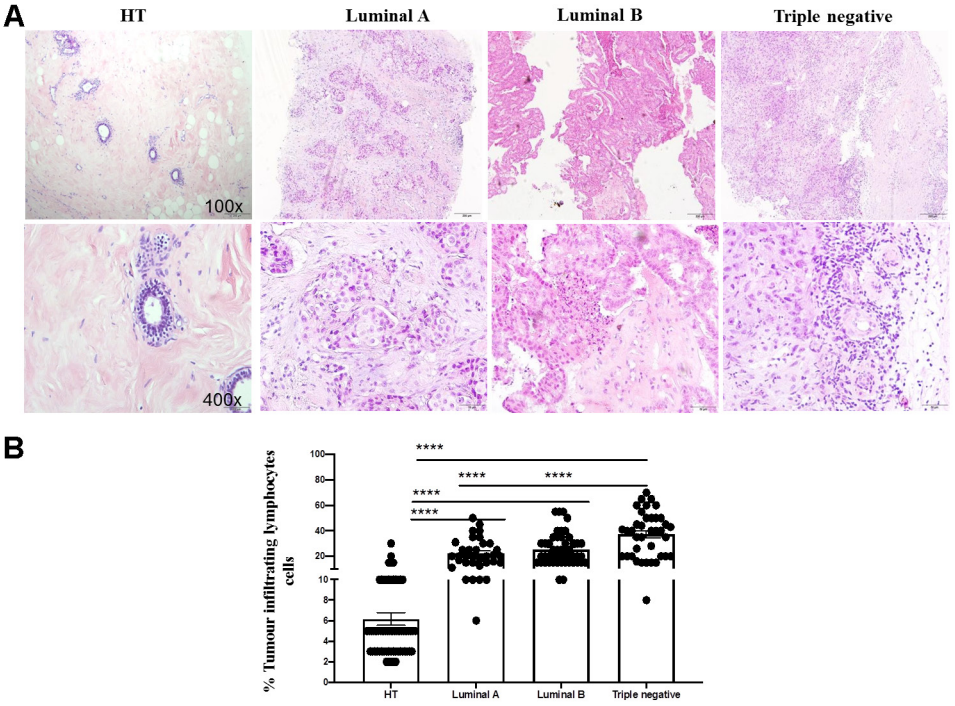

Figure 1. Micrographs (A) show tumor samples of luminal A, luminal B, and triple-negative cancer, and healthy control (HT) from mammary tissue gland stained with hematoxylin and eosin (H&E) to evaluate qualitatively tumor infiltrating lymphocytes (TILs). Graph B shows the percentage of TILs in tissue samples of healthy control and breast cancer. Forty independent tissue samples were processed in each group. Images were taken with an Olympus UPlan FI objective lens (Tokyo, Japan), 409/0.75, ∞/0.17, and the level of statistical significance is ****p<0.0001.

level of CD56 expression was associated with a more malignant subtype of breast cancer (Figure 4B, rs=0.54; p<0.0001). The highest value of CD56 expression was observed in triple-negative breast cancer subtypes. We analyzed the AUC (Area Under Curve) ROC (Receiver Operating Characteristics) curve to distinguish between different diagnoses. The ROC-AUC was 0.83 (CI 0.76–0.89); p<0.001) which means it has a very good measure of distinguishing diagnosis. The

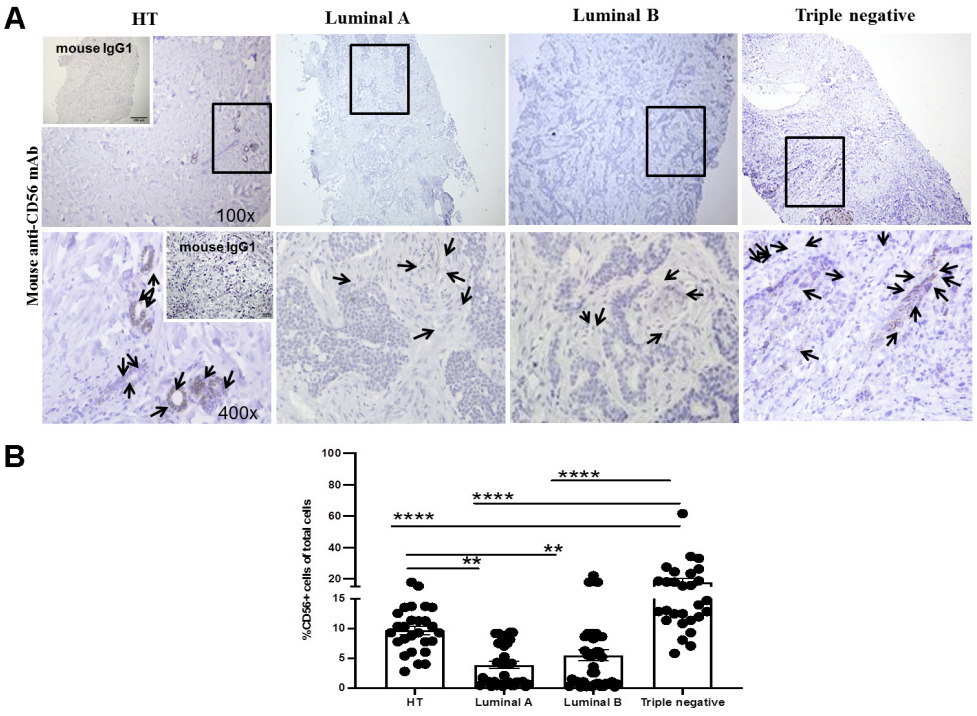

Figure 2. Expression of CD56 in paraffin-embedded mammary gland tissue sections of breast cancer subtypes (luminal A, luminal B, and triple-negative cancer) and healthy control group (HT) were analyzed. Immunohistology (A) was done with single-labeling mouse anti-CD56 mAb using indirect immunoperoxidase staining. CD56⁺ cells are present as brown by 3,3-diaminobenzidine (DAB) (black arrows). Isotype match control (mouse IgG1) is shown in inserts and the lined quadrangle area is present at higher magnification (×400 high-power field). The percentage of CD56⁺ cells of total cells using Alphelys Spot Browser 2 integrated system is shown in graph B. Around fifty independent tissue samples were investigated in each group. Images were taken with an Olympus UPlan FI objective lens (Tokyo, Japan), 409/0.75, ∞/0.17and the levels of statistical significance are ****p<0.0001 and **p<0.01.

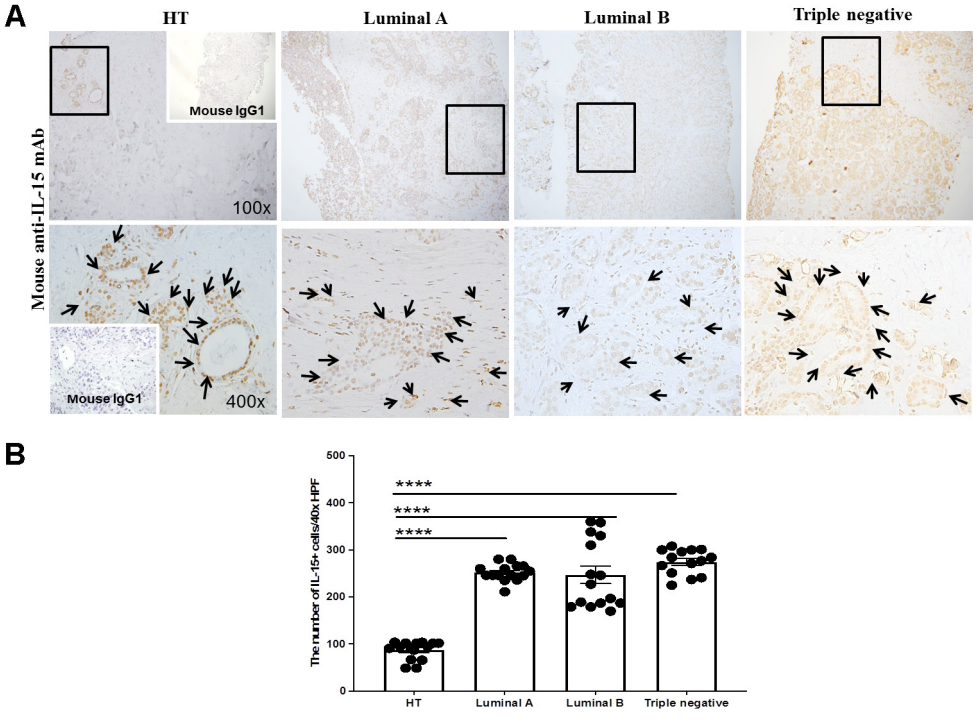

Figure 3. Immunohistological analyses of IL-15 expression in paraffin-embedded mammary gland tissue sections of luminal A, luminal B, triple-negative cancer, and healthy control group were performed. Immunohistology (A) shows single-labeling with mouse anti-IL-15 Ab by indirect immunoperoxidase staining. IL-15⁺ cells are visualized as brown by 3,3-diaminobenzidine (DAB) staining (black arrows). Isotype match control mouse IgG1 is present in inserts and the lined quadrangle shows higher magnification (×400 high-power field). The number of IL-15⁺ cells/400x HPF is shown in graph B by using Alphelys Spot Browser 2 integrated system. Twenty independent tissue samples were investigated in each group. Images were taken with an Olympus UPlan FI objective lens (Tokyo, Japan), 409/0.75, ∞/0.17, and level of statistical significance is ****p<0.0001.

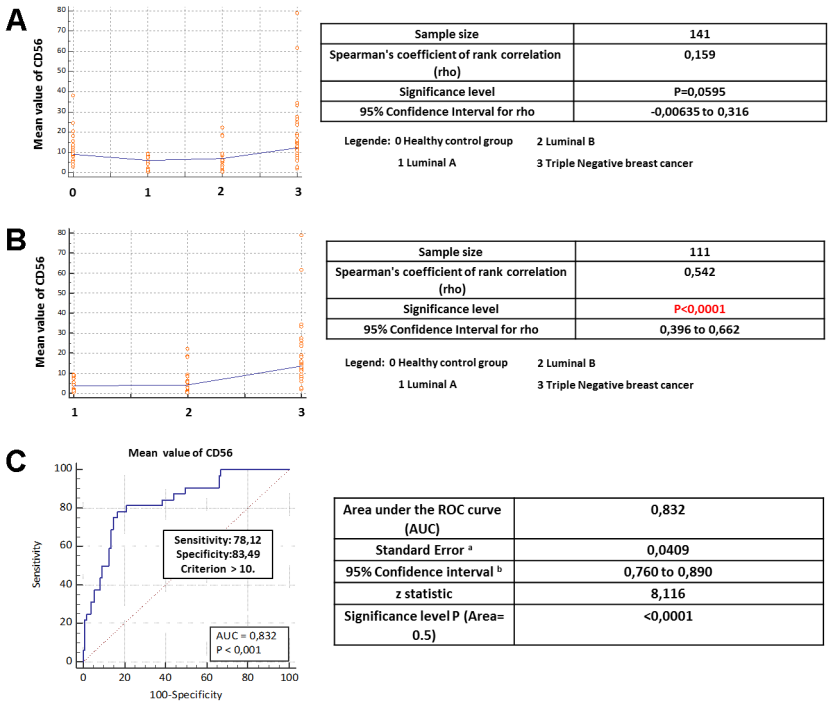

Figure 4. Scatter-diagram of the correlation between the mean value of CD56⁺ cells in the healthy control group and molecular subtypes of breast cancer (luminal A, luminal B, and triple-negative cancer). Table show Spearman's correlation coefficient (rho) of investigated variables (A). Scatter-diagram of the correlation between the mean value of CD56⁺ cells and different subtypes of breast cancer. The table shows Spearman's correlation coefficient (rho) of investigated variables (B). Graphic representation of the Area Under the ROC Curve (AUC) with criterion values for sensitivity and specificity. In the table, values for the Area Under the ROC curve (AUC) are shown (C).

criterion value of the CD56 was >10 with a sensitivity of 78.12 and specificity of 83.49. It connotes those patients with CD56 higher than 10 probably have triple-negative breast cancer.

Increased Apaf-1 and cytolytic mediator expression in luminal B and triple-negative breast cancer. The impaired apoptosis mechanism is associated with various pathologies, including different solid tumors [7, 52, 53]. Generally speaking, highly differentiated cells like skeletal myocytes, cardiomyocytes, and neurons show decreased levels of Apaf-1 (Apoptotic protease activating factor 1) protein expression impaling higher resistance of these cells to apoptosis in normal physiological conditions [52, 53] as well as in cancer where Apaf-1 downregulation promotes the survival of tumor cells [51, 52]. In contrast, in some tumors such as ovarian cancer, it was found the increased expression of a nonfunctional APAF-1 form that was unable to activate caspase-9 and form apoptosome that correlates with higher resistance to apoptosis [54]. Therefore, we can speculate that the presence of nonfunctional Apaf-1 might be responsible for the impaired apoptosis elicited by cytolytic mediators (TRAIL and Fas-L) causing the resistance of cancer cells. We analyzed the presence of Apaf-1 by immunohistology in all investigated groups. Positive staining (brown) for Apaf-1 was observed in the cells randomly distributed in the stroma tissue, cells near the glands, and tumor cells in breast cancer subtypes (Figure 5A). In contrast, healthy tissue samples were dominantly Apaf-1-negative (Figure 5A). Further, we observed cytoplasmatic Apaf-1 labeling in all investigated groups (Figure 5A). In addition, we determined the gene expression of cytolytic mediators (perforin, TRAIL, and Fas-L) in freshly isolated mammary core tissue samples by RT-qPCR (Figures 5B–5D). The expression of perforin mRNA was downregulated in luminal A and triple-negative breast cancer tissue and upregulated in the luminal B subtype, approximately five times (Figure 5B). The same trend was observed for TRAIL and Fas-L mRNA as they were upregulated in the luminal B subtype (Figures 5C, 5D).

Altered expression of activation CD69 receptor and CD107a in peripheral blood NK cell subsets of breast cancer patients. Human NK cells are effector immune cells characterized by cytotoxic activity and cytokine production as a main source of antiviral and antitumor response [29, 30, 32]. A pioneering study in human populations has shown that an increased level of lymphocytes' cytotoxic activity is associated with a lower incidence of cancer, in contrast to the decreased activity that is linked to an increased incidence of cancer because we performed a series of experiments to determine the activating status of peripheral NK cells in

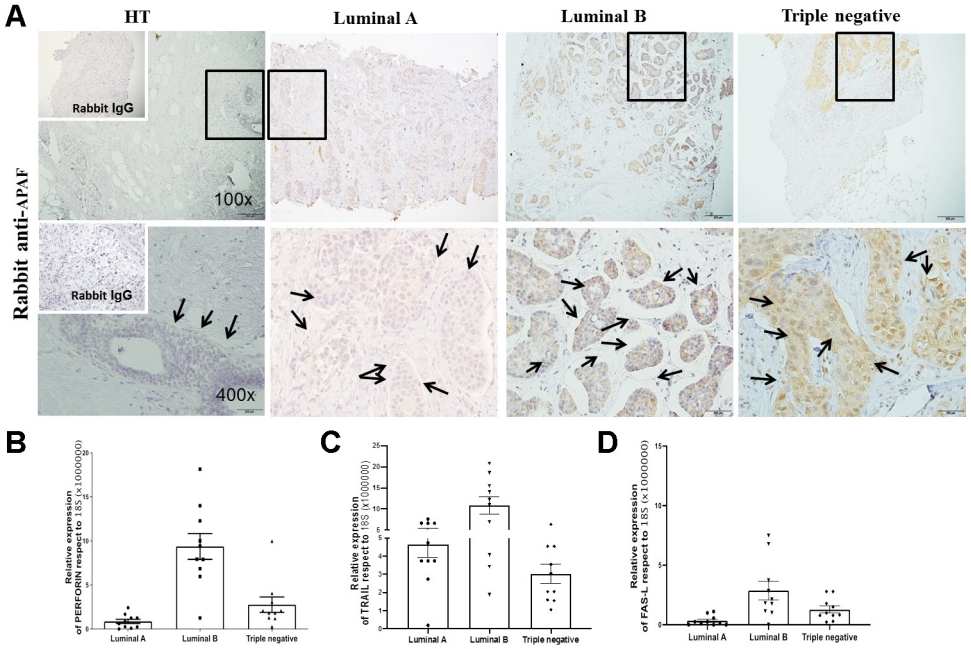

Figure 5. Apaf-1 expression in paraffin-embedded mammary gland tissue sections of breast cancer subtypes (luminal A, luminal B, and triple-negative cancer) and healthy control group were performed. Immunohistological labeling (A) using rabbit IgG anti-Apaf- 1 mAbs results in brown color development after using 3,3-diaminobenzidine (black arrows). The area of the lined quadrangle is shown at higher magnification (×400 HPF, lower row). Ten samples were present in each group. Graph B shows the mRNA expression of Perforin, TRAIL, and Fas-L determined by RT-qPCR in tumor tissue samples from different breast cancer subtypes (luminal A, luminal B, and triple-negative cancer). The results are expressed as fold changes of Perforin, TRAIL, and Fas-L mRNA levels relative to the housekeeping gene (18S). Twelve samples were analyzed per group.

molecular subtypes of breast cancer. Figure 6A shows the representative experiment of the analysis, the frequency of gated $CD3^{-}CD56^{+}$ cell population (NK cells; Region R2) was investigated within lymphocyte gate (Region R1) in the suspension of mononuclear peripheral blood cells from healthy donors and breast cancer patients (luminal A, luminal B, and triple-negative cancer). In the dot plot, the numbers represent the percentage of the particular analyzed marker. In the representative sample from healthy donors, $CD3^{-}CD56^{+}$ cell population was around 18.53% compared to the isotype control 0.014% (Figure 6A), which corresponds to 20% (18%; 23%) [median (25^{th} percentile; 75^{th} percentile)] of NK cells in eleven experiments analyzed. NK cells' frequency in luminal A [17% (14%; 18%)], luminal B [22% (17%; 25%)], and triple-negative breast cancer [23% (19%; 75%)] did not significantly differ from healthy control (Figure 6A). However, our results showed that the frequency of CD69 was significantly lower in women with luminal A breast cancer [2% (1%; 4%)] and triple-negative cancer [1% (1%; 3%)] than in healthy donors [7% (5%; 9%)] on $CD3^{-}CD56^{+}$ NK cells (Figure 6B, $^{**}p<0.01$). No differences of CD69 expression were found between the healthy donors and the luminal B subtype of breast cancer. Contrary, significantly higher percentage of CD107a degranulation marker on NK cells was found in luminal B breast cancer patients [29% (16%; 46%)], when compared to healthy donors [15% (8%; 20%)] and expressed as a median (25^{th}–75^{th} percentile of eleven experiments) (Figure 6C, $^{*}p<0.05$). In spite of that, no differences were found for CD107a expression in luminal A and triple-negative breast cancer patients compared to healthy donors and luminal B, respectively (Figure 6C).

Increased expression of NKG2A inhibitory receptor and decreased expression of NKp46 and NKG2C activating receptors on peripheral blood NK cell subsets in triple-negative breast cancer. To determine how luminal A, luminal B, and triple-negative breast cancer could influence the expression of activating and inhibitory receptors on NK cells to mediate the killing of tumor cells, we evaluated the NKp46, NKG2A, and NKG2C expression on peripheral $CD3^{-}CD56^{+}$ population in subtypes of breast cancer patients and healthy donors. Figure 7A shows the frequency of NKp46 activating molecules on NK cells by dot plots of the representative samples of healthy donors [56% (44%; 61%)], luminal A breast cancer [35% (24%; 46%)], luminal B breast cancer [25% (16%; 35%)], and triple-negative breast cancer [21% (18%; 22%)] and expressed as a median (25^{th}–75^{th} percentile of eleven experiments). The frequency of NKp46 significantly decreased in triple-negative breast cancer (Figure 7A, $^{****}p<0.0001$) and luminal B (Figure 7A, $^{***}p<0.001$) when compared to healthy donors. In luminal A breast cancer, the expression of NKp46 did not significantly differ among investigated groups. NKG2A expression on NK cells is present by

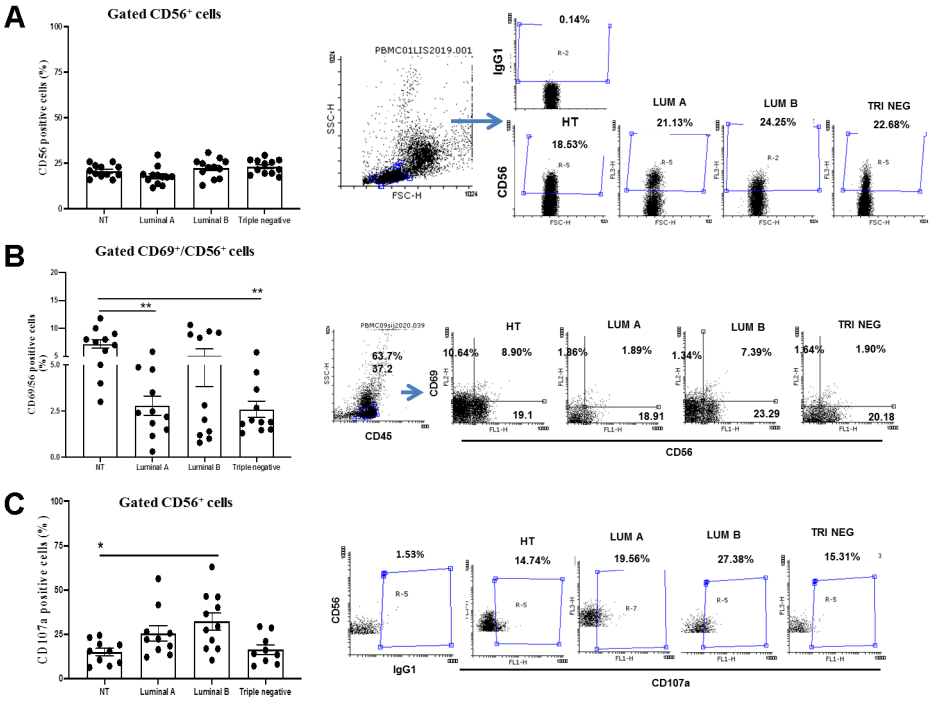

Figure 6. Flow cytometry of triple-labeled peripheral blood mononuclear cells (CD56⁺CD3⁻/CD107a⁺ or CD69⁺) from the healthy control group, luminal A, luminal B, and triple-negative cancer within the lymphocytes R1 gate. The R2 gate was set up with respect to CD3⁻CD56⁺ events in the R1 gate. The results are expressed in dot plots as the percentage of the CD56⁺ cell population with anti-CD69 mAb (B) or with anti-CD107a mAb (C) compared to isotype-matched controls. Eleven to twelve experiments were carried out per group and levels of statistical significance are shown as **p<0.01 and *p< 0.05. Abbreviations: FSC-forward-scatter; SSC-side-scatter

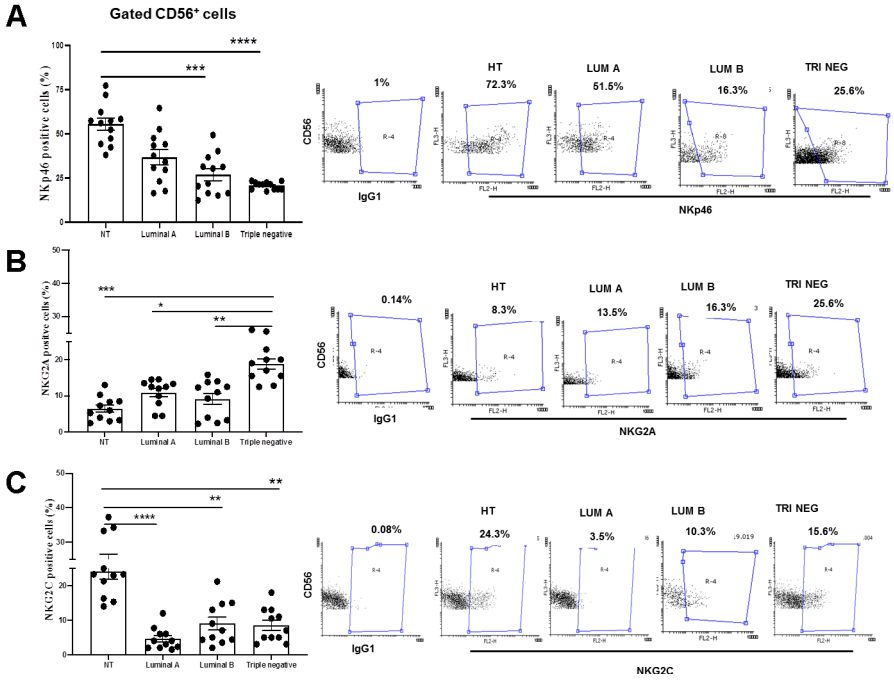

Figure 7. The percentages of the cell surface molecules NKp46 (A), NKG2A (B), and NKG2C (C) on gated CD56⁺/CD3⁻ cells were analyzed from the freshly isolated peripheral blood mononuclear cells of the healthy control group, luminal A, luminal B, and triple-negative cancer group. Dot plots illustrate the labeling of CD56⁺/CD3⁻ cells with antibodies of interest NKp46 (A), NKG2A (B), and NKG2C (C) in comparison to isotype-matched control. The results are present as the median of eleven to twelve independent experiments analyzed in each group. Levels of statistical significance are shown as ***p<0.0001, ***p<0.001, **p<0.01 and *p<0.05. Abbreviations: FSC-forward-scatter; SSC-side-scatter

dot plots of the representative samples of healthy donors [6% (3%; 8%)], luminal A breast cancer [12% (8%; 13%)], luminal B breast cancer [11% (3%; 13%)], and triple-negative breast cancer [19% (15%; 22%)] and indicated as a median of eleven experiments, Figure 7B. The expression of NKG2A inhibitory receptor on NK cells in investigated breast cancer subtypes, while it was significantly higher on NK cells of triple-negative breast cancer when compared to healthy donors (Figure 7B, ***$p<0.001$), luminal A (Figure 7B, **$p<0.01$) and luminal B (Figure 7B, *$p<0.05$). The expression of NKG2C activating receptor NKG2C on NK cells is present by dot plots of the representative samples of healthy donors [23% (16%; 33%)], luminal A breast cancer [4% (2%; 6%)], luminal B breast cancer [7% (4%; 14%)], and triple-negative breast cancer [7% (5%; 13%)] and expressed as a median of eleven experiments, Figure 7C). However, NKG2C percentage significantly decreased in luminal A (Figure 7C, ****$p<0.0001$), luminal B (Figure 7C, ** $p<0.01$), and triple-negative breast cancer (Figure 7C, **$p<0.01$) when compared to healthy donors (Figure 7C).

Discussion

The high heterogenicity of breast cancer presents a challenge to clinicians. At the present, used systemic therapy has significantly increased the survival rate of breast cancer patients, but relapse and distant metastases remain the cause of death [5–7, 9, 10]. The complex interaction between the immune system and tumor cells is of pivotal importance in controlling and eradicating cancer [7–10, 55].

Tumor-infiltrating lymphocytes (TILs) represent a potential biomarker for survival prediction in breast cancer patients [46–50] but the exact clinical importance is still unclear [56]. We focus our attention on TILs expression as well as on the NK cells' activation status in molecular subtypes of breast cancer at a systemic and local level. We set up this investigation using core tissue sample biopsies and peripheral blood samples of the same woman to analyze the cytotoxic status of NK cells in subtypes of breast cancer. This parameter represents the strength of our study because we used materials as previously mentioned before any chemotherapy, surgery, and radiotherapy.

Our results clearly indicate significantly decreased expression of TILs infiltration in normal mammary gland tissue samples compared to molecular subtypes of breast cancer (luminal A, luminal B, and triple-negative cancer) (Figure 1A). In agreement the with previous study, we confirmed the highest TILs infiltration in triple-negative cancer in comparison to hormone-positive receptor breast cancers (luminal A and luminal B) (Figure 1B). The luminal A subtype presents lower TILs infiltration in tumor stroma compared to luminal B and triple-negative cancer (Figure 1B). In addition, many studies have reported that an intense TILs infiltration is associated with the progression of the disease [5, 48, 49]. Increasing levels of TILs have been associated with improved therapy outcome in HER2+ breast cancer and/or triple-negative breast cancer patients [57]. Furthermore, we observed a highly similar distribution pattern of $CD56^+$ cells in the core tissue sample of healthy donor and breast cancer subtypes (Figure 2A). CD56 protein marker was found in the cytoplasm, on the cell membrane, and on cells lining blood vessels, compared to isotype-matched mouse IgG1 (Figure 2A). The number of $CD56^+$ cells was significantly increased in triple-negative breast cancer in comparison to the control group and luminal A and B breast cancer group (Figure 2B). Surprisingly, we found a significantly higher number of $CD56^+$ cells in the control group compared to luminal A and luminal B breast cancer (Figure 2B). None of the studies showed the correlation of NK cell expression between the molecular subtypes of breast cancer and ROC curve to distinguish between different diagnoses. We show that a high level of CD56 expression was associated with a more malignant subtype of breast cancer (Figure 4B). Also, we found that the CD56 marker is a very good measure for distinguishing diagnoses between triple-negative and hormone-positive subtypes of breast cancer (Figure 4C). A high frequency of NK cells or CD8-positive T cells has been reported to correlate with prolonged survival in patients with various cancers [48–50].

Our immunohistological analysis of CD56 and IL-15 could indicate that a higher infiltration of NK cells in the triple-negative tumors correlated with an abundant expression of $IL\text{-}15^+$ cells (Figures 3A, 3B). Our results show that IL-15 was significantly increased in all investigated groups of breast cancer compared to the control group probably due to abundant infiltration of immune cells especially macrophages (Figures 3A, 3B). IL-15 is a component of the inflammatory milieu in TME and could provide an appropriate activation signal for the survival of NK cells [37, 38]. However, we emphasized that this method is not a suitable solution for determining the biological role of secreted IL-15 form and its possible influence on the neighboring cells. Perhaps, also tumor cells represent a large source of IL-15 that lies hidden in the tumor stroma (Figure 3A) and do not have the possibility to affect NK cells' function and proliferation [37]. Some studies showed that induction using IL-15 in NK cells increased the expression of activating receptors (NKp30, NKp46, NKG2C, and NKG2D) and CD107a degranulation marker [58, 59]. This upregulation is associated with a concomitant increase in NK cell activity [58–60]. So, tumor-sequestrating IL-15 adopts the strategy to keep NK cells in a "sleep mode" [22, 37]. Our results show that despite the increased expression of IL-15 locally mRNA of cytolytic mediators (perforin, TRAIL, and Fas-L) was found four time reduced in luminal A and triple-negative breast cancer compared to luminal B (Figure 5B). This phenomenon suggests the poor cytotoxic capacity of infiltrating NK cells due to particular tumor microenvironment and sequestered IL-15 by tumors. Cytolytic mediators TRAIL and Fas-L have the ability to activate both the extrinsic and intrinsic apoptosis pathways and induce cell-killing via

apoptotic protease activating factor 1 (APAF-1)-mediated activation of the initiator caspase 9 and formation of apoptosome [52, 53]. APAF-1 is a key component of the apoptosome that is present in humans and mice [52] and assembled in response to cellular stress, including DNA damage, hypoxia, and oncogene activation [53, 54]. APAF-1 is closely related to several oncogenes and tumor suppressor genes, including B-cell lymphoma-2 (Bcl-2) and p53 [61]. A series of studies have shown that APAF-1 protein is an important apoptotic factor that is abnormally expressed in cancer tissues and is downregulated in more severe histological-grade tumors [53, 54]. We determined the APAF-1 expression only in tumor cells by immunohistology. In the healthy control group and luminal A breast cancer tissue, APAF-1 expression was lower compared to luminal B (Figure 5A). Abundant expression of APAF-1 was found in tumor cells of triple-negative breast cancer that could suggest the possible site of action for cytolytic mediators (Figures 5A, 5B) but their mRNA level was decreased.

Regarding above mentioned, we analyzed the distribution and activation status of peripheral blood NK cells in different breast cancer subtypes. Our results did not find any alteration in the percentage of $CD56^+$ cells in healthy donors and investigated breast cancer groups (Figure 6A) in agreement with a previous study [51, 59, 60]. Our result showed a significantly decreased frequency of NKp46 on $CD56^+$ NK cells in more malignant subtypes of breast cancer (luminal B and triple-negative breast cancer) compared to the healthy control group (Table 2), which could indicate a lower capacity of cellular killing. It's already known that the downregulation of NKp46 expression could be provoked by circulating tumor cells [31, 62]. A probable adopted mechanism of tumor cells to suppress NKp46 is by shedding soluble MHC class I chain-related molecules or by secreting prostaglandin E2 [31, 32]. Downregulation of another activating receptor NKG2C, found in different tumors, including breast cancer, ovarian cancer, and colorectal cancer [30, 32, 63], may result in an impaired immune response against tumor cells [64]. We found that breast cancer patients showed a decreased expression of NKG2C in all investigated subtypes of breast cancer, which may indicate a possible evasion mechanism for tumor cells to prevent the NK cell lysis (Figure 3A). In addition, the activation status of NK cells (CD69/CD56) was also significantly decreased in luminal A and triple-negative cancer compared to the healthy control group (Figure 6B). However, the degranulation marker CD107a did not significantly differ among the breast cancer group. Inhibitory receptor NKG2A was significantly increased in triple-negative cancer compared to the other investigated breast cancer group. Tolerogenic NK cells in malignant types of breast tumors result in weakened cellular immunity on a systemic level [64–66].

Our results showed that NK cells, present in breast cancer patients, decrease the expression of cytotoxic receptors NKp46 and NKG2C, degranulation marker CD107a, and activation marker CD69, which indicates that tumor cells somehow could bypass or escape NK cell surveillance. In addition, they upregulate the inhibitory NKG2A receptor by reducing their ability to kill. An increased immunosuppressive cytokine milieu could result in decreased expression of cytolytic mediators (TRAIL and Fas-L), which may be the reason for the absence of a cytotoxic immune response. Future mechanistic studies will be necessary to determine the role of the immunosuppressive NKG2A marker as well as the axis of action between NK cells and IL-15 in breast cancer patients.

Limitations of the study. A limitation of the current study is not so big number of patients involved. Although we found statistically significant results, future studies should be directed to extend these results and provide further confirmation and deeper molecular analysis of NK cell subpopulations in molecular subtypes of breast cancer. In conclusion, our results showed that NK cells in the more malignant breast cancer investigated groups display tolerogenic properties by alternating receptor repertoire promoting upregulation of NKG2A (Table 2). In addition, the cytolytic activity was found to be decreased in triple-negative breast cancer, although NK cells abundantly infiltrate tumor stroma, which is rich in IL-15 (Table 2). Our data suggest that reduced killing capacity may be caused by an impaired expression of TRAIL and Fas-L NK cell dysfunction could be a reasonable explanation for the higher cancer risk development [66, 67].

Moreover, our data provide evidence that $CD56^+$ cells could be a possible biomarker for distinguishing triple-negative from luminal A and luminal B breast cancer. This accentuates NK cells as a suitable target for triggering a potent anti-tumor response improving their cytotoxic function.

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