**RESEARCH ARTICLE**

# Impact of HLA Class I Antigen, Killer Inhibitory Receptor, and FCGR3A Genotypes on Breast Cancer Susceptibility and Tumor Stage

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**Abstract:** ***Background:*** The identification in breast cancer (BC) of novel genetic biomarkers regulating natural killer (NK) cell function, including the HLA, KIR, and CD16A (FCGR3A), may be still a challenge.

***Objective:*** We aimed to evaluate whether the combined effect of these polymorphisms has an impact on BC susceptibility and progression.

***Methods:*** 47 BC Italian patients and healthy individuals (39 females and 66 males/ females) were genotyped by Sanger sequencing (HLA-C exon 2-4 and FCGR3A-158V/F, 48L/R/H) and PCR-SSP typing (KIR genes).

***Results:*** HLA-C gene allele analysis showed the group C1, with HLA-C*07:02:01 allele, to be significantly associated with tumor progression (16.7% *vs.* 4.0%, $p$=0.04, OR=4.867), and instead, group C2, with HLA-C*05:01:01, was protective against disease susceptibility (0.0% *vs.* 7.2%, $p$=0.019, OR=0.087). In addition, we highlighted a significant reduction of the KIR2DS4ins in BC patients ($p_{corr.}$=0.022) and an increased combined presence of KIR2DL1 and KIR2DS1 genes in advanced BC patients compared to earlier stages (66.7% *vs.* 19.2%, $p$=0.002). The concurrent lack of KIR2DL2 and KIR2DS4 genes in the presence of HLA-C2 alleles was significantly associated with increased susceptibility to BC ($p$=0.012, OR=5.020) or with lymph node involvement ($p$=0.008, OR=6.375). Lastly, we identified different combinations of the FCGR3A-48/158 variants and KIR genes in BC patients compared to controls.

***Conclusion:*** Our findings suggest that in the development of BC probably exists a disorder of the NK innate immunity influenced by KIR/HLA-C gene content and FCGR3A-158 polymorphisms and that the combined analysis of these biomarkers might help predict genetic risk scores for tailored screening of BC patients in therapy.

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## 1. INTRODUCTION

Breast cancer (BC) is the most frequent malignancy in women worldwide and it represents the second most common cause of cancer deaths [1]. Besides genetic mutations, diet, age, environmental contaminants, and steroid hormones are involved in the pathogenesis of the disease. BC is characterized by a strong immune component supported by immune cells, cytokines, and growth factors. A high proportion of NK cells, neutrophils, and macrophages, and a lower frequency of cytotoxic T cells and $CD4^+$ cells were found in estrogen receptor (ER)-positive BC [2]. NK cells represent the first-line defense against transformed cells, and class I human leukocyte antigens (HLA) and killer-cell immunoglobulin-like receptors (KIRs) regulate their function. NK cells' cytotoxic activities are regulated by opposing signals delivered by different surface receptors that can activate or inhibit NK cell cytotoxic responses. KIRs are cell-surface molecules that are able to induce activation or suppression of NK cell response by interaction (REF) [3].

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Killer immunoglobulin-like receptors (KIRs) are transmembrane proteins encoded by multiple genes mapping to chromosome 19q13.4 [4-5]. Generally, two types of KIRs can be identified: the first inhibitory type has a long intracellular tail (KIRL), while the second stimulatory type is characterized by a short intracellular tail (KIRS). Worldwide population variation in the KIR *loci* and the relationship between KIR genes and their human leukocyte antigen (HLA) ligands were evaluated in the anthropology component of the 15th International Histocompatibility Workshop (IHIWS) [6]. KIR gene profiles are defined on the basis of the presence or absence of some KIR genes in A and B haplotypes. All haplotypes contain some framework genes (KIR2DL4, KIR3DL2, and KIR3DL3). The A haplotype contains several inhibitory KIR genes and only one activating KIR (KIR2DS4). Conversely, the B haplotype displays one or more activating KIR genes (KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS5, and KIR3DS1) [7].

The HLA-C antigens are the best-known KIR ligands. Based on polymorphisms at amino acid positions 77 and 80, in the α1-domain of the β chain, they can be divided into two groups: the HLA-C1 that carries a serine residue at position 77 with an asparagine at position 80, and the HLA-C2 that has an asparagine at position 77 and a lysine residue at amino acid 80 [8, 9].

A relationship between KIR genotype and cancer has been recently proposed [10-14]. Inhibitory KIRs, interacting with HLA class I antigens, regulate the NK cell cytotoxicity, including antibody-dependent cellular cytotoxicity (ADCC). The extent of this process relies on the binding affinity of the Fc portion of the IgG antibody to the CD16 receptor.

CD16A (FCGR3A) is a polymorphic activating receptor expressed on the surface of NK cells. It induces molecular signals that lead to the activation of the NK cell-killing machinery, resulting in the elimination of antibody-opsonized target cells [15]. Changes in the monoclonal antibody (mAb) affinity for CD16 polymorphisms can modulate the ADCC response as a function of the different IgG binding affinity of valine or phenylalanine polymorphisms at amino acid position 158 of FcγR molecule [16-18]. Indeed, the presence of the higher affinity genotype (V/V or V/F) enhances the NK cell binding affinity to human IgG1 antibody more than that of the lower affinity genotype (F/F) [19,20]. FCGR3A-158V/F and 48L/R/H polymorphisms have been extensively studied. The presence of either arginine or histidine enhanced the binding of IgG1, IgG3, and IgG4 to CD16 [21].

The importance of steroid hormones as prognostic markers in BC development is also widely shown. About a fifth of BCs (15-20%) overexpress the human epidermal growth factor receptor 2 (HER2), often associated with a more aggressive profile and decreased survival [22]. However, the use of HER2-mAbs, such as trastuzumab (herceptin), in addition to chemotherapy, has been successfully implemented in the treatment of BC [23-25]. Several publications indicate that NK and T lymphocytes strongly influence tumor development and response to anti-HER mAbs treatment [26-27]. Given that, the FCGR3A genotype might be a factor contributing to the anti-tumor activity of the antibody. Thus, it would be of interest to analyze FCGR3A-158V/F and the FCGR3A-48L/R/H gene polymorphisms in association with KIR genes and their HLA-C ligands to understand their contribution to BC susceptibility and staging.

## 2. MATERIALS AND METHODS

### 2.1. Population

*A cohort of 47 Italian patients* with breast cancer (46 females and 1 male, mean age: 64 years, ranging from 45 to 86) were enrolled in the Division of Medical Oncology of Tor Vergata University Hospital in Rome. Tumor specimens consisted histologically of ductal (69.2%) or lobular (15.4%) carcinomas. The histopathological assessment (pTNM) and grading, the expressions of estrogen receptor (ER) and progesterone receptor (PR), and HER2 status were determined by using immunohistochemical detection. The BC patients were mainly categorized according to TNM staging and grouped as early-stage (0-II) or advanced-stage (III-IV). The study was approved by the Internal Review Board (IRB) of the Polyclinic "Tor Vergata" Hospital, Rome, Italy (number 133/10). A written informed consent was obtained from all the subjects. All clinical investigations were conducted according to the Declaration of Helsinki principles. BC patients received adjuvant or neoadjuvant chemo-therapy, with trastuzumab in some cases. Hormonal therapy was planned in the case of estrogen receptor (ER) or progesterone receptor (PR) positive tumor.

We used a control group composed of 39 women (FCTRs, mean age: 62.7 years) and a mixed group of 66 male/female healthy subjects (MCTRs: 66.0 years) for KIR and FCGR3A genetic analysis. The reason for this choice follows the assumption that although BC rarely affects men (0.5-1% of all BC patients), its diagnosis is often delayed and it associates with worse outcomes. As regards the case-control analysis of HLA-C allelic contribution, we considered a historical group of 76 healthy individuals already HLA-typed for bone marrow transplantation in our laboratory. All subjects were ethnically matched, unrelated, and randomly selected, with no cancer and no history of any immunological disease.

### 2.2. DNA Extraction and KIR Genotyping

DNA was isolated from 1 ml of cryopreserved peripheral blood cell sample using the DNA Blood Midi kit (Qiagen, Hilden, Germany) by spin columns, according to the manufacturer's protocol. DNA purity and concentration were analyzed using an ultraviolet-visible spectrophotometer (DU530, Beckman Coulter Life Sciences, Brea, CA-US). KIR genotyping was performed using polymerase chain reaction with sequence-specific primers (PCR-SSP) for 16 KIR genes (*KIR2DL1, 2DL2, 2DL3, 2DL4, 2DL5A,*

*2DL5B,2DS1, 2DS2, 2DS3, 2DS4del* and *2DS4ins, 2DS5, 3DL1, 3DL2, 3DL3,* and *3DS1*) and 2 pseudo-genes (*2DP1* and *3DP1*) (KIR Typing Kit, MACS Molecular, Miltenyi Biotec, BergischGladbach, Germany), with a genomic DNA control for contamination, a β-actin positive control, and negative control. PCR products were analyzed on a 2% agarose gel electrophoresis containing ethidium bromide and photo-documented ultraviolet transilluminator (Supplemental Fig. **1**). KIR genotyping is utilized for the analysis of gene content and the categorization of A/B haplotypes, as well as for the prediction of NK cell reactivity in autologous and allogeneic NK cell-based immunotherapy.

### 2.3. HLA-C Allele Typing by PCR-SBT

HLA-C typing was performed by using sequence-based typing (SBT) with the AlleleSEQR HLA-C Plus sequence-based typing (SBT) kit (Abbot Molecular, Des Plaines, Illinois, USA), which analyzes the allelic polymorphisms in exons 2-4 of HLA-C gene. Allele assignments were evaluated using Assign™ SBT software (*Conexio Genomics, Fremantle, Western Australia*). These HLA-C alleles were also considered as belonging to C1 (*HLA-C*01,03,07,08,09,10,12,14*) and C2 (*HLA-C*02,04,05,06,15*) subsets, depending on the presence of asparagine (Asp) or lysine (Lys) at position 80, respectively. The study of the interactions between a specific KIR and subsets of HLA-C allotypes (C1 and C2) was established according to the Campbell KS's review scheme [28].

### 2.4. FCGR3A Genotyping by Sequence-Based Typing (SBT)

The genotyping of *FCGR3A-158G/T* (V/F) and *FCGR3A-48A/T/G* (L/R/H) polymorphisms was performed on genomic DNA by polymerase chain reaction (PCR)-SBT using primers previously described [19]. Briefly, PCR reactions were set up with 250 ng of genomic DNA per 50 µl reaction, and PCR products were purified and sequenced using Big Dye Terminator v1.1 Cycle Sequencing Kit on an ABI Prism 3130 Genetic Analyzer (PE Applied Biosystems, Foster City, CA). Typing was obtained by alignment of the processed sequences and the reference human CD16A gene on dedicated software.

### 2.5. Statistical Analysis

The frequencies of genes or haplotypes were calculated by direct counting. Individuals considered homozygous for a gene had two identical alleles, while heterozygous ones had two different alleles of a gene. Two-tailed Pearson $X^2$ test or Fisher's exact test were used for comparison of the gene frequencies of the patient group with controls, as appropriate. A *p*-value of 0.05 or less was considered to be significant and Bonferroni correction for multiple testing was examined. The odds ratio (OR) and its 95% confidence intervals (CI) were calculated. The association of KIR genes and HLA-C ligands with BC status was assessed using the binary logistic regression analysis. Comparison between groups and correlation between variables were examined by parametric (t-test/one-way ANOVA, Pearson's correlation), and non-parametric tests (Kruskal Wallis, Friedman test for repeated measures, and Spearman's test), as appropriate. The SPSS statistical package, version 19 for Windows, was used for data and statistical analysis, and graphic representations were obtained by using GraphPad Prism (v.6). FCGR3A allele and haplotype frequencies were assessed by an expectation-maximum (EM) algorithm. For multilocus genotypic data, the maximum likelihood was estimated using an EM algorithm when the gametic phase is not known. The Hardy-Weinberg equilibrium was calculated by the Guo and Thomson exact test. The analysis of molecular polymorphisms within the population was performed using the Arlequin V.3.0 population genetics software.

## 3. RESULTS

### 3.1. Patients

47 BC patients with different histological diagnoses of early or advanced invasive and metastatic cancer were considered for this study. Patients were treated with neoadjuvant chemotherapy (n=5), adjuvant chemotherapy (n=24), hormonal therapy (n=17), and trastuzumab in some cases (n=5). 26 patients were in an early stage of the disease (stage 0-II), while 21 patients were in advanced stages (stage III-IV). 27 patients had lymph node metastases (LNM). The histological classification of the disease mainly included ductal or lobular carcinomas. Overall, 10 patients relapsed and 2 patients died during the follow-up for liver injury and cardiovascular diseases (Table **1**).

### 3.2. HLA-C Allele Polymorphisms in BC Patients

We compared the distribution of HLA-C alleles and associated epitopes (C1:C80N and C2:C80K) of BC patients at different tumor stages with that of healthy controls. The incidence of the group C1, with *HLA-C*07:02:01* allele, was higher in more advanced tumors than in female controls (AF%:-16.7% *vs.* 4.0%, *p*=0.04, OR=4.867) or mixed subjects (*p*=0.02, OR=4.867). Also the group C2, with *HLA-C*04:01:01* allele, was positively associated with LNM+ tumors compared to mixed controls (28.6% *vs.* 14.5%, *p*=0.040, OR=2.364), while the *HLA-C*05:01:01* in the whole population of BC patients was significantly protective (0% *vs.* 7.2%, *p*=0.019, OR=0.087) to the MCTRs (Table **2**). However, the significance of the association of HLA-C alleles was not confirmed after Bonferroni's correction, may be because of the limited number of BC patients and the high HLA-C allele variability.

### 3.3. KIR/HLA Genes Distribution in the Total BC Patients

The analysis of KIR genes distribution between BC patients and controls highlighted a significant reduction of the *KIR2DS4ins* in BC patients (25.5% *vs.* 57.6%, *p*=0.001, OR=0.253), especially MCTRs, after

**Table 1. Clinical and biological features of the breast cancer (BC) patients.**

| Variables | N (%) |
|---|---|
| *Number patients* (n=47): | |
| - males | 1 (2.1) |
| - females | 46 (97.9) |
| *Median age*, years (range ) | 62 (45-87) |
| ≥50 | 30 (63.8) |
| ≤50 | 17 (36.2) |
| *Tumor Stage*: | |
| - early (0-II ) | 26 (55.3) |
| - advanced (III-IV) | 21 (44.7) |
| *Hormonal status* (n=34): | |
| ER+ | 25 (73.5) |
| PR+ | 23 (67.6) |
| ER-/PR- | 8 (23.5) |
| ER+PR+ | 22 (64.7) |
| HER2+ | 5 (14.7) |
| Triple negative | 5 (14.7) |
| *Ki67 index* (n=30): | |
| >20% | 17 (56.7) |
| <20% | 13 (43.3) |
| *Chemotherapy and hormonal therapy* n=34: | |
| Adjuvant (anthracyclines /taxanes) | 24 (70.6) |
| Neoadjuvant | 5 (14.7) |
| Hormonal therapy | 17 (50.0) |
| *Anti-HER2* (n=5): | |
| Trastuzumab | 4 (11.8) |
| Trastumab+pertuzumab | 1 (2.9) |
| *Metastasis site*: | |
| LNM+ | 27 (57.4) |
| LNM- | 20 (42.6) |
| Visceral | 6 (12.8) |
| *Tumor size*: | |
| T1-T2 | 34 (72.3) |
| T3-T4 | 8 (17.0) |
| NA (Tis *etc*) | 5 (10.6) |
| *Relapse*: | 10 (33.3) |
| *Diagnosis* (n=39): | |
| Ca ductal | 27 (69.2) |
| Ca lobular | 6 (15.4) |
| Other types (apocrine, mucinous *etc*.) | 6 (15.4) |

Bonferroni's correction (p=0.022) (Table **3**). The combined effect of KIR genes with their cognate HLA-C ligands evidenced the frequency of the *KIR2DL2* gene, not licensed by the *HLA-C1(HLA-C2+)*, to be significantly lower in BC patients than FCTRs group (28.6% *vs.* 57.9%, p=0.022, OR=0.291). This negative correlation was validated by univariable regression analysis (*p*=0.013, OR=0.291, 95%CI: 0.110-0.772). In the presence of *HLA-C2*, the concurrent lack of *KIR2DL2* and *KIR2DS4ins* was significantly higher in patients than in females (37.1% *vs.* 10.5%, *p*=0.012, OR=5.020) and mixed controls (37.1% *vs* 12.7%, *p*=0.010, OR=3.886), suggesting that this combination may increase the susceptibility to develop BC. Besides, the percentage of the *KIR2DS5* gene (haplotype B), in association with the *C2* allele group**,** was significantly lower in BC patients than in mixed controls (total BCs: 17.1% *vs.* 63.5%, *p*=0.0001, OR=0.119) (Table **4**).

### 3.4. Involvement of KIR Genes and HLA-C Epitopes in Tumor Progression

To distinguish the role of different biomarkers in breast malignancy, the influence of variables, such as the invasion type, clinical tumor stage, and the presence of immunohistochemistry markers, including ER, PR, and HER2, was considered. The incidence of the *KIR2DS1* gene in patients with advanced stages of the disease was significantly higher than in patients at early stages (66.7% *vs.* 26.9%, *p*=0.015; Table **3**), and interestingly, an increased coexistence of *KIR2DL1* and *KIR2DS1* genes was evidenced in these advanced BC patients (66.7% *vs.* 19.2%, *p*=0.002). On the contrary, the presence of *KIR2DL1* without *KIR2DS1* was instead higher in patients at early stages (18/26=69.2% *vs.* 6/21=28.6%, *p*=0.013). The frequency of the *KIR2DS4ins* gene was lower in patients with advanced cancer than MCTRs (14.3% *vs.* 57.6%, *p*=0.020, OR=0.226)**.** In the context of KIR/HLA-C combinations, we confirmed the negative association of *KIR2DL2/HLA-C2+* **(**20% *vs.* 57.9% FCTRs, *p*=0.016, OR=0.182) and *KIR2DS5* gene/*HLA-C2+* (20.0% *vs.* 63.5% MCTRs, *p*=0.003, OR=0.144) with advanced disease (Table **4**). The positive influence of the *HLA-C2* with *KIR2DL2*$^+$ NK cells combination is demonstrated by the significant difference in the frequency of the *KIR2DL2 HLA-C2/C2* and/or *HLA-C1/C2* combinations between patients with advanced tumors with respect to FCTRs (20.0% *vs.* 55.3%, *p*=0.031). The same relationship was found in BC patients with LN+ metastasis, where the AF of *KIR2DL2* genes in combination with the homozygous *HLA-C1 (C1/C1)* was significantly higher than that in both the controls (FCTRs, *p*=0.042, OR=4.250; MCTRs, *p*=0.042, OR=4.000). Always in this subset of LNM+ patients, the absence of *KIR2DL2* and *KIR2DS4ins* genes in the presence of *HLA-C2* was higher (42.9% *vs.* FCTRs:10.5%, p=0.008, OR=6.375) (Suppl. Table **1**).

### 3.5. Evaluation of KIR Polymorphisms in BC Patients Depending on Steroid Hormone Receptors

Taking into account the presence of specific steroid hormone receptors, ER$^+$ BC patients (n=25) had a lower frequency of *KIR2DS4ins* gene compared to controls, especially mixed ones (20.0% *vs.* MCTRs 57.6%, p=0.002, OR=0.174). All ER+, LNM+BC patients (n=16) carried the *KIR2DL3* gene (100.0% *vs.* FCTRs 76.9%, *p*=0.046, OR=10.28). Similar results were obtained when we evaluated the PR+ and LNM+ BC patients group (*KIR2DL3:* 100% *vs.* FCTRs 76.9%, *p*=0.049; *KIR2DS4ins*: 26.7% *vs.* MCTRs 57.6%,

**Table 2. HLA-C alleles in breast cancer patients (BC, n=35), female controls (FCTRs, n=38) and mixed controls (MCTRs, n=76).**

| HLA-C* | BC tot (2n=70) | | BC stage 0-II (2n=40) | | BC stage III-IV (2n=30) | | LNM+ (2N=42) | | FCTRs (2n=76) | | *P* val | OR= | MCTRs (2n=152) | | *P* val | OR= |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | n | AF(%) | n | AF(%) | n | AF(%) | n | AF(%) | n | AF(%) | | | n | AF(%) | | |
| 01:02 | 1 | 1.4 | 1 | 2.5 | 0 | 0.0 | 0 | 0.0 | 4 | 5.3 | ns | - | 4 | 2.6 | ns | - |
| 02:02 | 4 | 5.3 | 2 | 5.0 | 2 | 6.7 | 2 | 4.8 | 7 | 9.2 | ns | - | 6 | 4.0 | ns | - |
| 03:03 | 1 | 1.4 | 1 | 2.5 | 0 | 0.0 | 0 | 0.0 | 1 | 1.3 | ns | - | 3 | 2.0 | ns | - |
| 03:04 | 1 | 1.4 | 0 | 0.0 | 1 | 3.3 | 1 | 2.4 | 2 | 2.6 | ns | - | 4 | 2.6 | ns | - |
| 04:01:01 (C2) | 17 | 24.3 | 9 | 22.5 | 8 | 26.7 | 12 | 28.6 | 16 | 21.1 | ns | - | 22 | 14.5 | 0.040 | 2.364 |
| 05:01:01 (C2) | 0 | 0.00 | 0 | 0.0 | 0 | 0.0 | 0 | 0.0 | 3 | 4.0 | ns | - | 11 | 7.2 | 0.019 | 0.087 |
| 06:02:01 | 7 | 10.0 | 4 | 10.0 | 3 | 10.0 | 3 | 7.1 | 8 | 10.5 | ns | - | 12 | 7.9 | ns | - |
| 07:01:01 | 7 | 10.0 | 5 | 12.5 | 2 | 6.7 | 4 | 9.5 | 14 | 18.4 | ns | - | 28 | 18.4 | ns | - |
| 07:02:01 (C1) | 7 | 10.0 | 2 | 5.0 | 5 | 16.7 | 7 | 16.7* | 3 | 4.0 | 0.04 | 4.867 | 6 | 4.0 | 0.020 | 4.867 |
| 07:04:01 | 3 | 4.3 | 3 | 7.5 | 0 | 0.0 | 1 | 2.4 | 1 | 1.3 | ns | - | 2 | 1.3 | ns | - |
| 07:43 | 1 | 1.4 | 0 | 0.0 | 1 | 3.3 | 1 | 2.4 | 0 | 0.0 | ns | - | 0 | 0.0 | ns | - |
| 08:01 | 0 | 0.0 | 0 | 0.0 | 0 | 0.0 | 0 | 0.0 | 0 | 0.0 | ns | - | 1 | 0.7 | ns | - |
| 08:02:01 | 2 | 2.9 | 2 | 5.0 | 0 | 0.0 | 1 | 2.4 | 4 | 5.3 | ns | - | 7 | 4.6 | ns | - |
| 12:02 | 1 | 1.4 | 1 | 2.5 | 1 | 2.5 | 0 | 0.0 | 2 | 2.6 | ns | - | 1 | 0.7 | ns | - |
| 12:03:01 | 5 | 7.1 | 2 | 5.0 | 3 | 10.0 | 3 | 7.1 | 4 | 5.3 | ns | - | 12 | 7.9 | ns | - |
| 12:05 | 1 | 1.4 | 1 | 2.5 | 0 | 0.0 | 1 | 2.4 | 0 | 0.0 | ns | - | 0 | 0.0 | ns | - |
| 14:02 | 3 | 4.3 | 2 | 5.0 | 1 | 3.3 | 1 | 2.4 | 0 | 0.0 | ns | - | 6 | 4.0 | ns | - |
| 15:02:01 | 1 | 1.4 | 0 | 0.0 | 1 | 3.3 | 1 | 2.4 | 3 | 4.0 | ns | - | 10 | 6.6 | ns | - |
| 15:05 | 1 | 1.4 | 1 | 2.5 | 0 | 0.0 | 0 | 0.0 | 1 | 1.3 | ns | - | 2 | 1.3 | ns | - |
| 15:13 | 1 | 1.4 | 0 | 0.0 | 1 | 3.3 | 1 | 2.4 | 0 | 0.0 | ns | - | 0 | 0.0 | ns | - |
| 16:01 | 3 | 4.3 | 3 | 7.5 | 0 | 0.0 | 2 | 4.8 | 2 | 2.6 | ns | - | 5 | 3.3 | ns | - |
| 16:02:01 | 1 | 1.4 | 0 | 0.0 | 1 | 3.3 | 1 | 2.4 | 0 | 0.0 | ns | - | 2 | 1.3 | ns | - |
| 16:04 | 0 | 0.0 | 0 | 0.0 | 0 | 0.0 | 0 | 0.0 | 0 | 0.0 | ns | - | 2 | 1.3 | ns | - |
| 17:01,03 | 2 | 2.9 | 1 | 2.5 | 1 | 3.3 | 1 | 2.4 | 1 | 1.3 | ns | - | 6 | 4.0 | ns | - |
| C1 subgr. | 25 | 71.4 | 15 | 75.0 | 10 | 66.7 | 20 | 47.6 | 28 | 73.7 | ns | - | 48 | 63.2 | 0.080 | - |
| C2 subgr. | 25 | 71.4 | 13 | 65.0 | 12 | 80.0 | 22 | 52.4 | 28 | 73.7 | 0.14 | - | 43 | 56.6 | ns | - |

**Table 3. KIR allele frequencies (AF) in BC patients (n=47) in comparison with two healthy controls groups (female controls, FCTRs n=39 and mixed controls, MCTRs n=66).**

| KIR GENES | BREAST CANCER | | | | | | HEALTHY CONTROLS | | | | COMPARISONS | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | TOTAL (N=47) | | EARLY STAGE (0-II grade, N=26) | | ADVANCED STAGE (III-IV grade, N=21) | | FCTRs (N=39) | | MCTRs (N=66) | | TOTAL *VS.* CONTROLS | | ADVANCED *VS.* CONTROLS | |
| | N | AF% | N | AF% | | N AF% | N | AF% | N | AF% | *P*-value | OR= | *P*-value | OR= |
| 2DL1 | 45 | 95.7 | 25 | 96.2 | 21 | 100.0 | 39 | 100.0 | 64 | 97.0 | NS | NS | NS | NS |
| 2DL2 | 24 | 51.1 | 15 | 57.7 | 9 | 42.9 | 26 | 66.7 | 40 | 60.6 | NS | NS | NS | NS |
| 2DL3 | 43 | 91.5 | 23 | 88.5 | 20 | 95.2 | 30 | 76.9 | 53 | 80.3 | NS | NS | NS | NS |

**(Table 3) Contd….**

| KIR GENES | BREAST CANCER | | | | | | HEALTHY CONTROLS | | | | COMPARISONS | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | TOTAL (N=47) | | EARLY STAGE (0-II grade, N=26) | | ADVANCED STAGE (III-IV grade, N=21) | | FCTRs (N=39) | | MCTRs (N=66) | | TOTAL *VS.* CONTROLS | | ADVANCED *VS.* CONTROLS | |
| | N | AF% | N | AF% | N | AF% | N | AF% | N | AF% | *P*-value | OR= | *P*-value | OR= |
| 2DL4 | 47 | 100.0 | 28 | 100.0 | 19 | 100.0 | 39 | 100.0 | 66 | 100.0 | NS | NS | NS | NS |
| 2DL5A | 19 | 40.4 | 7 | 26.9 | 12 | 57.1 | 18 | 46.2 | 26 | 39.4 | NS | NS | NS | NS |
| 2DL5B | 22 | 46.8 | 11 | 42.3 | 11 | 52.4 | 25 | 64.1 | 41 | 62.1 | NS | NS | NS | NS |
| 2DS1 § | 21 | 44.7 | 7 | 26.9° | 14 | 66.7 | 18 | 46.2 | 37 | 56.1° | NS | NS | NS | NS |
| 2DS2 | 22 | 46.8 | 14 | 53.8 | 8 | 38.1 | 21 | 53.9 | 35 | 53.0 | NS | NS | NS | NS |
| 2DS3 | 16 | 34.0 | 10 | 38.5 | 6 | 28.6 | 13 | 33.3 | 34 | 51.5 | NS | NS | NS | NS |
| 2DS4del*003 | 38 | 80.9 | 23 | 88.5 | 16 | 76.2 | 30 | 76.9 | 56 | 84.8 | NS | NS | NS | NS |
| 2DS4ins*001-002 * | 12 | 25.5 | 8 | 30.8 | 3 | 14.3 | 14 | 35.9 ^ | 38 | 57.6 | 0.022* | 0.253 | 0.020 | 0.226 |
| 2DS5 | 16 | 34.0 | 7 | 26.9 | 10 | 47.6 | 15 | 38.5 | 22 | 33.3 | NS | NS | NS | NS |
| 3DL1 | 43 | 91.5 | 25 | 96.2 | 18 | 85.7 | 36 | 92.3 | 60 | 90.9 | NS | NS | NS | NS |
| 3DL2 | 47 | 100.00 | 28 | 100.0 | 19 | 100.0 | 39 | 100.0 | 66 | 100.0 | NS | NS | NS | NS |
| 3DL3 | 47 | 100.00 | 28 | 100.0 | 19 | 100.0 | 39 | 100.0 | 66 | 100.0 | NS | NS | NS | NS |
| 3DS1 | 19 | 40.43 | 7 | 26.9 | 11 | 52.4 | 18 | 46.2 | 29 | 43.9 | NS | NS | NS | NS |

**Note:** § KIR2DS1: III-IV grade *vs.* 0-II grade tumors, p= 0.015. * KIR2S4ins: Total BC pts vs mixed healthy controls (p=0.0014), with Bonferroni correction. (in bold) . ^Advanced stage *vs.* Females 14.3% *vs.* 35.9%, p=0.132 ns; ° KIR2DS1: less advanced tumors 26.9% *vs.* Mixed controls 54.5%, p=0.031. KIR2DS1. 40.7% male controls

**Table 4. KIR/HLA-C interaction frequencies in the study groups with comparisons.**

| KIR / HLA-C combinations | BREAST CANCER | | | | | | CONTROLS | | | | COMPARISONS | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | TOTAL (N=35) | | EARLY STAGE (N=20) | | ADVANCED STAGE (N=15) | | FEMALES (N=38) | | MIXED (N=63) | | TOTAL *VS.* CONTROLS | | ADVANCED *VS.* CONTROLS | |
| | N | AF% | N | AF% | N | AF% | N | AF% | N | AF% | *P*-value | OR= | *P*-value | OR= |
| 2DL1 HLA-C2+ | 24 | 68.6 | 12 | 60.0 | 12 | 80.0 | 28 | 73.7 | 43 | 68.3 | NS | NS | NS | NS |
| 2DL1 HLA-C1+ | 24 | 68.6 | 14 | 70.0 | 10 | 66.7 | 28 | 73.7 | 46 | 73.0 | NS | NS | NS | NS |
| 2DL2 HLA-C1+ | 14 | 40.0 | 10 | 50.0 | 4 | 26.7 | 17 | 44.7 | 28 | 44.4 | NS | NS | NS | NS |
| 2DL2 HLA-C2+ | 10 | 28.6 | 7 | 35.0 | 3 | 20.0 | 22 | 57.9 | 31 | 49.2 | 0.022 | 0.291 | 0.016 | 0.182 |
| 2DL2 HLA-C1C1 | 7 | 20.0 | 4 | 20.0 | 3 | 20.0 | 4 | 10.5 | 7 | 11.1 | NS | NS | NS | NS |
| 2DL2 HLA-C2C2 § | 3 | 8.6 | 2 | 10.0 | 1 | 6.7 | 8 | 21.1 | 11 | 17.5 | NS | NS | NS | NS |
| 2DL2 HLA-C1C2 § | 7 | 20.0 | 5 | 25.0 | 2 | 13.3 | 13 | 34.2 | 20 | 31.7 | NS | NS | NS | NS |
| 2DL3 HLA-C2 | 23 | 65.7 | 11 | 55.0 | 12 | 80.0 | 20 | 52.6 | 43 | 68.3 | NS | NS | NS | NS |
| 2DS1 HLA-C2* | 8 | 22.9 | 2 | 10.0* | 6 | 40.0* | 14 | 36.8 | 22 | 34.9 | NS | NS | NS | NS |
| 2DS1 HLA-C1 | 12 | 34.3 | 4 | 20.0 | 8 | 53.3 | 12 | 31.6 | 21 | 33.3 | NS | NS | NS | NS |
| 2DS4ins HLA-C2 | 4 | 11.4 | 4 | 20.0 | 0 | 0.00 | 11 | 28.9 | 15 | 23.8 | 0.084 | NS | 0.023 | 0.077 |
| 2DS4ins HLA-C1 | 8 | 22.9 | 6 | 30.0 | 2 | 13.3 | 10 | 26.3 | 15 | 23.8 | NS | NS | NS | NS |
| 2DS5 HLA-C2 | 6 | 17.1 | 3 | 15.0 | 3 | 20.0 | 12 | 31.6 | 40 | 63.5 | 0.0001 | 0.119 | 0.003 | 0.144 |
| 2DL2$^-$ 2DS4INS$^-$ C2+ | 13 | 37.1 | 4 | 20.0 | 8 | 53.3 | 4 | 10.5 | 8 | 12.7 | 0.012 | 5.020 | 0.002 | 9.71 |

**Note:** *KIR2DS1/HLA-C2: early tumors *vs.* advanced tumors p=0.05. §The comparison of KIR2DL2 HLA-C2/C2 plus KIR2DL2 HLA-C1C2 frequencies between BC patients with advanced tumors and FCTR was significant (p=0.0313).

*p*=0.045). Interestingly, the frequency of *KIR2DS4ins* in ER/PR double-positive patients (n=22) was significantly lower than that of mixed controls (4/22 18.2% *vs.* 38/66 57.6%, *p*=0.001, OR=0.164), and they showed a lower incidence of B haplotype in homozygosity compared to female controls (1/22 4.5% *vs.* FCTRs10/39 25.6%, *p*=0.045, OR=0.138). Noteworthy, *KIR2DS1* gene was more frequent in $HER2^+$ patients than in $HER2^-$ patients (5/5 100% *vs.* 12/28 42.9%, *p*=0.04, OR=14.52).

### 3.6. FCGR3A Polymorphisms in BC Onset and Clinical Outcome

Although no significant difference between FCGR3A 48 and 158 polymorphisms was found in terms of alleles, we observed the prevalent presence of a valine (G) in position 158 and a leucine (T) in position 48 in both BC patients and controls (Suppl. Table **2**). We noted a reduced incidence of the *158T/T* (FF) homozygous genotype in the whole group of BC patients (2.2% *vs.* 11.1% in FCTRs), including those with more aggressive tumors (0% *vs.* 11.1%, *p*=0.13). A significant negative relationship between 48 *G/T* (LR) genotype and the proliferative index (Ki-67) was observed, as indicated by Spearman's correlation (*p*=0.020, rs=-0.439). Besides, the FCGR3A-48,158 haplotype-based analysis showed a different distribution between patients and controls*: 48T-158G* was the most frequent haplotype in BC patients (HF=44.2%), while the *48T-158T* was more commonly identified in FCTRs (HF=44.4%) (Suppl. Table **3**).

### 3.7. Correlations Between FCGR3A Haplotypes and KIR Genes

When FCGR3A 48-158 haplotypes were combined with KIR genes by using Spearman's ranks method (Table **5**)**,** we made some interesting observations. 1. The *FCGR3A48A-158G* haplotype was found negatively linked with the *KIR2DL2 gene* (*p*=0.034, rs=-0.434) in LNM+ BC patients, while it was negatively associated with KIR2DS4del (*p*=0.029, rs=-0.365) and positively with *KIR2DS5* gene (*p*=0.043, rs=0.339) in the female control group; 2. The *48T-158T* haplotype was positively correlated either with *KIR2DL2* (*p*=0.006, rs=0.542) or with the *KIR2DS2* gene (*p*=0.026, rs=0.454 in LNM+ BC patients), while it was positively associated with *KIR2DS4ins* (*p*=0.038, rs=0.348) and negatively with *KIR2DS5* gene (*p*=0.041, rs=-0.343) in the controls. It must be noted that there was a significant correlation observed between the *48T-158T* haplotype and *KIR2DL2* (*p*=0.035, rs=0.500) in advanced stages of the disease, and with *KIR2DL3* (*p*=0.032, rs=0.431) in patients with early tumors. It is interesting to note that *KIR2DL2* and *KIR2DL3* genes are alleles of the same locus, which segregates in different haplotypes (B and A, respectively). In contrast, in relapsed BC patients, a positive relationship with the *KIR2DL1* gene (*p*=0.016, rs=0.674), on haplotype A, was also observed. 3. The *48T-158G* haplotype was the third haplotype detected in the studied patient population. While it was negatively correlated with the *KIR2DL1* gene (*p*=0.046, rs=-0.475) in more advanced tumors, it was positively associated with *KIR2DL5B* (*p*=0.009, rs=0.512) and *KIR2DS3* (*p*=0.012, rs=0.493) genes (adjacent loci in the B haplotype) in early-stage tumors. In FCTRs, this haplotype was negatively correlated to *KIR2DS4ins.*

## 4. DISCUSSION

Breast cancer is characterized by a microenvironment often infiltrated by immune cells, including tumor-infiltrating T cells (TILs), and to a lesser extent, NK cells [29]. Metastatic dissemination of cancer cells consists of an interplay between cancer cell-intrinsic factors (genetic and epigenetic diversification) and microenvironmental immunosuppressive determinants, such as metabolic, stromal, and immunological factors. Interindividual variability in the NK receptors repertoire is also influenced by KIR genes, HLA class I alleles, and FCGR3A (CD16A) genetic polymorphisms. The presence of steroid hormone receptors, including ER (estradiol E2), mediates immunostimulation or immunosuppression, particularly on NK cells in a time-dependent manner [30-32].

To date, the analysis of gene polymorphisms at the nucleotide level of the HLA-C locus, in combination with those of FCGR3A in breast cancer, has not been rated yet. For this purpose, we carried out a case-control study between BC patients and healthy controls to evaluate the impact of the KIR/HLA-C and FCGR3A genes on the pathogenesis and progression of breast cancer in the Italian population and correlate them with proliferation and some key clinical features of BC, including tumor stage and recurrence.

Based on our observations, first of all, the presence of *HLA-C*05:01:01* allele could be considered protective against BC, while the presence of *HLA-C*07:02:01* could play a role in BC progression. We can speculate that a differential expression of these alleles might influence the efficacy of the immune response toward cancer cells given that, as suggested by previous studies [33], different expressions of HLA-C7 and HLA-C5 antigens have been found. Low levels of *HLA-C*07* allele cell surface expression are due to a more restrictive peptide-binding pocket than the *HLA-C*05* allele, which has a flatter cleft that allows the binding of a larger range of peptides. This situation can stabilize the HLA-C molecule affecting its expression level on the cell surface. As a consequence, the HLA-C7 and HLA-C5 antigens may negatively and positively regulate T cell immunosurveillance, respectively. Instead, the predisposing effect of the *HLA-C*04:01:01* allele to lymph node metastasis could be due to its low cell surface expression [34]. These features might cause NK hyporesponsiveness predisposing to tumor invasiveness.

Defective NK cell cytotoxicity has been described in a variety of solid tumors, including breast cancer [29,35], which has been associated with increased frequency of CD56*bright* NK cells in peripheral blood [36]. Thus, while the interaction between inhibitory KIRs and their HLA class I ligands, by the process of

**Table 5. Spearman's test correlation between FCGR3A48-158 haplotypes and KIR genes in BC patients compared to female healthy controls (FCTRs).**

| FCGR3A | Grade 0-II BC patients (n=25) | *P*= | rs | FCTRs (n=36) | *P*= | rs |
|---|---|---|---|---|---|---|
| 48-158 haplotype | KIR gene | - | - | KIR gene | - | - |
| 48G-158G | 2DL5B | 0.046 | -0.411 | --- | - | - |
| 48T-158T | 2DL3 | 0.032 | 0.431 | 2DS4ins | 0.038 | 0.348 |
| - | - | - | - | 2DS5 | 0.041 | -0.343 |
| 48T-158G | 2DL5B | 0.009 | 0.512 | 2DS4ins | 0.000 | -0.565 |
| - | 2DS3 | 0.012 | 0.493 | - | - | - |
| FCGR3A | Grade III-IV BC patients (n=18) | *P*= | rs | FCTRs (n=36) | *P*= | rs |
| 48T-158T | 2DL2 | 0.035 | 0.500 | 2DS4ins | 0.038 | 0.348 |
| - | - | | - | 2DS5 | 0.041 | -0.343 |
| 48T-158G | 2DL1 | 0.046 | - 0.475 | 2DS4ins | 0.000 | -0.565 |
| FCGR3A | LNM* BC patients (n=24) | *P*= | rs | FCTRs (n=36) | *P*= | rs |
| 48A-158G | 2DL2 | 0.034 | - 0.434 | 2DS4del | 0.029 | -0.365 |
| - | - | | - | 2DS5 | 0.043 | 0.339 |
| 48T-158T | 2DL2 | 0.006 | 0.542 | 2DS4ins | 0.038 | 0.348 |
| - | 2DS2 | 0.026 | 0.454 | 2DS5 | 0.041 | -0.343 |
| - | 2DL2/HLA-C1 | 0.032 | 0.480 | - | - | - |
| 48T-158G | -- | | - | 2DS4ins | 0.000 | -0.565 |

"licensing" or education, allows NK cells to acquire full effector functions, a mismatch makes NK cells hypo-responsive. Also, the activating KIR-HLA class I licensing may influence NK cells' unresponsiveness to cancer cells. NK cells expressing activating KIR2DS1 in the presence of self-HLA-C2 ligands are poorly responsive toward cancer [37]. Our study indicates that, in BC patients, *KIR2DS4ins* and *KIR2DL2* genes, not licensed by HLA-C1 ligands, seem to be protective from the neoplasm onset. Conversely, the absence of both *KIR2DS4* and *KIR2L2* genes may increase the risk of BC occurrence. The protective role of *KIR2DS4ins* receptor could be due to its inhibitory action on NK response towards bacterial or viral infections, predisposing to a particular tumor microenvironment. Indeed, it is known that this KIR receptor recognizes recA peptides derived by pathogens**,** mainly in the context of HLA-C5 antigen [38]. Also, the protective role on BC pathogenesis of the inhibitory *KIR2DL2* receptor, unlicensed by C1 ligands, may be due to the NK cells' unresponsiveness to cancer cells. This finding was consistent with what has already been described in a recent study on Saudi women with breast cancer [39]. Our analysis evidenced that a simultaneous absence of the inhibiting *KIR2DL2*, not licensed by its cognate C1 ligand, together with the *KIR2DS4ins* gene, increases the risk of tumor progression by nine-fold.

Furthermore, we can also speculate that the activating *KIR2DS1* in association with the HLA-*C*07:02:01* allele might play a role in breast cancer aggressiveness, may be supporting a chronic over-stimulation of NK cells. The association of this gene with increased BC risk had already been highlighted in another investigation on Turkish patients with advanced BC compared to controls [40]. This correlation might depend on the influence on patients with advanced BC of the simultaneous presence of its equivalent inhibitory, *KIR2DL1* gene. On the contrary, the increased presence of *KIR2DL1* in the absence of the *KIR2DS1* counterpart, in advanced tumors, suggests a putative "antitumor role" of the *KIR2DL1* receptor. This information is also supported by an analysis of Ashoury E.[41]. Interestingly, BC patients with lymph node metastasis have shown a significantly higher frequency of the *KIR2DL2* gene in the presence of *HLA-C1/C1* homozygous (OR=4.25) than healthy controls, which, by the effect of the process of "licensing", could favor full pro-inflammatory effector functions.

On the basis of these findings, we could speculate that the activation of inflammation mediated by *KIR2DS1* could be turned off by *KIR2DL2/KIR2DS4*, but only when they are not licensed by the HLA-C1 ligand. Such results suggest that the susceptibility to BC transformation and progression might depend on the type of genotype combinations. Indeed, the involvement of activating KIRs in cancer pathogenesis has also been observed by other studies [in *chronic myeloid leukemia* (CML) and nasopharyngeal

carcinoma, respectively] [42,43]. Increases in the levels of innate immune response stimulation may contribute to an increased risk of some virus-associated cancers, maybe through an amplified inflammatory response triggered by NK cells (or other effector cells) expressing activating KIRs.

Examining the influence of KIR genes on lymph node status, in the context of hormone markers, we did not evidence any difference in the progression of disease between ER or PR-positive patients, even though in these patients, a prevalent frequency of the *KIR2DL3* and a reduction of *KIR2DS4ins* gene were specifically detected. It is possible to hypothesize that breast cancer hormones may influence in a different way some immune responses depending on genetic background (every HER-2+ BC patient in our study carried the KIR2DS1 gene and showed signs of metastasis).

Fcγ receptors are essential for the ADCC pathway, and FCGR3A functional gene polymorphisms may affect the killing function of immune effector cells. *FCGR3A158G/T* polymorphism is the most studied biomarker for ADCC, and several reports have already demonstrated its involvement in enhanced efficacy of monoclonal antibodies (mAb) therapy in solid tumors [17, 44, 45]. Furthermore, it has also been shown that affinity-modulating FCGR3A sequence variants play a major role in the binding of antibodies by CD16-CAR T cells, and can increase their activity [46].

One of the goals of this work was to define the impact of FCGR3A gene polymorphisms together with KIR/HLA-C interaction on BC pathogenesis and malignancy. While FCGR3A gene polymorphisms alone did not show a prominent role in the development of BC, the 48-158 haplotypes in the context of particular KIR genes had specific correlations in BC patients' subsets compared to female controls. A recent study by Zheng *et al*. illustrated another KIR/HLA interaction (HLA-G/KIR2DL4), which could resensitize breast cancer to trastuzumab treatment [47].

Our results suggest a hypothetical synergic effect of particular KIR genes with FCγR 48-158 variants responsible for ADCC efficiency. For instance, in LNM+ BC patients, the inhibitory receptor *KIR2DL2* gene was negatively related to *FCGR3A 48A-158G* with higher ADCC efficiency, but positively to *FCGR3A48T-158T* haplotype (lower ADCC response). This finding had already been shown by Muraro E. on a selected group of HER-2-positive breast cancer [48].

The impact of these HLA-C/KIR and FcγR variants on the immune microenvironment in breast cancer might help to predict the outcome of immunotherapy and provide novel targets for the treatment of this neoplasia.

## CONCLUSION

Although in a relatively small sample size, our study examined the influence of the genetic basis of KIR receptors, together with their HLA-C ligands, and FCGR3A genes for NK antitumor activity using high-resolution techniques. Our findings suggest that in the development of breast cancer exists a disorder of immune regulation, and that NK cells might represent a promising target for the development of immuno-therapeutic strategies for metastatic disease. The KIR-HLA and FCGR3A associations evidenced in this analysis constitute a hypothetical indication of genetic risk scores for tailored screening of BC patients. Further studies involving BC patients with advanced tumors are needed to verify the specific *in vivo* activation of NK cells. These results could potentially guide immunotherapy against breast cancer by specifically targeting NK cell clones with particular KIR-HLA-CD16A patterns favoring antitumor activity.

## LIST OF ABBREVATIONS

| | | |
|---|---|---|
| ADCC | = | Antibody-Dependent Cellular Cytotoxicity |
| AF | = | Allele Frequency |
| BC | = | Breast Cancer |
| CML | = | Chronic Myeloid Leukemia |
| ER | = | Estrogen Receptor |
| FCTRs | = | Female Controls |
| HER2 | = | Human Epidermal Growth Factor Receptor 2 |
| HLA | = | Human Leukocyte Antigens |
| KIR | = | Killer-Cell Immunoglobulin-Like Receptor |
| LNM | = | Lymph Node Metastases |
| MCTRs | = | Mixed Controls |
| NK | = | Natural killer |
| PCR | = | Polymerase Chain Reaction |
| PR | = | Progesterone Receptor |
| SBT | = | Sequence-Based Typing |
| TILs | = | Tumor-Infiltrating T Cells |

## AUTHORS' CONTRIBUTIONS

A.C. and A.A. designed the study, analyzed and interpreted the data. A.C. performed KIR and HLA-C genotyping and analyzed the results. T.D.B. performed the FCGR3A SBT method. AC, A.A., and G.S. wrote the manuscript. G.N., O.B., P.R., A.V., and F.P. critically revised the manuscript. G.S. provided funding. All the authors approved the final version.

## ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was approved by the Internal Review Board (IRB) of the Polyclinic "Tor Vergata" Hospital, Rome, Italy (number 133/10).

## HUMAN AND ANIMAL RIGHTS

No animals were used in this research. All procedures performed in studies involving human participants were in accordance with the 1975 Declaration of Helsinki, as revised in 2013.

## CONSENT FOR PUBLICATION

A written informed consent was obtained from all the subjects.

## STANDARD OF REPORTING

STROBE guidelines were followed.

## AVAILABILITY OF DATA AND MATERIAL

To protect patients' privacy, datasets generated and analyzed in this study will be available from the corresponding author on request and included as supplementary information files.

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## CONFLICTS OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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Declared none.

## SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's website along with the published article.

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