Trastuzumab induced a high rate of pathological Complete Response (pCR) in patients affected by locally advanced HER2-positive Breast Cancer (HER2-BC), by exploiting immune-mediated mechanisms as Antibody-Dependent Cell Cytotoxicity (ADCC) involving Natural Killer (NK) cells. Host’s immune genetics could influence the response to therapy, through the expression of variants that characterize NK receptors involved in ADCC effectiveness. Killer cell immunoglobulin-like receptors (KIRs) modulate NK cell activity through their binding to class-I Human Leukocyte Antigens (HLA). The impact of the KIR/HLA repertoire in HER2-BC is under study. We characterized KIR genotypes of 36 patients with locally advanced HER2-BC treated with neoadjuvant chemotherapy including trastuzumab. We monitored pCR achievement before surgery and Disease-Free Survival (DFS) and Overall Survival (OS) after adjuvant therapy. HLA, and Fc gamma receptor IIIa (FcγR3A) and IIa (FcγR2A) were genotyped through targeted PCR and Sanger sequencing in 35/36 patients. The KIR-HLA combinations were then described as functional haplotypes and divided in two main categories as inhibitory tel A and stimulatory tel B. Trastuzumab-dependent ADCC activity was monitored with an in vitro assay using HER2-BC model and patients’ NK cells. We observed a higher frequency of KIR activators in patients who achieved a pCR compared to partial responders. During the study of functional haplotypes, individuals carrying a tel B haplotype showed greater ADCC efficiency than tel A cases. In subjects with the tel A haplotype the presence of the favorite V allele in FcγR3A receptor improved their low ADCC levels. Regardless of the haplotypes detected, the presence of KIR3DL2/HLA-A03 or A11 was always associated with the FcγR3A V allele, and therefore correlated with greater ADCC efficiency. However, this particular KIR receptor appeared to harm DFS and OS. Indeed, patients with tel B
INTRODUCTION

At present, Neoadjuvant Chemotherapy for the treatment of locally advanced HER2-positive Breast Cancer (BC) includes trastuzumab (Herceptin), a humanized IgG1 monoclonal antibody (mAb) targeting human epidermal growth factor receptor-2 (HER2). The receptor triggers downstream signal by forming heterodimers with other HER family members [HER1 (also named Epidermal Growth Factor, EGFR), HER3 and HER4], leading to auto- and/or trans-phosphorylation of definite tyrosine residues within the cytoplasmatic domain of these receptors, thus providing signaling cascades promoting cell proliferation and survival. By blocking the extracellular ligand domain of HER2, trastuzumab antagonizes the functional activity of the receptor and the cleavage of the extracellular domain of HER2 leading to a downmodulation of the function and formation of HER heterodimers. In addition, accumulating evidence suggested a relevant role of trastuzumab in the engagement of immune cells, such as Natural Killer (NK) cells expressing the Fc receptor CD16, which through antibody-mediated cellular cytotoxicity (ADCC) may result in the killing of target cells expressing HER2 (1). Trastuzumab is used in about 20 to 30% of BC, achieving high rates of pathological Complete Response (pCR) (2). The achievement of a pCR after NC has been associated with long-term survival, thus becoming a surrogate endpoint and a potential prognostic biomarker (3). Moreover, trastuzumab treatment contributed to the induction of an improved Disease-Free Survival (DFS) when used in the adjuvant setting for 1 year (4). We and others showed that a higher proficiency of both, innate and adaptive host immunity, characterized patients with BC achieving a pCR compared to partial responders (5–7). This evidence suggests that a better knowledge of the host immunity in this setting may be relevant for the identification of suitable biomarkers able to predict pCR and with possible prognostic significance.

Several studies have reported a correlation between clinical response and polymorphisms of Fc gamma receptor IIIa (FcγR3A, or CD16A) on NK cells, which recognizes the Fc portion of mAbs mediating ADCC as trastuzumab, bound to the tumor cells (8). As we noticed previously, patients carrying the FcγR3A-V-allele (9), responsible for a stronger affinity to Fc, showed an increased ADCC activity compared to the F allele (10, 11). The importance of ADCC effect in BC treated with trastuzumab was also demonstrated through the evidence of a dramatic reduction of its efficacy in knockout mice for FcγR3A (12).

NK cell activation depends also on the interaction with other receptors like the killer cell immunoglobin-like receptors (KIRs), which bind the peptide-binding region of several class-I Human Leukocyte Antigens (HLA class-I) (13). Several studies have demonstrated that the absence of HLA class-I on tumor cells, or their decreased expression, damped the HLA-inhibitory KIRs interaction thus improving NK cell activation, including the ADCC activity (14, 15). KIR positivity is acquired late during NK cell maturation, resulting expressed mainly by the most mature CD16bright CD56dim NK cells. KIR family members consist of 13 activating and inhibitory genes and 2 pseudogenes (KIR2DP1, and KIR3DP1), which separate the KIR genotype into two halves: the centromeric and the telomeric half. KIR gene region shows a difference in KIR gene contents and polymorphisms, and functions only in the presence of specific cognate HLA ligands, currently still not completely described (16), thus leading to a vast KIR functional repertoire where the inhibitory variants are prevalent (16–18). The number and type of KIR genes define different KIR haplotypes, which have been classified into two main groups, termed “A” and “B” genotypes (16). The A genotype is mainly constituted of the inhibitory receptors and the only activating 2DS4 KIR, while the B genotype shows a variable number of activating KIR.

Accumulating evidence suggested that different KIR/HLA gene combinations and HLA expression levels can influence tumor prognosis and treatment response (19). Studies employing the anti-KIR mAb lirilumab in combination with rituximab demonstrated a higher ADCC efficiency mediated by NK cells against lymphoma in vitro and in vivo, due to the interruption of the binding between inhibitory KIRs and their ligand and the consequent inhibitory signal (20, 21). Interestingly, using KIR-ligand-mismatched NK subsets Ehlers and colleagues demonstrated a stronger degranulation of NK cells against BC cells in the presence of trastuzumab (1). Moreover, Terszowski et al. demonstrated that both KIR/HLA interaction and the FcγR3A V-allele may act synergistically to improve NK cell activation in vitro (11, 22). However, the influence of particular KIR haplotypes in the efficiency of neoadjuvant trastuzumab for the treatment of BC remains unaddressed (10).

On these grounds, the present study aimed at exploring the possible correlation of KIR/HLA haplotypes with the induction of a pCR after NC in a cohort of patients affected by locally advanced BC overexpressing HER2 and treated with trastuzumab. We focused in particular on the efficiency of the ADCC mediated by NK cells and assessed the potential synergic impact of KIR haplotype and FcγR3A polymorphisms.

Keywords: KIR, HLA, breast cancer, trastuzumab, ADCC
Moreover, we evaluated whether the identification of such a predictive haplotype could have a prognostic impact in the same cohort of patients also in the follow-up after surgery and adjuvant chemotherapy.

MATERIALS AND METHODS

Patients and Biological Samples

The present study analyzed the impact of genetic KIR/HLA combination performed in blood samples obtained from 36 patients affected by locally advanced HER2-overexpressing breast carcinoma (defined as not susceptible of conservative surgery at diagnosis; UICC, International Union Against Cancer, stage II to III) and included in the phase II CRO Clinical Trial, NCT02307227 (9). HER2 status was assessed by immunohistochemistry (IHC) and chromogenic in situ hybridization or fluorescence in situ hybridization in the case of IHC 2+. Almost all patients (34/36, 94.4%) showed overexpressed HER2 oncoprotein with a strong IHC score (IHC 3+); 2 patients (2/36, 5.6%) showed a weakly positive IHC score (IHC 2+) but were ISH positive for HER2 gene amplification. All patients had the following clinical features: Eastern Cooperative Oncology Group (ECOG) performance status of 0 or 1; baseline left ventricular ejection fraction greater than 50%; adequate organ function (bone marrow function: status of 0 or 1; baseline left ventricular ejection fraction greater than 50%); transaminases <2.5 times ULN, alkaline phosphatase ≤2.5 times ULN, serum creatinine ≤1.5 times ULN). Patients received neoadjuvant chemotherapy with trastuzumab (loading dose 4 mg/kg intravenously, then 2 mg/kg weekly) and concomitant weekly Paclitaxel (80 mg/m2) for 3 cycles, followed by clinical evaluation and, in case of clinical response, 3 more cycles to obtain a pCR. After neoadjuvant chemotherapy, patients underwent primary surgery (mastectomy or conservative treatment) as well as axillary node dissection. In selected patients based on tumor pathological features, post-mastectomy radiation was performed to the chest wall and in addition at the axillary region when they showed more than 3 positive lymph nodes. After surgery, adjuvant chemotherapy with 3 more cycles of trastuzumab and paclitaxel (12 weeks) was planned, and trastuzumab alone every 3 weeks was continued for a total of 1 year, together with hormonal therapy for 5 years in the case of Estrogen Receptor (ER) and/or Progesterone Receptor (PgR) positive tumor. The instrumental evaluation was performed at baseline and every 12 weeks. Patients’ follow-up was monitored for a maximum of 120 months. This study was conducted according to the ethical principles of the Declaration of Helsinki and approved by the local Ethical Committee (Comitato Etico Indipendente del CRO di Aviano, May 29, 2006). Written informed consent was obtained from all patients.

Blood samples were collected from each patient at diagnosis, at the 12th and 24th week of neoadjuvant treatment, and 2, 6, and 12 months of follow-up. Samples were transported at room temperature and processed within 5 hours. Genomic DNA was purified using the DNA extraction kit (EZ1 DNA Blood 350 µl kit, Qiagen, Valencia, CA) from blood samples obtained at diagnosis from all patients. Peripheral blood mononuclear cells (PBMCs) were freshly isolated from heparinized blood of patients by Ficoll-Hypaque gradient (Lymphoprep, Fresenius Kabi Norge Halden) using standard gradient separation. Cells were washed in PBS (Biomerieux), counted using Trypan blue (viability >90%), and viable frozen (90% heat-inactivated Fetal Bovine Serum [Gibco®, Life Technologies] and 10% DMSO) at −80°C for 24 h and then in liquid nitrogen until use. After thawing in RPMI-1640 medium (Sigma-Aldrich) with 3 µg/ml Deoxyribonuclease (Sigma-Aldrich), cells were washed in PBS (Biomerieux) and counted again to check the viability.

ADCC Assay, Flow Cytometry and Analysis of FcγR Receptor Polymorphisms

The trastuzumab-dependent ADCC efficiency, the NK cells number quantification, and the immunogenetic analysis of FcγR polymorphisms were evaluated as already described (9). Briefly, the cytotoxic activity mediated by trastuzumab was quantified in a Calcein release assay, using the HER2/neu-overexpressing breast cancer cell line MDA-MB453, as target cells, and as effectors, PBMCs obtained from patients at diagnosis (n=34), and at the 12th (n=31), and the 24th (n=28) week of treatment. Calcein-acetomethoxy (AM) (Molecular Probes, Eugene, Oregon, USA)-labeled 10,000 target cells (target cells/patients PBMCs, ratio 30:1) were plated in triplicates into 96-well plates after treatment with trastuzumab at 20 µg/ml for 1 hour in ice (Roche, Basel, Switzerland). After 4 h at 37°C and 5% CO2, the release of Calcein (excitation = 485 nm; emission = 530 nm) was measured with a fluorescence plate reader (SpectraFluor Plus, Tecan, Männedorf, Switzerland). Maximal and spontaneous Calcein release values were obtained by adding either 100 µl lysis buffer (NaBO3 0.025 M, Triton X-100 0.1%, pH 9) or HBSS, to wells containing 1 × 10,000 labeled target cells. The percentage of calcein release (CalR) was calculated as follow: CalR (%) = (experimental CalR – F0/Fmax – F0) x100, where Fmax referred to the maximal calcein released after the lysis of cells by adding 100 µl lysis buffer to the medium, and F0 referred to spontaneous calcein released from cells in the medium.

NK cell percentage from PBMCs count was obtained by flow cytometry protocols, across multiple time points: at diagnosis; 12th-24th-week; 2-, 6- and 12-months follow-up. NK cells were labeled with α-CD3 phycoerythrin-texas red (mouse IgG1, clone UCHT1; Beckman Coulter), α-CD16 FITC (mouse IgG1, 3G8; Beckman Coulter), and α-CD56 PE (mouse IgG1 k, B159; BD Biosciences) antibodies and quantified on Cytoomics FC500 flow cytometer (Beckman Coulter, Fullerton, CA, USA) with the CXP software (Beckman Coulter).

Finally, the percentage of cell lysis [lysis (%)], was assessed by using the CalR (%) normalized for 10,000 NK cells: Normalized ADCC=(CalR(%)×10,000) /[30*10,000*NKcell(%)], where 10,000 is the number of target cells in each well, 30 corresponds to the Effector: Target ratio and NK cell(%) is the percentage of NK cells quantified at the specific time point.

Genotyping at the FcγR locus was performed on genomic DNA by polymerase chain reaction (PCR) followed by direct
sequencing to determine Single Nucleotide Polymorphisms (SNPs) variants at FcγR3A-158 and FcγR2A-131. The FcγR3A-158, indicated as V>F variant, was investigated through a nested PCR first using the forward primer 5′-TTGAAAGGCCATGCTCAGTAAT-3′ and the reverse primer 5′-AGGCTGTTGCTACAGAACCCTA-3′ to amplify a fragment of 1699 bp; and then the forward 5′-TTACAGAATGGCACAAGGCAG-3′ and the reverse 5′-TCTCCTCCCACTCAACTTCC-3′ primers, to generate a 238 bp fragment. The FcγR2A-131, H>R variant, was analyzed through a single PCR using the forward primer 5′-CTGGTCAAGGTCACTCTTCC-3′ and the reverse 5′-CAATTGCTGCTATGGGC-3′ (277 bp fragment). The PCR products were purified and directly sequenced using the BigDye Terminator sequencing kit and an ABI Prism 3100 sequencer (both from Applied Biosystems, Foster City, CA).

**PCR-SSP KIR Typing**

Genomic DNA was used to determine the genotype of the 13 functional KIR genes and the 2 pseudogenes by sequence-specific primer (SSP) polymerase chain reaction (PCR). Our laboratory had developed the procedure to consent multiplex PCR combinations of KIR pairs (17). Primers were assigned to 15 multiplex reactions, resulting in 30 amplicons that allowed the detection of all KIR genes as previously reported (17). The amplified products were analyzed on a 4% agarose gel and photo-documented.

**Frequencies of Alleles, Genotypes, and Haplotypes**

KIR gene profiles were determined by the presence or absence of each KIR gene. KIR2DL1, KIR2DL2/KIR2DL3, KIR2DL4, KIR2DS2, KIR2DS3, KIR2DS4 (-full and -del variant), KIR2DS5, KIR2DL5, KIR2DS1, KIR3DL1/KIR3DS1, KIR3DL2, and KIR3DL3, in a given individual. All genotypes contained KIR2DL4, KIR3DL2, and KIR3DL3 as framework genes. Carrier frequencies of KIR genes and genotypes were calculated as their percentage of the total number of individuals. The frequencies of genotypes or haplotypes were calculated by direct counting, alleles duplicated on a single haplotype were not included, and absence was counted as a distinct allele. The composition and frequencies of haplotypes were determined using the Haplotype Analysis software (Forest Genetics and Forest Tree Breeding, Georg-August University Goettingen, Germany), distributed by the authors Eliades N-G., Eliades D. G)

In the assessment of the KIR genotype, group B genotypes were individually determined by direct counting of the individual who tested positive for a specific (pair of) gene. Patients included in the study were all Caucasian. Differences in frequencies were estimated by using Yates-corrected Chi-square, Fisher’s exact test, and Jonckheere-Terpstra trend test for categorical variables. Haplotypes were determined by using the Haplotype Analysis software (Forest Genetics and Forest Tree Breeding, Georg-August University Goettingen, Germany). The Simpson Diversity Index was applied to evaluate the variability of haplotypes within pCR and partial pathological response (pPR) groups. T-test, Anova, and Kruskal-Wallis tests were used for non-parametric values. Kaplan-Meier method was used to analyze disease-free survival (DFS) and overall survival (OS) time from surgery (MedCal software package). A p-value of <0.05 was considered significant.

**RESULTS**

**Patients and Tumor Characteristics**

Thirty-six women (median age 46 years, range 24–72, 23 (63.9%) under age 50) affected by a HER2-overexpressing locally
advanced breast cancer were consecutively included in the present study. Table 1 shows the major clinical parameters of the global case study. Almost one-half of patients had a hormone receptor-negative tumor (47.2%), and the majority were classified as stage IIB (63.9%). Twenty patients (20/36, 58.3%) had undergone radiotherapy; breast-only radiotherapy was administered in 16 patients, 4 patients with more than 3 positive-lymph nodes received additional radiotherapy for axilla. A patient undergoing axillary radiotherapy had completed the subsequent adjuvant chemotherapy protocol consisting of 3 cycles of Paclitaxel and trastuzumab and then trastuzumab alone for one year, the remaining 3 patients received another treatment with anthracycline-containing chemotherapy(ECx4). All tumors showed a ductal histotype (not shown). According to Response Evaluation Criteria in Solid Tumors (RECIST) criteria, a total of 16 pCR (44.4%) were achieved after neoadjuvant chemotherapy, the breast-conserving surgery rate was 38.9% (14/36). The induction of a pCR statistically was not correlated with younger than 50 years (P=0.471). Due to the potential prognostic impact of pCR and to show a reduced number of different haplotypes compared to pCR (Simpson Diversity Index: 0.06 for pPR and 0.03 for pCR). KIR haplotypes were grouped in centromeric (cen) and telomeric (tel) following the recommendations from the 2011 KIR workshop (24). Briefly, cent A was characterized by the presence of 2DL3 and 2DL1, and cent B by at least one of the genes 2DS2, 2DL2. Tel A was characterized by 3DL1 and only one activating KIR gene, the 2DS4, while tel B had several activating genes (i.e. 3DS1, 2DS1, 2DS3, and/or 2DS5).

KIR Haplotyping
We determined the KIR gene for all 13 KIR genes, including the KIR2DL4full and KIR2DL4del variants, and 3 framework KIRs.

The frequency of KIR genes was compared between 16 pCR and 20 pPR cases (Table 2 and Figure 1). Framework genes (KIR3DL3, KIR3DL2 and KIR2DL4) were presented in all cases. KIR2DL1 and KIR2DL3 (centromeric region) were present in all pCR cases, KIR2DS3 and KIR2DL5 (both centromeric and telomeric) and KIR2DS1, KIR2DS3, KIR2DS5, and KIR3DS1 (centromeric) were more presents in the pCR cases, while KIR2DS4full variants (telomeric) showed a lower frequency in the pCR cases compared to pPR (Figure 2). However, none of the KIR genes reach a significant difference in the frequencies between patients achieving a pCR response compared to those with pPR.

We observed 16 different KIR haplotypes in the population, of them 5 were present only in pCR (i.e. haplo-6, haplo-10, haplo-12, halo-15, haplo-16) and 4 were present only in pPR (i.e. haplo-1, haplo-2, haplo-4, haplo-13) (Table 3). The distribution of overall haplotypes was different between the two groups and pPR showed a reduced number of different haplotypes compared to pCR (Simpson Diversity Index: 0.06 for pPR and 0.03 for pCR). KIR haplotypes were grouped in centromeric (cen) and Telomeric (tel) following the recommendations from the 2011 KIR workshop (24). Briefly, cent A was characterized by the presence of 2DL3 and 2DL1, and cent B by at least one of the genes 2DS2, 2DL2. Tel A was characterized by 3DL1 and only one activating KIR gene, the 2DS4, while tel B had several activating genes (i.e. 3DS1, 2DS1, 2DS3, and/or 2DS5).

Cent A genotype in homozygous (AA) was found in 43.7% (7/16) of pCR and 50.0% (10/20) of pPR. Tel A in homozygous (AA) in 37.5% (6/16) pCR and 65.0% (13/20) in pPR, respectively.

These data indicated a trend towards a higher number of activator KIR genes (tel B) in patients achieving a pCR; the difference between the two groups is nearly statistically significant (Fisher exact test, p=0.1787) (Figure 1).

Comparisons of KIR Genes and Their Cognate HLA Ligands Between pCR and pPR Groups
All HLA genes characterizing the KIR-HLA functional units were analyzed in 35 patients (one HLA typing in the pCR group did not amplify with the standard PCR used) (Table 4). Results showed the lowest correlation between pCR and pPR for HLA-A*11 and HLA-C*04 (Chi-square 1.4583 and 0.8929, respectively). The Odds ratio calculated was 3.800 (95%CI 0.68-21.13, P=0.127), and 2.02 (95%CI 0.65-6.25, P=0.22) for HLA-A*11 and HLA-A*C04, respectively.

Comparison of the numbers of KIR-HLA ligands combinations showed an increased frequency of 2DS3+/HLA-CI+ (chi-square 3.055), 2DS5+/HLA-C2+ (chi-square 1.6406), 3DS1+/HLA-Bw4+ (chi-square 0.9537) and 2DS1+/HLA-C2+ (Chi-square 0.7595) in the pCR group compared to pPR patients (Table 4). Overall these combinations exerted an activating NK function and KIR genes were included in the tel B genotype. Only the 3DL2+/HLA-A03+ or -A11+ combination led to a slight increase in the frequency of inhibitor-based interactions in the...
### TABLE 2 | Comparison of the frequencies of KIR genes in NC treatment response.

| KIR gene | CR (n=16) | RP (n=20) | Chi-square | P value* |
|----------|-----------|-----------|------------|----------|
| **A haplotype associated** | | | | |
| 2DL1 | 16 (100.0%) | 18 (90.0%) | – | – |
| 2DL3 | 16 (100.0%) | 18 (90.0%) | – | – |
| 3DL1 | 15 (93.8%) | 20 (100.0%) | – | – |
| 2DS4 | 15 (93.8%) | 20 (100.0%) | – | – |
| 2DS4full | 4 (25.0%) | 8 (40.0%) | 0.2387 | 0.625150 |
| 2DS4del | 13 (81.3%) | 17 (88.0%) | 0.1606 | 0.688614 |
| 2DS4fuldel | 15 (93.8%) | 20 (100.0%) | – | – |
| **B haplotype associated** | | | | |
| 2DS1 | 7 (43.8%) | 6 (30.0%) | 0.3728 | 0.541483 |
| 2DS2 | 9 (56.3%) | 10 (50.0%) | 0.0355 | 0.850554 |
| 2DS3 | 5 (31.3%) | 2 (10.0%) | 1.6406 | 0.20024 |
| 2DS5 | 6 (37.5%) | 5 (25.0%) | 0.2912 | 0.589465 |
| 3DS1 | 8 (50.0%) | 7 (35.0%) | 0.4663 | 0.493329 |
| 2DL2 | 9 (56.3%) | 10 (50.0%) | 0.0355 | 0.850554 |
| 2DL5 | 10 (62.5%) | 7 (35.0%) | 2.2900 | 0.130213 |
| 2DS4full | 4 (25.0%) | 8 (40.0%) | 0.2387 | 0.625150 |
| 2DS4del | 13 (81.3%) | 17 (88.0%) | 0.1606 | 0.688614 |
| 2DS4fuldel | 15 (93.8%) | 20 (100.0%) | – | – |

2DS4full: KIR2DS4 full-length variant, 2DS4del: KIR2DS4 deleted variant, 2DS4fuldel: both KIR2DS4 full-length and deleted variant.

*not valuable for statistic analysis,*P Yates’ corretion, value < 0.05 was considered as statistically significant.

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**FIGURE 1** | The histogram shows the average number of activating KIR genes for each individual as a function of the pathological Complete Response (pCR) (n=16) and pathological Partial Response (pPR) (n=20) to Neoadjuvant Chemotherapy (NC) treatment (P=0.125). The number of activating KIRs appeared higher in patients who achieved pCR. P-value was calculated using the ANOVA test. Bar height and error bars represent the mean ± standard error mean (SEM) for the data set.
pCR group (chi-square 1.7262) (Table 4). None of these combinations alone reached a statistical significance.

**Comparisons of KIR/HLA Functional Haplotypes Between pCR and pPR Groups**

Based on the above-reported results we performed haplotype analysis of functional KIR/HLA gene combinations including the KIR2DL5 gene, whose HLA ligand is unknown, and the 2 framework genes (i.e. KIR2DL4 and KIR3DL3).

We found 27 different haplotypes (Table 5): 11 haplotypes and 13 haplotypes were uniquely found in the samples of pCR and pPR groups, respectively; 11 haplotypes were shared between pCR and pPR patients. Results indicated a reduction in the number of haplotypes shared between pCR and pPR groups using functional haplotyping (n=11) compared to KIR gene haplotyping (n=25) (Tables 5, 3). We added the extension – Func to the centromeric and telomeric genotypes to underline the KIR+/HLA+ functional variants.

Cent A-Functional genotypes in homozygous (AA- Func) were found in 60.0% (9/15) of patients achieving a pCR and in 65.0% (13/20) of patients achieving a pPR, moreover, 2 patients with pPR showed a Cent B- Functional genotype in homozygous (BB- Func).

Tel A- Functional genotypes in homozygous (Tel AA- Func), without any functional activating KIR genes in the telomeric region, excepted the KIR2DS4full/HLA-A11, were found in 46.7% (7/15) of pCR, and 70.0% (14/20) of pPR cases.

By analyzing the haplotypes associated only with patients achieving pCR and pPR separately, we observed that 3DL2+/HLA-A03+ or A11+ were associated specifically with pCR carrying the Tel AA- Func genotype (Fisher exact test 0.008). Moreover, while KIR3DL2+/HLA-A03+ or A11+ was observed in 37.5% (3/8 tel B- Func) of unique pCR, KIR3DL2+/ligand+ was observed in 50.0% (3/6 tel B- Func) of unique pPR patients and was absent in patients with haplotype shared between pCR and pPR groups. Overall KIR3DL2+/ligand was observed in 40% (6/15) of patients with pCR and 15% (3/20) of those with a pPR (all carrying a tel B- Func genotype) (Fisher exact test p= 0.1157).

**Effects of tel A-F Genotype, KIR3DL2+/HLA-3 or 11 and FcR Combinations on ADCC Efficiency**

To examine whether functional KIR/HLA genes included in the telomeric tel region and FcR polymorphisms influence ADCC efficacy *in vitro*, we determine the correlation between specific KIR/FcR combinations and normalized ADCC levels obtained in vitro using PBMCs of the corresponding patients (9).

We observed that patients carrying the telomeric tel B- Func genotype, which includes several activating KIR, had a better ADCC efficacy than patients with the tel A- Func genotype [21.53 (95%CI 13.09-30.01) vs 12.92 (7.08-18.74), t-test p=0.07] (Figure 3A) and almost all combined with the FcγRIII V-allele (Tel B-F 12/14, 85.7%; Tel-A 7/19, 36.8%, Fisher exact test P= 0.0113). The consequence was altogether a more evident effect of FcγRIII V-carrier on *in vitro* NK cytolysis; higher cytolysis was observed in patients carrying the tel A-Func genotype in the presence of FcγRIII V-allele (F/F 4.6 [95%CI -4.33-13.55]; V/F 17.06 [95%CI 8.70-25.43]; V/V 21.9 [8.3-35.6] Jonckheere-Terpstra trend test, p=0.008) (Figure 3B).

The effect of FcR polymorphisms on ADCC was also evident by combining the FcγR3A and the FcγR2A polymorphic variants. Fcγ receptors differ in their affinity for the antibody Fc-fragment and cell-type expression; FcγR2A is mainly expressed on NK cells.
and monocytes, while the FcγR2A is expressed on monocytes, platelets, neutrophils, macrophages, and dendritic cells, but not on lymphocytes. FcγR3A had two variants, the FcγR3A-V and FcγR3A-F, and NK cells expressing the V variant demonstrated a stronger binding affinity for IgG-FC than the F-variant. FcγR2A has two variants (R131 and H131), which differ at amino acid position 131 in the extracellular domain with the FcγR2A-H variant showing a higher binding affinity for IgG1 and IgG2 than the R-variant (25). We observed a gradual increase in the ADCC efficacy, from FcγR3A-F/FcγR2A-H to FcγR3A-V/FcγR2A-R combination of FcγRIII and FcγR2A variants, that resulted statistically significant only in patients lacking the activating KIRs (tel A-F) (Figure 3C).

Interestingly, all patients carrying matched KIR3DL2+/ligand+ showed the FcγR3A V-carrier (n=9); PBMCs collected from these patients before treatment showed a higher ADCC efficacy compared with PBMCs collected from patients lacking this combination (Figure 4A). Thus, the highest increase in ADCC efficacy was noticed for PBMCs obtained from patients having the combination of both Tel A-Func and KIR3DL2+/ligand compared to those carrying the Tel A-Func with other KIR/HLA combinations (Figure 4B). The increase in cytolsis was also associated with the presence of the FcγR3A-V allele (Figure 4C). These results underlined the key role played by NK cell receptors as FcγR3A-V variant and specific KIR receptors in determining a better trastuzumab ADCC efficacy, while suggesting a minor role for receptors presented by other ADCC mediators, as the FcγR2A, predominantly expressed on monocytes and neutrophils.

### Analysis of CD16 and CD56 Expression Levels on NK Cells During Adjuvant Treatment According to patients KIR tel and KIR3DL2+/HLA Genotypes

We then investigated the potential contribution of KIR tel and KIR3DL2+/HLA genotypes on biological and clinical parameters measured after surgery during adjuvant chemotherapy and at follow-up.

As shown in Figures 5A, B, the expression levels of CD16, a marker of NK cell mature status, increased overtime in particular in patients carrying the tel B genotype, while the change in the CD56 expression levels on NK cells was inconsistent. The presence of KIR3DL2+/HLA-A03 or A11 gene combinations on the CD16 and CD56 expression levels had no substantial effect during the time.

### Influence of Haplotype tel and KIR3DL2+/HLA-3 or 11 on Clinical Outcome

Kaplan-Meier survival analysis showed no difference in DFS time for tel B genotype carriers compared to tel A carriers (Figure 6A). Interestingly, the absence of the functional tel KIR3DL2+/HLA-A3 or 11 combinations seemed to decrease the DFS compared to other KIR/HLA combinations (Figure 6B). The presence of both Tel B genotype and KIR3DL2+/HLA-A3 or 11 genes reduces DFS compared to Tel-B lacking the KIR3DL2+/HLA-A3 or 11 combinations (HR: 3.85, 95%CI 0.72-20.5, p = 0.1141 (Figure 6C).
Finally, the haplotype including the functional tel B genotype and the KIR3DL2+/ligand was significantly associated with a poorer OS compared to tel B haplotype without the 3DL2+/ligand as the reference (P = 0.0328, Figure 7), or Tel A without the 3DL2+/ligand combination as the reference (HR: 2.66, 95%CI 0.47-14.8, P=0.26).

**DISCUSSION**

Response to trastuzumab treatment in cancer is mainly mediated by ADCC (1), as a consequence, a reduced DFS has been hypothesized to be related to a less NK cell cytotoxic efficacy (8). However, the impact of KIRs involved in ADCC regulation in trastuzumab-treated BC has been poorly explored (26).

Key findings disclosed herein supported a role of the immune genetic background of KIRs in the efficacy of trastuzumab treatment in HER2-positive BC. Indeed, although preliminary, our data showed that the KIR functional repertoire could be associated both with the response to neoadjuvant therapy and with DFS and OS in the adjuvant setting. KIR haplotypes, HLA ligands for KIRs, and combinations of KIR and HLA were investigated in 35 patients with HER2-positive locally advanced BC patients treated with trastuzumab both given as NC together with paclitaxel, and for 1 year after surgery. In this cohort of patients, no correlation was highlighted between the induction of a pCR and clinical parameters as hormone receptor status, age, and tumor stage. ER and PgR are expressed by a low number of cases, too exiguous to evaluate a possible correlation with a clinical response as reported elsewhere (3). Young age (<50 years) is considered as a favorable predictive factor for pCR in BC (27). Consistently, eleven patients aged under 50 years showed a higher rate of pCR compared with 5 patients in the older group, but the different frequencies did not reach a statistical significance. Finally, the tumor stage could not be considered an influencing variable in our study since all patients were characterized by a locally advanced stage (Stage II-III).

### TABLE 4 | Comparisons of KIR genes and their cognate HLA ligands between CR and PR group.

| KIR Ligands | pCR n=30 | pPR n=40 | Chi-square | P* |
|-------------|----------|----------|------------|-----|
| HLA Bw4     |          |          |            |     |
| Ile80       | 10       | 11       | 27.50%     | 0.0694 | 0.792147 |
| T80         | 6        | 8        | 20.00%     | 0.0911 | 0.762725 |
| Bw6         | 14       | 21       | 52.50%     | 0.0083 | 0.809150 |
| HLA-A’11    | 5        | 2        | 5.00%      | 1.4883 | 0.227195 |
| HLA-A’23    | 0        | 4        | 10.00%     | –      | –         |
| HLA-A’24    | 3        | 2        | 5.00%      | 0.1122 | 0.737676 |
| HLA-A’32    | 2        | 4        | 10.00%     | 0.0004 | 0.950861 |
| HLA-A’03’   | 2        | 2        | 5.00%      | 0.0497 | 0.823558 |
| HLA-B’51    | 4        | 7        | 17.50%     | 0.0202 | 0.868915 |
| HLA-B’48:01 | 0        | 0        | 0.00%      | –      | –         |
| HLA-B’73:01 | 0        | 0        | 0.00%      | –      | –         |
| C1          | 10       | 14       | 35.00%     | 0.0119 | 0.913715 |
| C2          | 10       | 11       | 27.50%     | 0.0694 | 0.792147 |
| HLA-C’04    | 9        | 7        | 17.50%     | 0.8929 | 0.346995 |
| HLA-C’04:01 | 3        | 4        | 10.00%     | 0.0000 | 1.000000 |
| HLA-C’05:01 | 1        | 1        | 2.50%      | 0.2681 | 0.604625 |
| HLA-C’01:02 | 1        | 1        | 0.00%      | –      | –         |
| HLA-C’14:02 | 0        | 0        | 0.00%      | –      | –         |
| HLA-C’16:01 | 1        | 1        | 2.50%      | 0.2681 | 0.604625 |

Finally, the haplotype including the functional tel B genotype and the KIR3DL2+/ligand was significantly associated with a poorer OS compared to tel B haplotype without the 3DL2+/ligand as the reference (P = 0.0328, Figure 7), or Tel A without the 3DL2+/ligand combination as the reference (HR: 2.66, 95%CI 0.47-14.8, P=0.26).
non-Hodgkin lymphoma (28); the presence of a stimulatory tel KIR2DS1 strongly potentiated rituximab-mediated ADCC in the ADCC mechanism. For example, the presence of the activating tumors treated with targeted-antibodies acting through the cytotoxicity thereby contributing to trastuzumab-mediated expressed by the tel B haplotype could favor the NK cell presence of the inhibitory functional KIR3DL2/HLA-A03 or A11 combination was more frequent in pCR patients. KIR haplotypes can be divided into two regions (i.e. centromeric, cen, and telomeric, tel) and in two main groups based on the different numbers and types of KIR genes. The “A” type includes mainly inhibitory KIRs while the type “B”, is characterized by the presence of at least one activating KIR receptor among KIR2DS1, KIR2DS3, KIR2DS5, and KIR3DS1 (16). Interestingly, we observed that individuals with a tel B KIR stimulatory haplotype at the time of diagnosis (including those with a KIR3DL2+ligand combination) showed slightly higher ADCC activity in vitro than those with a tel A KIR inhibitory haplotype. We hypothesized that the prevalence of activating KIRs expressed by the tel B haplotype could favor the NK cell cytotoxicity thereby contributing to trastuzumab-mediated ADCC efficiency. Similar results have been reported for other tumors treated with targeted-antibodies acting through the ADCC mechanism. For example, the presence of the activating KIR2DS1 strongly potentiated rituximab-mediated ADCC in non-Hodgkin lymphoma (28); the presence of a stimulatory tel B genotype increased the anti-GD2-mediated ADCC in neuroblastoma (29). However, in other cases, the presence of inhibitory KIRs was shown to be necessary to obtain a clinical benefit with the tumor-specific antibody (30, 31).

There may be a synergistic effect between the KIR haplotype and high-affinity FcyR variants, which are responsible for a higher affinity of CD16-positive NK cells to the Fc fragment of the monoclonal antibody (29). Patients with these specific Fc genetic variants had shown stronger ADCC efficacy and superior event-free survival (29). Similarly, we found an impact of the FcγR3A polymorphism in patients characterized by the tel A KIR haplotype. In particular, the presence of at least one FcγR3A V-allele (V/V or V/F genotypes), responsible for a stronger affinity of NK cells to trastuzumab, induced a higher ADCC efficiency compared to cases showing a FcγR3A F/F genotype within the tel A population, while no difference was observed for patients carrying a tel B haplotype. This observation was independent of the FcγR2A genotype since FcγR3A V-carriers showed a similar high ADCC, in the presence of both the H allele and the R allele of the FcγR2A. These data suggested that, among individuals carrying the stronger inhibitory KIR tel A genotype (showing lower NK cell activity and the R allele of the FcγR2A). These data suggested that, among individuals carrying the stronger inhibitory KIR tel A genotype (showing lower NK cell activity and the R allele of the FcγR2A). These data suggested that, among individuals carrying the stronger inhibitory KIR tel A genotype (showing lower NK cell activity and the R allele of the FcγR2A). These data suggested that, among individuals carrying the stronger inhibitory KIR tel A genotype (showing lower NK cell activity and the R allele of the FcγR2A).
FcγR3A locus significantly improved the trastuzumab-mediated ADCC activity (9).

Regardless of the tel A and tel B haplotypes, we noted a prevalence of the functional KIR3DL2+/HLA ligand combination, present in both haplotypes, in patients achieving a pCR. KIR3DL2 is also known as CD158k, and is the only one in the KIR family to be expressed as a disulfide-linked homodimer (32). The known HLA-specific ligands for this receptor are HLA-A3, -A11, and the free heavy chain form of HLA-B27 (16). Furthermore, KIR3DL2 binds to CpG oligonucleotides.
particularly abundant in microbial genomes, transporting them to the Toll-Like Receptor 9 (TLR9) to mount an innate immune response, including NK cell activation (33). Interestingly, the KIR3DL2 carrier patients in our cohort also had the FcγR3A V-allele, and, accordingly, exhibited greater ADCC efficiency than the KIR3DL2+ and FcγR2A F/F individuals. These data agreed with the findings described by Sun et al, that reported a higher NK cell-mediated cytolysis of Multiple Myeloma cells dependent
on the mAb isatuximab in the presence of the KIR3DL2+, HLA-A3/A11+, and FcγR3A V markers (34).

Therefore, in the neoadjuvant setting, the characterization of the functional KIR repertoire together with the analysis of the FcγR3A polymorphism could favor the early identification of those patients who will respond better to trastuzumab, possibly contributing to the design of a more personalized therapy.

We investigated the KIR repertoire with biological and clinical parameters measured at follow-up. We noticed that individuals carrying the stimulatory tel B haplotype showed an increase in CD16 expression on NK cells 1 year after surgery, while there was no difference in patients with an inhibitory tel A haplotype. Likewise, Isitman and colleagues reported a higher frequency of CD16+ cells in the absence of inhibitory KIRs, thus suggesting that NK cells that mainly express inhibitory KIRs are poorer ADCC effectors than NK cells lacking these receptors (35). The increase of the CD16+ cell rate over time after treatment indicated the enrichment of an important fraction of mature NK cells, the only population of these cells expressing KIR receptors and functionally equipped to trigger ADCC in HER2-expressing tumor cells in the presence of trastuzumab. This is in agreement with other studies showing that the number of intratumoral and circulating CD16+ NK cells increased during mAb-based treatment as CD16-ligation positively affects NK cell survival and proliferation, and may be associated with a favorable patients’ outcome (36, 37). The role of these CD16+ cells is further supported by the observation that clinical response correlated with the presence of specific activating Tel B KIR genotype, while the CD56 expression on NK cells was found unchanged after treatment.

However, when comparing DFS we found no significant difference between BC patients with different KIR haplotypes. Conversely, the presence of the inhibitory KIR3DL2 appeared to be associated with worse DFS and OS, while patients with a tel B haplotype in the absence of KIR3DL2 showed the best prognosis. In particular, the expression of some other inhibitory KIR receptors correlated with a poor prognosis also in other pathologies, as previously observed also by our group.
Interestingly, KIR3DL2 expression on T-cells is a marker for several cutaneous T-cell lymphomas as Sézary syndrome and mycosis fungoides (40). Indeed, this KIR receptor is expressed not only on NK cells, but also by a small percentage of CD4+ and CD8+ T lymphocytes, and in lymphomatous T cells, and it represents a promising therapeutic target (40). In this context, KIR3DL2-targeted monoclonal antibody, IPH4102, has recently been developed.

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**FIGURE 6** | Kaplan-Meier survival analysis showed improved Disease-free survival (DFS) time for tel B genotype carriers compared to tel A carriers (A) and absence of the functional tel KIR3DL2+/HLA-A*03 or A*11 combinations compared to other KIR/HLA combinations (B). (C) Co-presence of Tel B genotype and KIR3DL2+/HLA-A*03 or A*11 genes reduce DFS compared to Tel-B lacking the KIR3DL2+/HLA-A*03 or A*11 combinations (HR: 3.85, 95%CI 0.72-20.5, p = 0.1141).
for the treatment of these diseases demonstrating a safe profile and encouraging clinical activity in a phase I clinical trial (41). In our cohort of BC patients, we noted a worse outcome in the presence of KIR3DL2, thus suggesting that this molecule may be eligible as a target for further treatments in this setting as well as in others, although our data are preliminary for this purpose.

Our study has several limitations, as the small cohort of patients that did not allow us to perform further subgroup analyses evaluating the contribution of different KIR/ligand combinations, and the lack of a control group of healthy women to compare the KIR repertoires and the ADCC efficiency. Moreover, it should be noted that the treatment of patients also included paclitaxel, which is known to act on NK cells, modifying their cytolytic potential (42).

In conclusion, our data are preliminary but have suggested a potential predictive role for a specific KIR genotype, namely tel B, in identifying patients who will achieve pCR after NC, and have supported a negative prognostic impact of KIR3DL2/HLA-A03 or A11 in the adjuvant setting. Prospective future studies, involving larger series and multiple centers, need to be performed to confirm the present findings. Moreover, clinical interventions in patients with unfavorable KIR profiles could be envisaged by using NK immunomodulatory drugs or mAb, like the IPH4102 targeting the KIR3DL2+ cells, with standard chemotherapy.

ETHICS STATEMENT
The studies involving human participants were reviewed and approved by phase II CRO Clinical Trial, NCT02307227, Centro di Riferimento Oncologico, Aviano, Italy. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS
EM and VD conceived the study and drafted the manuscript. EM and MDZ performed the experiments. GM, DL, SSc, and SSp collected and analyzed clinical data. SM performed surgical operations and contributed to analyzing data. TP performed histopathological diagnosis and immunohistochemistry. RD and AS supervised the study and reviewed the manuscript. VDR performed the statistical analysis and supervised the study. All authors read and approved the final manuscript.

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DATA AVAILABILITY STATEMENT
The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.
REFERENCES

1. Ehlers FAI, Beelen NA, van Gelder M, Evers TMJ, Smidt ML, Kooreman LFS, et al. ADCC-Inducing Antibody Trastuzumab and Selection of KIR-HLA Ligand Mismatched Donors Enhance the NK Cell Anti-Breast Cancer Response. *Cancers (Basel)* (2021) 13:3232. doi: 10.3390/cancers1332322

2. Cortazar P, Zhang L, Unth C, Mehta K, Costantino JP, Wolmark N, et al. Pathological Complete Response and Long-Term Clinical Benefit in Breast Cancer: The CTNeoBC Pooled Analysis. *Lancet* (2014) 384:164–72. doi: 10.1016/S0140-6736(13)62422-8

3. Gianni L, Zhang L, Unth C, Mehta K, Costantino JP, Wolmark N, et al. Neoadjuvant and Adjuvant Trastuzumab in Patients With HER2-Positive Locally Advanced Breast Cancer (NOAH): Follow-Up of a Randomised Controlled Superiority Trial With a Parallel HER2-Negative Cohort. *Lancet Oncol* (2014) 15:640–7. doi: 10.1016/S1470-2045(14)70080-4

4. Gianni L, Eiermann W, Semiglazov V, Lluch A, Tjulandin S, Zambetti M, et al. NeoAdjuvant and Adjuvant Trastuzumab in Patients With HER2-Positive Locally Advanced Breast Cancer (NOAH): Follow-Up of a Randomised Controlled Superiority Trial With a Parallel HER2-Negative Cohort. *Lancet Oncol* (2014) 15:640–7. doi: 10.1016/S1470-2045(14)70080-4

5. Muraro et al. KIR/HLA and Trastuzumab Response

6. doi: 10.1038/74704

7. Beano A, Signorino E, Evangelista A, Brusa D, Mistrangelo M, Polimeni MA, et al. Natural Killer Cells as Monotherapy and in Combination With Anti-CD20 Antibodies. *Blood* (2014) 123:678–86. doi: 10.1182/blood-2013-08-519199

8. Moretta L, Moretta A. Killer Immunoglobulin-Like Receptors. *Genetics, Function, and Translation. Immunity* (2011) 35:653–7. doi: 10.1016/j.immuni.2011.11.007

9. Anaya JC, Chenoweth AM, Wines BD, Hogarth PM. The Human Fc Gamma RIIL (CD32) Family of Leukocyte FcR and Its Pathobiological Functions. *Front Immunol* (2019) 10:464. doi: 10.3389/fimmu.2019.00464

10. Muraro et al. KIR/HLA and Trastuzumab Response

11. Morales-Estevez C, de la Haba-Rodriguez J, Manzanares-Martin B, Porras-Quintel A, Rodriguez-Ariza A, Moreno-Vega A, et al. KIR Genes and Their Ligands Predict the Response to Anti-EGFR Monoclonal Antibodies in Solid Tumors. *Front Immunol* (2016) 7:561. doi: 10.3389/fimmu.2016.00561

12. Chou H-H, Kuo W-L, Yu C-C, Tsai H-P, Shen S-C, Chu C-H, et al. Impact of Age on Pathological Complete Response and Locoregional Recurrence in Locally Advanced Breast Cancer After Neoadjuvant Chemotherapy. *BioMed J (2019)* 42:66–74. doi: 10.1080/08820139.2016.1208219

13. Makanga DR, Jullien M, David G, Legrand N, Willem C, Dubreuil L, et al. Low Number of KIR Ligands in Lymphoma Patients Favors a Good Rituximab-Dependent NK Cell Response. *Oncoimmunology* (2021) 10:1936932. doi: 10.1080/2162402X.2021.1936932

14. Siebert N, Jensen C, Troschke-Meurer S, Zumpe M, Jüttner M, Ehlert K, et al. Neutrophil Bloodstream Patients With High-Affinity FCGR2A-3A and Stimulatory KIR 2DS2 Treated by Long-Term Infusion of Anti-CD20 Antibody CH14.18/CHO Show Higher ADCC Levels and Improved Event-Free Survival. *Oncoimmunology* (2016) 5:e1235108. doi: 10.1080/2162402X.2016.1235108

15. Erbe AK, Wang W, Carmichael L, Hoeftes A, Groyewicz B, Revik P, et al. Follicular Lymphoma Patients With KIR2DL2 and KIR3DL1 and Their Ligands (HLA-C1 and HLA-Bw4) Show Improved Outcome When Receiving Rituximab. *J Immunother Cancer* (2019) 7:70. doi: 10.1186/s40425-019-0538-8

16. Manzanares-Martin B, Cebrían Aranda A, Del Puerto-Nevado L, González R, Solanes S, Gómez-España MA, et al. Improved Selection of Patients With Metastatic Colorectal Cancer to Benefit From Cetuximab Based on KIR Genotypes. *J Immunother Cancer* (2021) 9:e001705. doi: 10.1136/jitc-2020-00170

17. Pende D, Baissoni R, Canioni C, Verdiani S, Falco M, di Donato C, et al. The Natural Killer Cell Receptor Specific for HLA-A Allotypes: A Novel Member of the PSF/P70 Family of Inhibitory Receptors That is Characterized by Three Immunoglobulin-Like Domains and Is Expressed as a 140-kD Disulphide-Linked Dimer. *J Exp Med* (1996) 184:505–18. doi: 10.1084/jem.184.2.505

18. Sivori S, Falco M, Carlomagno S, Romeo E, Soldani C, Bensussan A, et al. A Novel KIR-Associated Function: Evidence That CpG DNA Uptake and Leading to Their Clinical Exploitation. *Front Immunol* (2010) 10:1179. doi: 10.3389/fimmu.2019.01179

19. Caggiani L, Toffoli G, De Re V, Orzes N, Spina M, De Zorzi M, et al. KIR/HLA Combination Associated With the Risk of Complications in Celiac Disease. *Int J Biol Markers* (2011) 26:221–8. doi: 10.5301/ijbm.2011.8903

20. Moretta L, Moretta A. Killer Immunoglobulin-Like Receptors. *Cytokine* (2004) 16:626–33. doi: 10.1016/j.cyto.2004.07.010

21. Kohrt HE, Thielen A, Marabelle A, Sagiv-Barfi I, Sola C, Chauc F, et al. Anti-KIR Antibody Enhancement of Anti-Lymphoma Activity of Natural Killer Cells as Monotherapy and in Combination With Anti-CD20 Antibodies. *Blood* (2014) 123:678–86. doi: 10.1182/blood-2013-08-519199

22. Haydarouglu H, Oguzkun Balci S, Pehlivan S, Ozdilk K, Gundogan E, Okan V, et al. Effect of Cytokine Genes in the Pathogenesis and on the Clinical Parameters for the Treatment of Multiple Myeloma. *Immunol Invest* (2017) 46:10–21. doi: 10.1080/08820139.2016.1208219

23. De Re V, Caggiani L, De Zorzi M, Talamin R, Racanelli V, D’ Andrea M, et al. Genetic Diversity of the KIR/HLA System and Outcome of Patients With Metastatic Colorectal Cancer Treated With Chemotherapy. *PloS One* (2014) 9:e84940. doi: 10.1371/journal.pone.0084940

24. Malmberg K-J, Michaelsson I, Parham P, Ljunggren H-G. Killer Cell Immunoglobulin-Like Receptor Workshop: Insights Into Evolution, Genetics, Function, and Translation. *Immunity* (2011) 35:653–7. doi: 10.1016/j.immuni.2011.11.007
Shuttling to Early Endosomes is Mediated by KIR3DL2. Blood (2010) 116:1637–47. doi: 10.1182/blood-2009-12-256586

34. Sun H, Martin TG, Marra J, Kong D, Keats J, Macé S, et al. Individualized Genetic Makeup That Controls Natural Killer Cell Function Influences the Efficacy of Isatuximab Immunotherapy in Patients With Multiple Myeloma. J Immunother Cancer (2021) 9:e002958. doi: 10.1136/jitc-2021-002958

35. Isitman G, Tremblay-McLean A, Lisovsky I, Bruneau J, Lebouché B, Routy JP, et al. NK Cells Expressing the Inhibitory Killer Immunoglobulin-Like Receptors (iKIR) KIR2DL1, KIR2DL3 and KIR3DL1 Are Less Likely to Be CD16+ Than Their iKIR Negative Counterparts. PloS One (2016) 11: e0164517. doi: 10.1371/journal.pone.0164517

36. Capuano C, Pighi C, Battella S, De Federicis D, Galandrini R, Palmieri G. Harnessing CD16-Mediated NK Cell Functions to Enhance Therapeutic Efficacy of Tumor-Targeting Mabs. Cancers (Basel) (2021) 13:2500. doi: 10.3390/cancers13102500

37. Arnould L, Gelly M, Penault-Llorca F, Benoit L, Bonnetain F, Migeon C, et al. Trastuzumab-Based Treatment of HER2-Positive Breast Cancer: An Antibody-Dependent Cellular Cytotoxicity Mechanism? Br J Cancer (2006) 94:259–67. doi: 10.1038/sj.bjc.6602930

38. He Y, Bunn PA, Zhou C, Chan D. KIR 2d (L1, L3, L4, S4) and KIR 3DL1 Protein Expression in Non-Small Cell Lung Cancer. Oncotarget (2016) 7:82104–11. doi: 10.18632/oncotarget.13486

39. Dębska-Zielkowska J, Moszkowska G, Zielińska M, Zielinska H, Dukat-Mazurek A, Trzonkowski P, et al. KIR Receptors as Key Regulators of NK Cells Activity in Health and Disease. Cells (2021) 10:1777. doi: 10.3390/cells10071777

40. Schmitt C, Marie-Cardine A, Bensussan A. Therapeutic Antibodies to KIR3DL2 and Other Target Antigens on Cutaneous T-Cell Lymphomas. Front Immunol (2017) 8:1010. doi: 10.3389/fimmu.2017.01010

41. Bafort M, Porcu P, Marie-Cardine A, Battistella M, William BM, Vermeer M, et al. IPH4102, a First-in-Class Anti-KIR3DL2 Monoclonal Antibody, in Patients With Relapsed or Refractory Cutaneous T-Cell Lymphoma: An International, First-in-Human, Open-Label, Phase 1 Trial. Lancet Oncol (2019) 20:1160–70. doi: 10.1016/S1470-2045(19)30320-1

42. Tsavaris N, Kosmas C, Vadiaka M, Kanelopoulos P, Boualamatis D. Immune Changes in Patients With Advanced Breast Cancer Undergoing Chemotherapy With Taxanes. Br J Cancer (2002) 87:21–7. doi: 10.1038/sj.bjc.6600347

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.

The editor PL declared a past co-authorship with one of the authors VR at the time of review.

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