HLA-G and HLA-F protein isoform expression in breast cancer patients receiving neoadjuvant treatment

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The immunosuppressive human leukocyte antigens HLA-G and HLA-F are expressed on trophoblast and malignant cells. Four membrane-bound and three soluble HLA-G protein isoforms have been described, which have different immunosuppressive potentials. HLA-F has three transcript variants, resulting in three different protein isoforms. The aim of this study was to evaluate the prognostic and predictive value of HLA-G and HLA-F protein isoform expression patterns in patients with breast cancer. Core biopsies were taken at diagnosis in patients with HER2+ (n = 28), luminal B-like (n = 49) and triple-negative (n = 38) breast cancers who received neoadjuvant chemotherapy. Expression levels of HLA-F and -G were correlated with the pathological complete response (pCR). Protein expression was determined by Western blot analysis, using two antibodies for each HLA, specific for different isoforms. The protein expression of HLA isoforms did not significantly differ between breast cancer subtypes. However, some initial indications were found for an association between the soluble HLA-G6 protein isoform and pCR in HER2+ breast cancer. The study provides preliminary evidence for the evaluation of HLA-G isoform expression, in particular HLA-G6, as a possible new marker for pCR in HER2+ breast cancer.

The immune system plays an important role in many cancers. In breast cancer (BC), the importance of the immune system has been established in several clinical scenarios. For example, first-line triple-negative advanced breast cancer patients treated with the programmed cell death-ligand 1 (PD-L1) antibody atezolizumab had an improved overall survival when tumour-infiltrating lymphocytes (TILs) showed PD-L1 expression. The number of TILs has also been shown to be an independent predictor of the response to neoadjuvant chemotherapy (NACT). NACT was initially administered in patients with inoperable breast cancer in order to reduce tumour size. Patients who responded well to NACT, as indicated by a decrease in tumour size, had improved overall (OS) and disease-free survival rates. The administration of NACT, in patients with operable breast cancer as well, became a standard treatment in view of higher rates of breast-conserving surgery and the opportunity it provides to monitor treatment success in vivo. Treatment success with NACT can range from a minimal reduction in tumour size to a total absence of tumour tissue. The total absence of cancer cells, described as a pathological complete response (pCR), is an important marker for improved overall survival.

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It is therefore of particular importance to understand the mechanisms underlying the response to NACT in order to evaluate valid response markers. The mechanisms behind the immune system's contribution to the outcome of treatment are currently under investigation. The present study focuses on the role of human leukocyte antigens G and F (HLA-G and HLA-F) in this context.

The HLA-G protein is a non-classical major histocompatibility antigen, mainly expressed on extravagillous trophoblasts of the placenta and various types of carcinoma, such as lung cancer, gastric cancer and prostate cancer. HLA-G predominantly mediates immune evasion and immune suppression by inhibiting cells of the adaptive and innate immune system—e.g., natural killer cells (NK cells), T lymphocytes and B lymphocytes—interacting with inhibitory receptors such as leukocyte immunoglobulin-like receptors B1 and B2 (LILRB1 and LILRB2) or killer cell immunoglobulin-like receptor 2DL4 (KIR2DL4). HLA-G can be expressed as four alternatively spliced membrane-bound isoforms (HLA-G1 to HLA-G4). Additionally, in contrast to classical HLA class I genes, HLA-G can also occur in the form of three soluble HLA-G isoforms (HLA-G5 to HLA-G7). HLA-G1 and the soluble HLA-G5 represent the complete extracellular protein structure, which is composed of three alpha domains (α1–α3) connected with β2 microglobulin (β2M). The other isoforms are not associated with β2M and differ in their extracellular protein structure. The soluble isoforms show a high degree of structural similarity with the membrane-bound isoforms, but preserve intron 4 (HLA-G5 and -G6) or intron 2 (HLA-G7), leading to a loss of the transmembrane domain. It is hypothesized that these seven protein isoforms have distinct immunosuppressive potentials. It is known that both full-length isoforms (HLA-G1 and HLA-G5) interact with the HLA-G-specific receptor via the α1 or α3 domain. The α1 domain binds to the KIR2DL4 receptor, whereas the α3 domain is recognized by LILRB1, LILRB2 and CD88. Isoforms that lack the α3 domain, such as HLA-G3, -G4 and -G7, do not interact with these receptors.

HLA-F is the second, less well known member of the non-classical HLA class I family. The protein structure of HLA-F is similar to that of HLA-G and consists of a leader peptide encoded by exon 1, three alpha domains encoded by exons 2, 3 and 4, a transmembrane domain (exon 5) and a cytoplasmic tail, encoded by exons 6 and 7. In comparison with other HLA genes, the cytoplasmic tail is shorter and varies in length between the three isoforms, which are encoded by three mRNA transcript variants. HLA-F is mainly restricted to the intracellular parts of the cell, but can also be expressed on the cell surface of activated B cells and extravillous trophoblasts. It has been suggested that cell surface expression depends on the transport of HLA-F protein to the cell membrane, mediated by its cytoplasmic tail. Similar to HLA-G, HLA-F also interacts with inhibitory receptors LILRB1 and -B2, as well as the activating KIR receptor KIR3DS1.

Due to the different immunosuppressive effects of the HLA-G isoforms and the diverse role of HLA-F, these proteins could be of interest for the investigation of pathogenesis and progression and as a therapeutic target for breast cancer. The primary aim of the study was therefore to analyse the distribution of HLA-G and HLA-F across the molecular subgroups of breast cancer. Investigating the association between HLA-G and HLA-F expression and pathological complete response (pCR) after NACT was an additional exploratory aim of the study.

Methods

Patient selection. Patients were recruited within the iMODE-B study (Imaging and Molecular Detection of Breast Cancer). Patients were eligible for inclusion if they had an indication for a diagnostic biopsy due to a suspicious breast lesion. The main aim of the iMODE-B study is to identify molecular markers at the time of the first breast cancer diagnosis that are predictive of the prognosis and treatment response.

A total of 1422 participants were recruited into the iMODE-B study from 2007 to 2017. Patients were excluded in the following hierarchical order (Fig. 1): 75 were not diagnosed with BC (controls as per iMODE-B protocol); 149 patients were diagnosed with ductal carcinoma in situ (DCIS); clinical staging data were missing for 117 patients; and 70 participants had bilateral breast cancer. The study population of iMODE-B patients who were diagnosed with BC at the primary diagnosis therefore comprised 1011 patients. Of these, 345 patients were treated with NACT, among whom 135 participants were excluded due to missing fresh-frozen tissue from the breast cancer core biopsy. The molecular subtypes were classified as follows, in accordance with the St. Gallen guidelines:

- Luminal B-like: hormone receptor (HR)-positive, human epidermal growth factor receptor 2 (HER2)-negative and grading G3.
- Triple-negative breast cancer (TNBC): HR-negative and HER2-negative.
- HER2-positive: HR-positive/negative [estrogen receptor (ER) and/or progesterone receptor (PR) ≥1%] and HER2-positive.

To achieve tumour-specific protein results, patients with core biopsies with tumour amounts ≤50% were also excluded from the study cohort. The final study population therefore consisted of 115 patients who were treated with NACT in the iMODE-B study (Fig. 1). Within this cohort, 38 patients were diagnosed with triple-negative breast cancer, 49 with luminal B-like breast cancer and 28 participants with HER2-positive breast cancer.

Pathological complete response (pCR) to NACT was defined in accordance with the semiquantitative scoring system presented by Sinn et al. All of the patients provided written informed consent, and the study was approved by the ethics committee of the Medical Faculty of Friedrich Alexander University of Erlangen–Nuremberg.

Clinical data. Patient data were collected prospectively, and a web-based database was used for documentation, as described previously. Patient and tumour characteristics, detailed treatment data and epidemiological...
cal data were documented. Follow-up treatments and disease characteristics were collected for up to 10 years after the primary diagnosis. All histological tumour data, such as tumour size, axillary lymph-node status, grading, ER status, PR status and HER2 status were documented. The data are monitored as part of the review process for certification of the breast cancer center and are audited annually. Data obtained from these collection and auditing processes were used in the analysis presented here.

**Sample collection and preparation.** Breast cancer biopsies were fresh-frozen in liquid nitrogen immediately after the core biopsy was taken and stored at −80 °C until further use. Only fresh-frozen biopsies of patients with tumour proportions of more than 50% in the diagnostic core biopsies were chosen for analysis. One fresh-frozen breast cancer core biopsy from each patient was used for protein extraction.

**Protein extraction and quantification.** Protein was extracted from fresh-frozen breast cancer core biopsies using RIPA protein lysis buffer (Sigma Aldrich, Taukirchen, Germany), consisting of 1 mM Na3VO4.
1.5 mM NaF, 1 mM phenylmethylsulfonyl fluoride (PMSF) and protein inhibitor cocktail (pepsstatin, leupeptin and chymostatin each 10 µg/mL). The tissues were homogenized with protein lysis buffer using the Precellys' 24-tissue homogenizer (Bertin Instruments, Frankfurt am Main, Germany) with reinforced tubes (MK28-R hard tissue grinding kit, 2 mL reinforced tubes with screw cap and skirt; VWR, Darmstadt, Germany) with an interval of three times 30 s at 4500 rpm. Afterwards, the tubes were centrifuged at high speed for 1 min. The supernatant was used for further analysis. Protein concentrations were measured using the EZQ protein quantitation kit (Thermo Fisher, Darmstadt, Germany) in accordance with the manufacturer’s instructions. Egg albumin served as the protein standard, provided by the manufacturer.

Western blot analysis of HLA-G and HLA-F isoforms. In order to analyse the isoform-specific expression pattern of HLA-G and HLA-F, protein samples (15 µg per lane) were separated using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). To determine the different isoforms of HLA-G, two commercially available antibodies were used that recognize the membrane-bound isoforms (monoclonal anti-HLA-G mouse antibody, clone 4H84; Abcam, Cambridge, United Kingdom, 1:100) and soluble isoforms (monoclonal anti-HLA-G mouse antibody, clone 5A6G7; Thermo Fisher, Darmstadt, Germany, 1:100), which have both been used in several studies34–37. The corresponding bands for the HLA-G isoforms were detected at 38 kDa, as well their glycosylated and β2-microglobulin-associated forms at around 50 kDa for HLA-G1, which were used for quantification36,39, 30 kDa for HLA-G2; 22 kDa for HLA-G3; 49 kDa for HLA-G5 (HLA-G5 (36 kDa), associated with β2-microglobulin (13 kDa))40; and 27 kDa for HLA-G6. For the HLA-F analysis, two different commercially available antibodies were used that recognize the N-terminal end of the HLA-F protein (polyclonal anti-HLA-F rabbit antibody; Aviva Systems Biology, San Diego, California, USA) and the C-terminal end (polyclonal anti-HLA-F rabbit antibody; Sigma Aldrich, Taufkirchen, Germany). The corresponding bands for the HLA-F isoforms were detected at 50 kDa for HLA-F1 and 30 kDa for HLA-F3. An anti-mouse HRP-labeled antibody was used as a secondary antibody (monoclonal anti-mouse goat antibody conjugated with horse radish peroxidase; Sigma Aldrich, Taufkirchen, Germany, 1:4000). Protein bands were quantified using ImageJ. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal protein control (rabbit anti-GAPDH polyclonal antibody, clone 0411, diluted 1:10,000; Santa Cruz Biotechnology, Heidelberg, Germany), and placental tissue was used as a reference sample for each blot. The isoform expression pattern was determined semiquantitatively by normalizing to GAPDH relative to the reference sample (HLA isoform/GAPDH ratio).

Statistical analysis. The primary objective was to compare the protein levels of the isoforms HLA-F1, HLA-F3, HLA-G1, HLA-G2, HLA-G3, HLA-G5 and HLA-G6 between molecular subtypes (three subtypes: HER2-positive, luminal B-like and triple-negative). A Kruskal–Wallis test was performed for each of these biomarkers. Median values and interquartile ranges (25th percentile and 75th percentile) are presented. Nonparametric methods were used, as the biomarker data were skewed with many low values. High and low levels of protein expression were determined by median. A secondary, exploratory objective was to compare patients who achieved a pCR with patients who did not achieve a pCR in relation to the isoforms. A Wilcoxon rank sum test was performed for each biomarker. All of the tests were two-sided, and a P value of < 0.05 was regarded as statistically significant. P values were not corrected for multiple testing. Calculations were carried out using the R system for statistical computing (version 3.4.0; R Development Core Team, Vienna, Austria, 2017).

Ethical approval. Approval for the study was obtained from the ethics committee of the Faculty of Medicine at Friedrich Alexander University of Erlangen–Nuremberg and all of the relevant local ethics committees. All procedures were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Informed consent. Written informed consent was obtained from the patients as part of the inclusion criteria before they entered the study.

Results
Patient characteristics. The study comprised 115 breast cancer core biopsies from patients who received neoadjuvant treatment for breast cancer, with a mean age at diagnosis of 56.7 years (standard deviation, SD, 13.8 years), 45 of whom (39.1%) achieved pCR. Patient characteristics including tumour and lymph-node stage, tumour grade, ER, PR, molecular subtype and pCR are shown in Table 1.

Expression pattern of HLA-F and HLA-G isoforms in molecular subtypes. The initial aim was to analyse the distribution of the HLA-F and HLA-G isoforms between the molecular subtypes. Western blot analysis was therefore performed with two different antibodies for each HLA, representing different isoforms in relation to their molecular weight. Antibodies were used to detect HLA-F1 and HLA-F3 isoforms, the membrane-bound isoforms HLA-G1, HLA-G2 and HLA-G3, and the soluble isoforms HLA-G5 and HLA-G6 (Table 2). No expression was detected for HLA-G2. In contrast, a high level of expression was noted for HLA-G1 in all subtypes and HLA-G3 in luminal B-like and triple-negative breast cancer core biopsies. However, HLA-F and HLA-G isoform expression did not differ significantly between the molecular subtypes (Table 2 and Fig. 2).
Association of HLA-f and HLA-G protein isoform expression with pCR status. In addition, HLA-F and HLA-G isoform distribution was analysed in patients with ("pCR") and without pCR ("no pCR"). However, no differences were observed between these two cohorts for any of the isoforms analysed (Table 3).

For luminal B-like breast cancer samples, an overall low percentage (12–22%) of patients who achieved pCR was observed, independently of the expression of HLA-F and HLA-G isoforms, in comparison with the pCR rates in the HER2+ and triple-negative BC subcohorts (46–86% and 32–71%, respectively) (Table 4). When the molecular subtypes were divided into those with low and high levels of isoform expression relative to the median, it was found that 86% of the patients with HER2+ and HLA-G6 low tumours had a pCR, in comparison with

| Characteristic | Mean or n | SD or % |
|---------------|-----------|---------|
| Age (years)   | 56.7      | 13.8    |
| Tumour stage  |           |         |
| cT1           | 32        | 27.8    |
| cT2           | 59        | 51.3    |
| cT3           | 11        | 9.6     |
| cT4           | 13        | 11.3    |
| Lymph-node status |         |         |
| cN0           | 88        | 77.9    |
| cN+           | 25        | 22.1    |
| Tumour grade  |           |         |
| G2            | 5         | 4.3     |
| G3            | 110       | 95.7    |
| Estrogen receptor |         |         |
| Negative      | 45        | 39.1    |
| Positive      | 70        | 60.9    |
| Progesterone receptor |        |         |
| Negative      | 69        | 60.0    |
| Positive      | 46        | 40.0    |
| Molecular subtype |        |         |
| HER2-positive | 28        | 24.3    |
| Luminal B-like | 49      | 42.6    |
| Triple-negative | 38     | 33.0    |
| pCR           |           |         |
| No            | 70        | 60.9    |
| Yes           | 45        | 39.1    |

Table 1. Patient and tumour characteristics. Mean and standard deviation (SD) are shown for continuous characteristics, frequency (n) and percentage (%) for categorical characteristics. cT clinical staging, cN clinical nodal status, HER2 human epidermal growth factor receptor 2, G2 and G3 grading, pCR pathological complete response.

| Biomarker | HER2+ (n = 28) | Luminal B-like (n = 49) | Triple-negative (n = 38) | P value |
|-----------|----------------|------------------------|--------------------------|---------|
| HLA-F1    | 0.26 (0.09, 0.46) | 0.27 (0.19, 0.44) | 0.27 (0.15, 0.5) | 0.82    |
| HLA-F3    | 0.58 (0.10, 2.23) | 0.57 (0.15, 2.66) | 0.92 (0.26, 2.11) | 0.76    |
| HLA-G1    | 2.92 (1.33, 5.97) | 2.85 (1.93, 4.54) | 2.69 (1.5, 8.86) | 0.80    |
| HLA-G2    | 0.00 (0.00, 0.00) | 0.00 (0.00, 0.00) | 0.00 (0.00, 0.00) | 0.92    |
| HLA-G3    | 1.48 (0.74, 3.87) | 2.16 (0.74, 3.96) | 2.57 (1.37, 4.99) | 0.17    |
| HLA-G5    | 1.87 (0.79, 3.22) | 1.37 (0.50, 3.18) | 1.17 (0.35, 5.95) | 0.72    |
| HLA-G6    | 0.18 (0.10, 0.45) | 0.19 (0.11, 0.47) | 0.19 (0.05, 0.37) | 0.73    |

Table 2. HLA-G and -F isoform distribution in the molecular subtypes. Protein bands from Western blots were quantified using ImageJ. GAPDH was used as an internal protein control, and placental tissue was used as a reference sample for each blot. The isoform expression pattern was determined semiquantitatively by normalizing to GAPDH relative to the reference sample (HLA isoform/GAPDH ratio). The median and interquartile range (25th percentile, 75th percentile) of the HLA isoform/GAPDH ratio were calculated for each molecular subtype, and P values were calculated using the Kruskal–Wallis test. HER2 human epidermal growth factor receptor 2, HLA human leukocyte antigen.
only 46% in the high-level HLA-G6 group (Table 4). In contrast, the triple-negative subtype cohort showed a high pCR rate (62% and 68%, respectively) when HLA-F3 and HLA-G6 expression levels were also defined as “high” (Table 4).

**Discussion**

HLA-G is one of the “non-classical” HLA antigens (class Ib) and is thought to have immunosuppressive effects. Isoform-specific differences have not previously been investigated. Large studies on HLA-G expression, as conducted by de Kruijf et al., mostly evaluated using immunohistochemistry, have reported a positive correlation between HLA-G protein expression, shorter disease-free survival and advanced disease stages. In the present study, however, correlation analyses between isoform-specific HLA-G and HLA-F expression and overall survival were not yet possible, as the study also included patients with recently diagnosed breast cancer. However, it was found that in the luminal B-like breast cancer subtype, only a small percentage of patients achieved pCR with...
As already mentioned, the α1 domain inhibits the cytolytic activity of NK cells (CD56dim)—via the KIR2DL4 pCR42. Since König et al. measured both soluble HLA-G isoforms (G5/G6) at once, these data are at least partly in plasma samples from patients with ER+ breast cancer before receiving NACT, they observed a significant correlation between soluble HLA-G, as well as total soluble HLA-G serum levels in breast cancer patients before and after NACT. In used an enzyme-linked immunosorbent assay (ELISA) to measure HLA-G5/G6 together in vesicular-derived HER2+ status11. In the present study of HER2+ breast cancer, showing higher pCR rates with high HLA-G1 due to the small cohort size. These data are not in line with current observations. Dong et al. reported significantly or HLA-F3 expression and low pCR rates were observed, but these findings did not reach statistical significance with only a single antibody.

With regard to triple-negative BC, relationships between HLA-G and clinical parameters such as low HLA-G6 expression and pCR rates were observed, but these findings did not reach statistical significance due to the small cohort size. These data are not in line with current observations. Dong et al. reported significantly high HLA-G protein expression using immunohistochemistry in non-luminal breast cancer subtypes (HER2+ and TNBC)46, whereas He et al. did not observe a significant association between high HLA-G expression and HER2+ status41. In the present study of HER2+ breast cancer, showing higher pCR rates with high HLA-G1 expression, however, none of the membrane-bound HLA-G isoforms revealed any significant association with pCR. Several studies on overall HLA-G expression, evaluated using immunohistochemistry, have reported an association between HLA-G protein expression and shorter disease-free survival and advanced disease stage in 41–70% of cases41. In addition, de Kruijf et al. found that HLA-G was an independent prognostic marker for reduced overall survival in patients with HLA class I negative breast cancer44. These data indicate that membrane-bound HLA-G may be well suited for use as a prognostic marker for overall survival as well as disease-free survival, rather than as a predictor for pCR in breast cancer core biopsies. The reason for this controversial finding might be the use of the anti-HLA-G antibody 4H84, which is known to recognize HLA class Ia molecules to a certain degree as well, and also the small sample size in the different subgroups.

A possible mechanism behind the high level of HLA-G isoform secretion and low pCR rates after NACT is the immunosuppressive potential of this particular HLA-G isoform, which is mediated by its α, and α, domains. As already mentioned, the α1 domain inhibits the cytolytic activity of NK cells (CD56dim)—via the KIR2DL4 receptor, for instance—whereas the α2 domain interacts with the LILRB1 and LILRB2 receptors, inhibiting NK cells, T cells, and B cells, as well as dendritic cells and macrophages32. NACT is able to enhance the general immune response, as is evident from increased immune cell infiltration into the stroma38. This suggests that soluble isoforms may contribute to an immunosuppressive environment in the proximity of tumours and inhibit the approach of immune cells. In addition, soluble HLA-G isoforms may also act indirectly by inducing HLA-G5-positive or HLA-G6-positive immune cells, which exert immunosuppressive effects. This indirect, secondary mechanism is already well described for the isoforms HLA-G1 and -G5, which are able to induce the generation of HLA-G-positive regulatory T cells (Tregs) and tolerogenic dendritic cells (DC-10), as well as HLA-G-positive macrophages (via trogocytosis)32,43. These indirect mechanisms may be responsible for a decreased immune

|                    | HER2+ (n = 28) | Luminal B-like (n = 49) | Triple-negative (n = 38) |
|--------------------|---------------|------------------------|------------------------|
| Low HLA-F1         | 5 (36%)       | 9 (64%)                | 19 (79%)               |
| High HLA-F1        | 5 (36%)       | 9 (64%)                | 22 (88%)               |
| Low HLA-F3         | 5 (33%)       | 10 (67%)               | 21 (84%)               |
| High HLA-F3        | 5 (42%)       | 7 (58%)                | 20 (83%)               |
| Low HLA-G1         | 6 (46%)       | 7 (54%)                | 19 (86%)               |
| High HLA-G1        | 4 (29%)       | 10 (71%)               | 20 (83%)               |
| Low HLA-G2         | 7 (32%)       | 15 (68%)               | 33 (85%)               |
| High HLA-G2        | 3 (50%)       | 3 (50%)                | 8 (80%)                |
| Low HLA-G3         | 6 (35%)       | 11 (65%)               | 21 (88%)               |
| High HLA-G3        | 4 (36%)       | 7 (64%)                | 18 (78%)               |
| Low HLA-G5         | 4 (36%)       | 7 (64%)                | 20 (83%)               |
| High HLA-G5        | 6 (35%)       | 11 (65%)               | 18 (82%)               |
| Low HLA-G6         | 2 (14%)       | 12 (86%)               | 21 (88%)               |
| High HLA-G6        | 7 (54%)       | 6 (46%)                | 20 (80%)               |

Table 4. Descriptive correlation of HLA-G and HLA-F isoforms with low and high levels of expression and pathological complete response status in the subtypes. Protein bands from Western blots were quantified using ImageJ. GAPDH was used as an internal protein control, and placental tissue was used as a reference sample for each blot. The isoform expression pattern was determined semiquantitatively by normalizing to GAPDH relative to the reference sample (HLA isoforms/GAPDH ratio). The frequencies of high and low levels of expression were determined using the median of the HLA isoform/GAPDH ratio. HLA human leukocyte antigen, HER2 human epidermal growth factor receptor 2, pCR pathological complete response.
response during and after NACT. This hypothesis is supported by Dong et al., who reported that a high level of HLA-G expression was inversely associated with TIL infiltration in breast cancer 45.

The impact of HLA-F expression in cancer is a matter of controversy. Harada et al. reported that HLA-F expression is significantly associated with tumour size and a poorer clinical outcome in breast cancer 47. However, in another study by Zhang et al. on gastric cancer, the authors were unable to show any correlation between HLA-F expression and parameters such as overall survival and tumour stage 48. In contrast, Ishigami et al. observed a strong correlation with 5-year survival rates and the degree of cancer cell invasion in gastric cancer 49. The findings of the present study may suggest that there is an impact of expression of HLA-F protein isoforms F1 and F3 and pCR in patients with luminal B-like breast cancer. Expression of HLA-F1/F3 was associated with low rates of pCR (12–21%); however, a significant association was not detected due to the small cohort size in luminal B-like cases. These findings are consistent with those of Harada et al., who used immunohistochemistry to measure HLA-F in the tumour node and at the invasive front of the tumour and found that HLA-F was a marker for poorer outcomes in clinical stage II breast cancer 47. Similar results have been observed in triple-negative breast cancer, where low HLA-G6 and HLA-F3 protein expression was associated with higher pCR rates. These data are partly in line with the observations by Yau et al., in which low mRNA HLA-F expression in combination with other genes served as a poor prognostic marker in TNBC 50.

However, Western blot analysis has limitations. It is subject to variabilities such as temperature changes and washing during performance 51. Additionally, film-based detection does not always allow detection of a broad dynamic range. To use Western blotting for quantitative protein detection, it is advisable to determine the linear and quantitative dynamic range of each HLA molecule, and this might generate more significant correlations with pathological clinical data 52.

An additional limitation of the present study is the limited number of patients, especially for the intrinsic subtypes. The small size of the cohort is also a weakness in relation to the pCR rates obtained in the study. The study shows for the first time that HLA-G6 may be a possible predictive marker for pCR in HER2+ breast cancer. However, this finding needs to be statistically verified in a larger cohort. In addition to verifying the results, enlarging the study cohort could allow adequate statistical analysis to fully determine the correlation between HLA-F and HLA-G isoform expression and pCR.

The exclusion of patients with less than 50% tumour cells in the tissue is a weakness of this study, since a high level of stromal content is associated with a poorer clinical outcome 53. This subpopulation of patients was excluded—as HLA-G and -F are mainly expressed in tumour, but not in stromal tissue—in order to maximize the tumour tissue content within the lysate. Tumour stromal tissue contains a low number of immune cells 54, indicating a weak immune response. A poor immune response might be induced by soluble factors such as HLA-G5 or -G6 creating an immunosuppressive environment around the tumour tissue. This was observed by Dong et al., in a study in which high HLA-G expression was associated with low immune cell infiltration 45.

In summary, this study suggests that HLA-G and HLA-F may play an immunoregulatory role in breast cancer, on the basis of specific analysis of isoforms and splicing variants. Some of the isoforms have different effects on the immune system. It also needs to be taken into consideration that not all of these isoforms are membrane-bound and that some exist as soluble variants, such as HLA-G6. This implies that (breast) cancer cells with high levels of HLA-G6 synthesis not only have a negative impact on TILs, but are also able to act on the surrounding tissue and possibly on the entire immune system.

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Author contributions
F.M.W. performed the experimental work. F.M.W., H.H., P.A.F. and M.R. wrote the main manuscript text and prepared all figures and tables. L.H. performed the statistical analysis. R.E. and A.H. carried out the pathological examination of the breast core biopsies. P.G., A.H., S.M.J., C.H. and M.W. were responsible for patient care, patient examinations, clinical data acquisition, and recruitment into the iMODE study. R.S.-W. performed mammography as well as biomaterial sampling of the breast core biopsies. A.B.E. provided analytical equipment and technical supervision. M.W.B., P.A.F. and M.R. supervised the study. All of the authors reviewed the manuscript.

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Competing interests
The authors declare no competing interests.

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