


Predictive Role of CD36 Expression in HER2-Positive Breast Cancer Patients Receiving Neoadjuvant Trastuzumab

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Abstract

Background: Despite huge efforts to identify biomarkers associated with long-term clinical outcomes in patients with early-stage HER2-positive breast cancer (HER2+ BC) treated with (neo)adjuvant anti-HER2 therapy, no reliable predictors have been identified so far. Fatty acid uptake, a process mediated by the transmembrane transporter CD36, has recently emerged as a potential determinant of resistance to anti-HER2 treatments in preclinical HER2+ BC models. **Methods:** Here, we investigated the association between baseline intratumor CD36 gene expression and event-free survival in 180 patients enrolled in the phase III trial Neoadjuvant Lapatinib and/or Trastuzumab Treatment Optimization (NeoALTTO), which randomly assigned stage II-III HER2+ BC patients to receive neoadjuvant lapatinib, trastuzumab, or lapatinib-trastuzumab in combination with chemotherapy. To this aim, we selected NeoALTTO trial patients for whom pretreatment whole transcriptomic data were available. The main study results were validated in an independent cohort of patients enrolled in the neoadjuvant phase II trial NeoSphere. **Results:** In 180 NeoALTTO patients, high intratumor CD36 expression was independently associated with worse event-free survival in patients treated with trastuzumab-based therapy (hazard ratio [HR] = 1.72, 95% confidence interval [CI] = 1.20 to 2.46), but not with lapatinib-based (HR = 1.02, 95% CI = 0.68 to 1.53) or trastuzumab-lapatinib-based (HR =

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1.08, 95% CI = 0.60 to 1.94) therapy. Among 331 NeoSphere patients evaluated, high CD36 expression was independently associated with worse patient disease-free survival in both the whole study cohort (HR = 1.197, 95% CI = 1.002 to 1.428) and patients receiving trastuzumab-based neoadjuvant therapy (HR = 1.282, 95% CI = 1.049 to 1.568). **Conclusions:** High CD36 expression predicts worse clinical outcomes in early-stage HER2+ BC treated with trastuzumab-based neoadjuvant therapy.

In the phase III Neoadjuvant Lapatinib and/or Trastuzumab Treatment Optimization (NeoALTTO) trial, neoadjuvant trastuzumab-lapatinib-based therapy increased pathologic complete response (pCR) rates compared with trastuzumab- or lapatinib-based treatment (1). In parallel, the phase II NeoSphere trial demonstrated increased pCR rates in patients treated with neoadjuvant trastuzumab-pertuzumab therapy compared with patients receiving trastuzumab- or pertuzumab-only treatment (2). Based on results of the NeoSphere trial as well as of other studies in which trastuzumab-pertuzumab-based neoadjuvant therapy was associated with excellent pCR rates and good safety profile (3-5), dual HER2 blockade has become the standard-of-care preoperative therapy for the majority of stage II-III HER2+ BC patients (6). However, dual HER2 blockade in the NeoALTTO and NeoSphere trials did not improve long-term clinical outcomes, such as event-free survival (EFS) or disease-free survival (DFS) (7,8). In the adjuvant setting, the ALTTO trial failed to show a survival advantage from adding lapatinib to trastuzumab-based therapy (9), whereas adding pertuzumab to adjuvant trastuzumab-based therapy in the Aphinity trial resulted in statistically significant improvement of invasive DFS in HER2+ BC patients with lymph node involvement (10).

Different tumor-related biomarkers, including HER2 mRNA levels (11), HER2 protein levels (12), HER2 enrichment according to PAM50 classification (11), ESR1 expression levels (11), exon 9 mutations of PIK3CA gene (12), tumor infiltrating lymphocytes (TILs) (13-15), and the 41-gene classifier TRAR (16), have been associated with the likelihood of achieving pCR in patients receiving neoadjuvant anti-HER2 therapies. However, except for TILs, no reliable predictors of long-term clinical outcomes in this setting have been identified so far.

HER2+ BC is a lipogenic malignancy (17), and HER2-induced enhancement of fatty acid (FA) biosynthesis sustains tumor cell growth, proliferation, and metastasization (18,19). More recently, FA uptake, as mediated by the transmembrane transporter CD36, has emerged as a metabolic process associated with acquired resistance to anti-HER2 agents in preclinical HER2+ BC models (20). Here, we investigated the association between CD36 gene expression and the prognosis of HER2+ BC patients enrolled in the NeoALTTO trial, and we confirmed the main study findings in an independent cohort of patients enrolled in the NeoSphere trial.

Methods

Patient Population

The NeoALTTO trial (NCT00553358) is a multicenter, randomized, open-label, phase III study that investigated the antitumor activity of lapatinib, trastuzumab, or lapatinib-trastuzumab in combination with preoperative chemotherapy in stage II-III HER2+ BC patients (1,8). The NeoSphere trial (NCT00545688) is a multicenter, randomized, phase II study that investigated the antitumor activity of trastuzumab plus docetaxel, pertuzumab plus trastuzumab plus docetaxel, pertuzumab plus trastuzumab without chemotherapy, or pertuzumab plus docetaxel in stage II-III HER2+ BC (2,7). Details regarding the NeoALTTO and NeoSphere trials are reported in [Supplementary Methods](#) (available online).

Study Design

In the present analysis, we aimed to investigate the association between CD36 expression, as evaluated from transcriptomic data, and EFS, defined as the time between patient random assignment and the occurrence of the first event (ie, breast cancer relapse after surgery, second primary malignancy, patient death, or failure to complete neoadjuvant therapy because of disease progression). As a secondary objective, we evaluated the association between CD36 expression and pCR, defined as the absence of invasive tumor cells in the breast at time of surgery. The NeoSphere trial cohort was used as a validation cohort to confirm the association between CD36 expression and DFS, defined as the time between surgery and the first documentation of progressive disease or death. pCR in the NeoSphere cohort was defined as the absence of invasive tumor cells in the breast and axilla at surgery.

Tumor Sample Processing and Gene Expression Analysis

Gene expression analysis in NeoALTTO tumor samples was performed through ClariomS platform. RNA concentration in each sample was determined by ND-1000 spectrophotometer (NanoDrop). RNA quality was checked using TapeStation 2200 (Agilent) and the RNA integrity number. For the analysis of samples from the NeoSphere trial, RNA was extracted from pretreatment formalin-fixed, paraffin-embedded (FFPE) core biopsies, and gene expression profiling was carried out with Affymetrix U133 Plus 2.0 gene chips, as previously described (21). Details are reported in the [Supplementary Methods](#) (available online).

Results

Study Population

The present analysis included 180 NeoALTTO patients ([Figure 1](#)). As reported in [Table 1](#), this cohort was representative of the whole NeoALTTO study population in terms of clinically relevant patient- and tumor-related characteristics. As in the whole NeoALTTO cohort (8), patient EFS was similar in the 3 treatment arms (log-rank test $P = .41$; data not shown). The distribution of CD36 expression levels in the whole study cohort and each treatment subcohort is reported in [Supplementary Figure 1](#) (available online). Baseline CD36 expression did not statistically significantly differ according to lymph node status, estrogen receptor (ER) status, tumor size, or pCR status at surgery in either the whole study cohort or individual treatment arms, whereas CD36 expression was lower in patients older than 50 years in the trastuzumab-alone cohort ([Supplementary Figure 2](#), available online).

Association Between CD36 Gene Expression and EFS in Whole Patient Cohort

We assessed the association between CD36 expression and patient EFS in a multivariable Cox regression model including clinically relevant patient-, tumor- and treatment-related variables

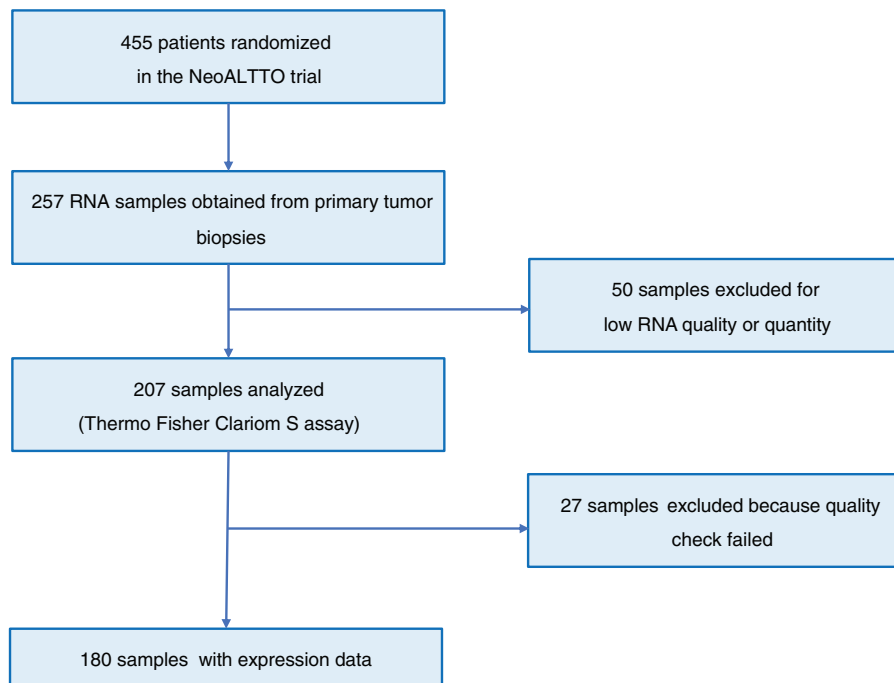


Figure 1. Study flow chart. Out of 455 patients randomly assigned in the Neoadjuvant Lapatinib and/or Trastuzumab Treatment Optimization (NeoALTTO) trial, tumor RNA was retrieved from 257 tumor biopsy specimens and was analyzed by Thermo Fisher Clariom S assay in 207 patients. After excluding 27 samples at quality check analysis, a total 180 RNA samples were used for this study.

(age at enrollment, tumor size, nodal involvement, ER status, pCR status at surgery, and treatment arm) (1). In this model, achieving pCR was associated with better EFS (hazard ratio [HR] = 0.35, 95% confidence interval [CI] = 0.16 to 0.76, $P = .01$) [in line with data from the whole NeoALTTO cohort (8)], whereas CD36 expression was not statistically significantly associated with EFS (HR = 1.22, 95% CI = 0.95 to 1.57, $P = .12$) (data not shown).

Then, we hypothesized that CD36 expression might be associated with clinical outcomes according to specific anti-HER2 treatments. To test this hypothesis, we fitted a multivariable Cox model that included the same clinic-pathological variables as well as the first-order interaction between baseline CD36 levels and treatment arm. This analysis revealed an interaction between CD36 expression and the type of treatment in explaining the observed EFS, with high CD36 expression levels being independently associated with worse EFS only in patients receiving trastuzumab-based therapy (continuous scale, HR = 1.72, 95% CI = 1.20 to 2.46, $P = .003$) (Figure 2). This model also confirmed the well-established association between pCR and better EFS. The inclusion of CD36 expression in the multivariable model improved its global predictive capability; indeed, the C-statistics value of a model including only clinical variables and treatment arm was 0.64 (95% CI = 0.56 to 0.71), which rose to 0.67 (95% CI = 0.61 to 0.74) after including CD36 expression and its interaction with treatment arm.

Association Between CD36 Expression and EFS in Individual Treatment Arms

Then, we performed additional analyses to explore the observed association between CD36 expression and EFS in patients randomly assigned to individual treatment arms. At univariate analysis, high-baseline CD36 expression was associated with statistically significantly worse EFS in patients receiving trastuzumab-based therapy (HR = 1.73, 95% CI = 1.20 to 2.49, $P = .03$) but not in patients treated with lapatinib (HR = 1.01, 95% CI = 0.69 to 1.47, $P = .98$) or lapatinib-

trastuzumab (HR = 1.02, 95% CI = 0.54 to 1.95, $P = .94$). The predictive role of baseline CD36 expression in trastuzumab-treated patients was confirmed by bivariate models adjusting the role of CD36 for each of the following covariates: pCR status (HR = 1.69, 95% CI = 1.18 to 2.41, $P = .004$), ER status (HR = 1.75, 95% CI = 1.20 to 2.54, $P = .004$), tumor size (HR = 1.77, 95% CI = 1.20 to 2.60, $P = .004$), lymph node status (HR = 1.74, 95% CI = 1.20 to 2.51, $P = .003$), and age (HR = 1.78, 95% CI = 1.21 to 2.63, $P = .004$) (Table 2).

When CD36 expression was dichotomized according to median CD36 levels in the whole study cohort, we found a trend toward worse EFS in patients with high CD36-expressing tumors (Figure 3, A), and this difference reached statistical significance in the trastuzumab-alone arm (log-rank $P = .03$) (Figure 3, B). Based on the well-established association between pCR and better long-term clinical outcomes in early-stage HER2+ BC (8,22), as well as on our findings showing that both CD36 expression and pCR status are independently associated with EFS (Figure 2), we divided patients into 2 groups according to CD36 expression and pCR status: (group A) high CD36/no pCR (bad prognosis group), (group B) low CD36/any pCR status (yes or no), or high CD36/pCR (good prognosis group). We found statistically significantly lower 7-year EFS in group A compared with group B patients in both the whole patient cohort (53%, 95% CI = 40% to 65% vs 76%, 95% CI = 66% to 83%, respectively; log-rank test $P = .002$) and in the trastuzumab-alone cohort (47%, 95% CI = 26% to 66% vs 79%, 95% CI = 62% to 88%, respectively; log-rank test $P = .006$) (Figure 3, C and D). Notably, combining CD36 expression and pCR status resulted in a better separation of Kaplan-Meier EFS curves compared with CD36-alone classification.

Interplay Between CD36 Expression and Intratumor Immunity

TILs were available in 159 of 180 (88.3%) NeoALTTO patients included in our analysis. High-baseline TILs were associated with a lower risk of disease relapse (HR = 0.976, 95% CI = 0.957 to

Table 1. Tumor and patient characteristics in the NeoALTTO study population and in this study cohort

| Characteristic | NeoALTTO cohort, No. (%) (n = 455) | Study cohort, No. (%) (n = 180) | P |
|-------------------------------------|---------------------------------------|------------------------------------|------------------|
| Age | | | |
| <50 y | 222 (49) | 101 (56) | .10 ^a |
| ≥50 y | 233 (51) | 79 (44) | |
| Tumor size | | | |
| T2 (≤5 cm) | 274 (60) | 106 (59) | .76 ^a |
| T3-T4 (>5 cm) | 181 (40) | 74 (41) | |
| Lymph node status | | | |
| N0 | 123 (27) | 42 (23) | .34 ^a |
| ≥N1 | 332 (73) | 138 (77) | |
| ER status | | | |
| Positive | 216 (47) | 91 (51) | .48 ^a |
| Negative | 239 (53) | 89 (49) | |
| Planned conservative surgery | | | |
| Yes | 130 (29) | 45 (25) | .36 ^a |
| No | 325 (71) | 135 (75) | |
| Treatment arm | | | |
| Trastuzumab | 149 (33) | 66 (37) | .31 ^a |
| Lapatinib | 154 (34) | 65 (36) | |
| Combination | 152 (33) | 49 (27) | |
| pCR | | | |
| Yes | 160 (35) | 51 (28) | .10 ^a |
| No | 295 (65) | 129 (72) | |
| Events | 127 (28) | 53 (29) | |
| Median follow-up (IQR), y | 6.7 (5.7-6.8) | 6.7 (6.1-6.8) | .32 ^c |
| TILs median %, (range) ^b | 12.5 (0-95) | 12.5 (0-95) | |

^aThe P value of the χ^2 test is indicated. ER = estrogen receptor; IQR = interquartile range; NeoALTTO = Neoadjuvant Lapatinib and/or Trastuzumab Treatment Optimization; pCR = pathologic complete response; TILs = tumor-infiltrating lymphocytes.

^bTIL assessment was available for 376 out of 455 and for 159 out of 180 patients in the NeoALTTO cohort and in this study cohort, respectively.

^cP value of the Wilcoxon test.

0.995, $P = .01$), in line with previously published data (13). CD36 expression did not correlate with TILs in either the whole study cohort or in individual treatment arms (Supplementary Figure 3, A, available online). Similarly, baseline CD36 expression did not correlate with any of 14 tumor-infiltrating immune cells, including CD8+, regulatory T cells, or naïve or activated-memory CD4+ T cells, as estimated by transcriptomic data deconvolution analysis (Supplementary Figure 3, B, available online).

Then, we fitted a multivariable Cox regression model to investigate if the association between high CD36 expression and worse EFS was independent of TILs and other relevant variables. In this model, high CD36 expression maintained a statistically significant association with worse EFS, whereas high TILs were associated with better EFS (Supplementary Figure 4, available online). Bivariate analysis adjusting CD36 expression for TILs in the trastuzumab-alone cohort confirmed an independent, bad predictive role of high CD36 expression in this subcohort (HR = 1.61, 95% CI = 1.06 to 2.44, $P = .02$) (Table 2).

Validation of the Predictive Role of CD36 Expression in the NeoSphere Trial

To confirm the role of high CD36 expression in an independent cohort of HER2+ BC patients treated with neoadjuvant anti-HER2 therapy, we investigated the association between baseline intratumor CD36 expression and DFS in 331 patients enrolled in

the phase II NeoSphere trial. This clinical cohort was representative of the whole NeoSphere study population (Supplementary Table 1, available online). The distribution of CD36 expression levels in our NeoSphere study cohort and in the “joined trastuzumab cohort,” which included patients treated with neoadjuvant chemotherapy plus trastuzumab, chemotherapy plus trastuzumab plus pertuzumab, or trastuzumab plus pertuzumab, is reported in Supplementary Figure 5 (available online). Supplementary Table 2 (available online) summarizes similarities and differences between the NeoALTTO and the NeoSphere study cohorts in terms of patients included, clinical outcomes and methods used to quantify CD36 expression.

At univariate analysis, high-baseline CD36 expression showed a statistically significant association with worse DFS in the whole study cohort (HR = 1.192, 95% CI = 1.003 to 1.416, $P = .046$) and in the joined trastuzumab cohort (HR = 1.272, 95% CI = 1.047 to 1.546, $P = .02$). In individual treatment subcohorts, we observed similar trends, although statistical significance was not reached, likely due to low number of disease relapse events (Supplementary Table 3, available online).

Multivariable analysis adjusting the role of baseline CD36 expression for clinically relevant variables (patient age, tumor size, lymph node status, ER status, and pCR status) revealed that CD36 expression was the only covariate to be independently associated with patient DFS both in the whole study cohort (HR = 1.197, 95% CI = 1.002 to 1.428, $P = .047$) and in the joined trastuzumab cohort (HR = 1.282, 95% CI = 1.049 to 1.568, $P = .02$) (Supplementary Table 3, available online).

CD36 dichotomization based on its median value confirmed that patients with high-baseline intratumor CD36 expression had statistically significantly worse DFS compared with patients with low CD36 expression, and this was observed both in the whole study cohort and the joined trastuzumab cohort (Figure 4, A and B). As in the NeoALTTO cohort, CD36 expression was not associated with the probability to achieve pCR (OR = 1.015, 95% CI = 0.983 to 1.048, $P = .38$). Patients with tumors expressing high CD36 levels and failing to achieve pCR had worse DFS compared with the remaining patients in both the whole NeoSphere cohort and the joined trastuzumab cohort (Figure 4, C and D). However, differently from the NeoALTTO cohort, the NeoSphere cohort combining high CD36 expression with the failure to achieve pCR did not result in a better separation of DFS curves compared with the evaluation of CD36 alone.

Discussion

We reported an unfavorable and independent association between high intratumor CD36 expression and worse EFS in HER2+ BC patients treated with neoadjuvant trastuzumab-based therapy in the context of the randomized, phase III trial NeoALTTO. The main study findings were validated in an independent cohort of HER2+ BC patients enrolled in the randomized, phase II trial NeoSphere. Our results consistently indicate a negative predictive role of a metabolic gene, namely CD36, which is not a classical downstream effector of the HER2 pathway, in HER2+ BC patients receiving trastuzumab-based neoadjuvant therapy.

In a recently published study, CD36 overexpression emerged as a mechanism of acquired resistance to lapatinib in preclinical models of human HER2+ BC, and high intratumor CD36 expression was associated with worse overall survival in a cohort of HER2+ BC patients enrolled in the NeoALTTO trial (20). However, the clinical relevance of these results was limited by the following factors: 1) the association between CD36

| Variable | b | SE | P value | HR (95% CI) | Favors better EFS | Favors worse EFS |
|---|-------|------|---------|---------------------|-------------------|------------------|
| Age (≥ 50 vs < 50 years) | -0.13 | 0.31 | .67 | 0.88 (0.48 to 1.61) | | |
| Tumor size (> 5 cm vs ≤ 5 cm) | -0.03 | 0.30 | .92 | 0.97 (0.54 to 1.74) | | |
| Lymph node status ($\geq N1$ vs $N0$) | 0.43 | 0.37 | .24 | 1.53 (0.75 to 3.15) | | |
| ER status (negative vs positive) | 0.27 | 0.31 | .38 | 1.31 (0.72 to 2.39) | | |
| pCR (Yes vs No) | -1.08 | 0.41 | .01 | 0.34 (0.15 to 0.75) | | |
| Single agent L vs single agent T | 3.94 | 2.11 | .06 | - | | |
| Combination arm vs single agent T | 3.45 | 2.61 | .19 | - | | |
| CD36 | 0.54 | 0.18 | .003 | - | | |
| CD36* ^a treatment arm | -0.52 | 0.27 | .06 | - | | |
| | -0.47 | 0.35 | .18 | - | | |
| CD36 in single-agent L | - | - | - | 1.02 (0.68 to 1.53) | | |
| CD36 in single-agent T | - | - | - | 1.72 (1.20 to 2.46) | | |
| CD36 in combination arm | - | - | - | 1.08 (0.60 to 1.94) | | |

Figure 2. Multivariable penalized Cox regression model for event-free survival (EFS) in the whole study cohort. Hazard ratios (HR) and 95% confidence intervals (CIs) in relation to EFS are shown for each variable. A 30-week landmark analysis was performed, including 169 patients and 51 events. b = beta coefficient estimate; CD36*^atreatment arm = interaction term between CD36 expression and treatment arm; ER = estrogen receptor; L = lapatinib; pCR = pathologic complete response; SE = standard error; T = trastuzumab.

Table 2. Bivariate Cox regression models for EFS in patients randomly assigned to trastuzumab arm (n = 66 and 20 events)

| Models | HR (95% CI) | P |
|---|---------------------|------|
| Bivariate model ^a with pCR and CD36 | | |
| pCR (yes vs no) | 0.44 (0.13 to 1.49) | .19 |
| CD36 | 1.69 (1.18 to 2.41) | .004 |
| Bivariate model with ER status and CD36 | | |
| ER status (neg vs pos) | 1.16 (0.48 to 2.85) | .74 |
| CD36 | 1.75 (1.20 to 2.54) | .004 |
| Bivariate model with tumor size and CD36 | | |
| Tumor size (> 5 cm vs ≤ 5 cm) | 1.19 (0.45 to 3.16) | .72 |
| CD36 | 1.77 (1.20 to 2.60) | .004 |
| Bivariate model with lymph node status and CD36 | | |
| Lymph node status ($\geq N1$ vs $N0$) | 0.89 (0.34 to 2.34) | .82 |
| CD36 | 1.74 (1.20 to 2.51) | .003 |
| Bivariate model with age and CD36 | | |
| Age (≥ 50 vs < 50 y) | 1.25 (0.48 to 3.24) | .65 |
| CD36 | 1.78 (1.21 to 2.63) | .004 |
| Bivariate model with TILs and CD36 | | |
| TILs | 0.98 (0.95 to 1.01) | .30 |
| CD36 | 1.61 (1.06 to 2.44) | .02 |

^aFor the model including pCR and CD36 as covariates, a 30-week landmark analysis was performed including 63 patients and 20 events. CI = confidence interval; ER = estrogen receptor; EFS = event-free survival; HR = hazard ratio; pCR = pathologic complete response; TILs = tumor-infiltrating lymphocytes.

expression and local or distant tumor relapse (eg, EFS or DFS) was not evaluated; 2) multivariable analysis adjusting for clinically or biologically relevant variables was not performed; 3) CD36 was only analyzed as a dichotomous variable, not as a continuous one; and 4) the role of CD36 in individual treatment arms was not investigated.

In our NeoALTTTO trial cohort, high-baseline intratumor CD36 expression, evaluated as a continuous variable, was independently associated with worse EFS in patients treated with

trastuzumab-based therapy, but not in patients receiving lapatinib- or trastuzumab-lapatinib-based treatment. In the NeoSphere validation cohort, high-baseline CD36 expression, evaluated as a continuous variable, was independently associated with worse DFS in the whole study cohort and the subset of patients treated with neoadjuvant trastuzumab-based therapy. The association between high CD36 expression and worse EFS (NeoALTTTO) or DFS (NeoSphere) in patients receiving neoadjuvant trastuzumab-based therapy was confirmed when CD36 was evaluated as a dichotomous variable. Together, these findings indicate that CD36 gene expression is a new and reliable biomarker that predicts poorer long-term clinical outcomes in early-stage HER2+ BC patients treated with neoadjuvant trastuzumab-based therapy.

In a recent study, CD36 expression in tumor-infiltrating CD8⁺ T lymphocytes was shown to promote CD8⁺ T-cell exhaustion, and it was associated with higher risk of tumor progression and lower survival of tumor-bearing mice and cancer patients (23). These results suggest that CD36 expression not only in cancer cells but also in specific intratumor immune cell subsets can affect tumor growth and progression and tumor-related outcomes. Because the antitumor activity of trastuzumab is in part mediated by the activation status of tumor-infiltrating immune cells (24,25), we hypothesized that the observed association between high CD36 expression and worse clinical outcomes specifically in patients receiving neoadjuvant trastuzumab could result from hampered adaptive immunity-mediated antitumor activity of trastuzumab in CD36-overexpressing tumors. To study if intratumor CD36 expression is associated with the presence or activation status of specific tumor-infiltrating immune cells, in the NeoALTTTO cohort we investigated the correlation between CD36 expression and TILs, or between CD36 and the estimates of 14 tumor-infiltrating immune cells. However, we did not find a correlation between CD36 expression and TILs or other immune cell subsets. Of note, CD36 expression maintained an independent association (ie, regardless of TILs) with patient EFS in the whole study cohort and in patients treated with neoadjuvant trastuzumab-alone therapy. Although these results do not allow to definitely exclude a role of CD36 expression in specific immune

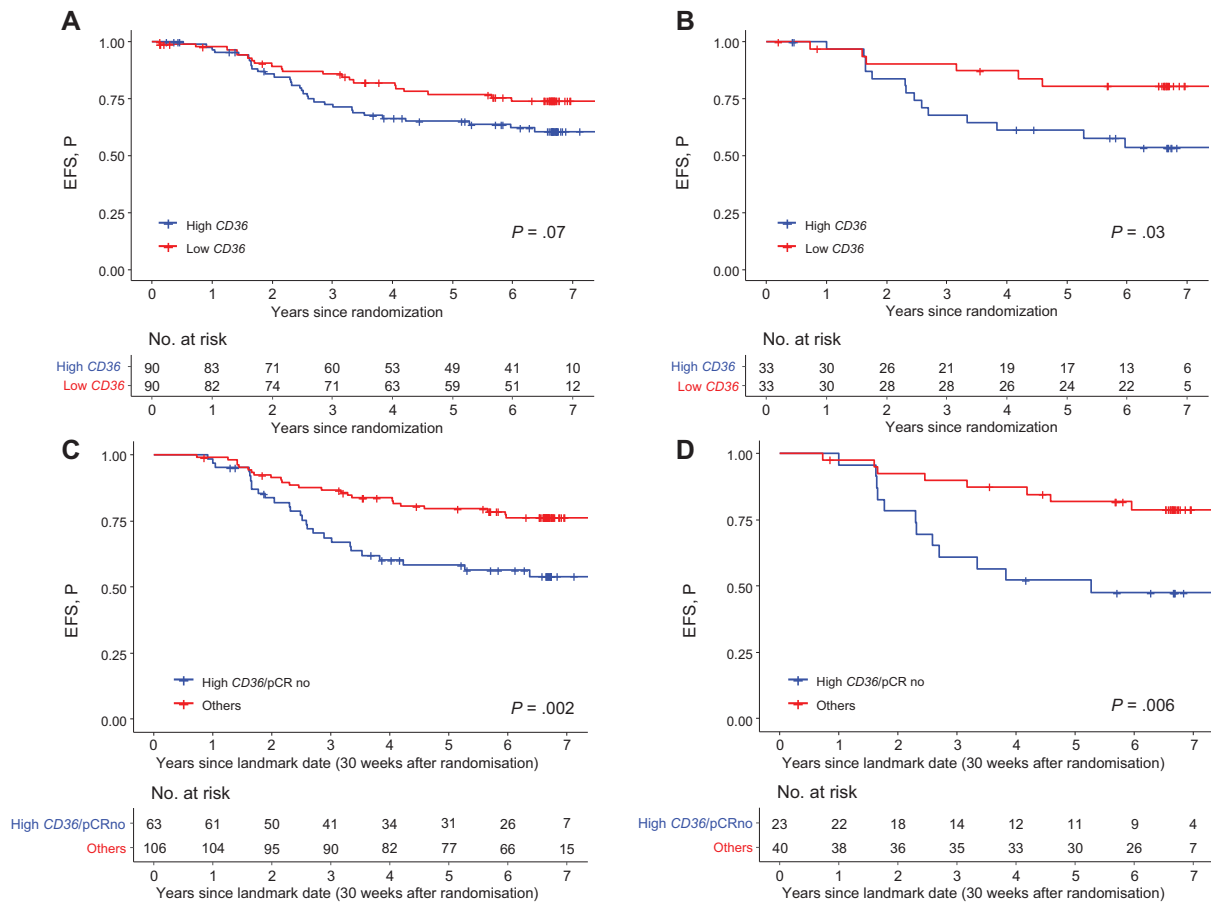


Figure 3. Event-free survival (EFS) probability according to baseline CD36 expression and pathologic complete response (pCR) status. Kaplan-Meier curves representing patient EFS according to CD36 expression, considered as a dichotomous variable, in the whole study cohort (A) ($n = 180$; 53 events) and in patients treated with trastuzumab-alone therapy (B) ($n = 66$; 20 events). Kaplan-Meier curves representing patient EFS according to both CD36 expression, considered as dichotomous variable, and pCR status in the whole study cohort (C) ($n = 180$; 53 events) and in patients treated with trastuzumab-alone therapy (D) ($n = 66$; 20 events). In this analysis, patients were grouped according to both pCR status and CD36 expression in the following 2 cohorts: high CD36/no pCR; others: low CD36/any pCR status (yes/no) or high CD36/pCR. A 30-week landmark analysis was performed, including 169 patients and 51 events. The P value of the log-rank test for the indicated comparisons is reported. Median CD36 levels were used as a threshold for the definition of CD36 categories (low and high).

cell subsets, we speculate that the observed bad predictive role of high CD36 expression might at least in part reflect CD36-mediated uptake of extracellular FA by cancer cells, consistent with recently published preclinical data (20). Future studies combining evaluations with single-cell resolution (eg, single-cell RNA-seq or immunohistochemistry analyses) will clarify if the expression of CD36 in specific tumor-infiltrating immune cell subsets, such as lymphocytes and macrophages, is associated with changes in their functional status.

Our findings could have implications in the context of the current debate on escalation or deescalation of anti-HER2 therapies in early-stage HER2+ BC patients, which is a highly relevant clinical issue in the perspective of treatment personalization (26). Indeed, high-baseline intratumor CD36 levels identify patients who might benefit from more aggressive therapies, such as dual HER2 blockade in the context of (neo)adjuvant therapies or postneoadjuvant T-DM1 in patients with high-baseline CD36 expression who fail to achieve pCR during neoadjuvant therapy. On the other hand, neoadjuvant dual HER2 blockade or postneoadjuvant T-DM1 could be less useful in patients with tumors expressing low CD36 levels. Prospective studies could be initiated to escalate or de-escalate (neo)adjuvant anti-HER2 treatments on the basis of high or low baseline CD36 tumor expression.

The main limitation of this study is that we included only a subset of NeoALTTO and NeoSphere patients, that is, those patients for whom transcriptomic data from basal tumor biopsies were available. However, our study cohorts were representative of the NeoALTTO and NeoSphere population in terms of clinical characteristics (1,2,8). Another limitation is the fact that CD36 gene expression was measured through different platforms in the 2 study cohorts, which implies that the CD36 gene expression threshold used to discriminate patients with CD36-high vs CD36-low tumors in the NeoALTTO trial cannot be generalized to other clinical cohorts (including the NeoSphere cohort), in which CD36 expression was quantified through different methods. Strengths of our study include 1) the novelty of the results, which establish high-CD36 gene expression as a new biomarker that independently predicts worse clinical outcomes in early-stage HER2+ BC patients treated with trastuzumab-based neoadjuvant therapy; 2) the main study findings were obtained in a cohort of patients enrolled in a phase III randomized trial (NeoALTTO); and 3) NeoALTTO cohort results were validated in a cohort of patients enrolled in the randomized, phase II registration trial NeoSphere.

In conclusion, high-CD36 gene expression is independently associated with worse clinical outcomes in early-stage HER2+ BC patients treated with neoadjuvant trastuzumab-based therapy. Along with the recently proposed mechanistic role of CD36

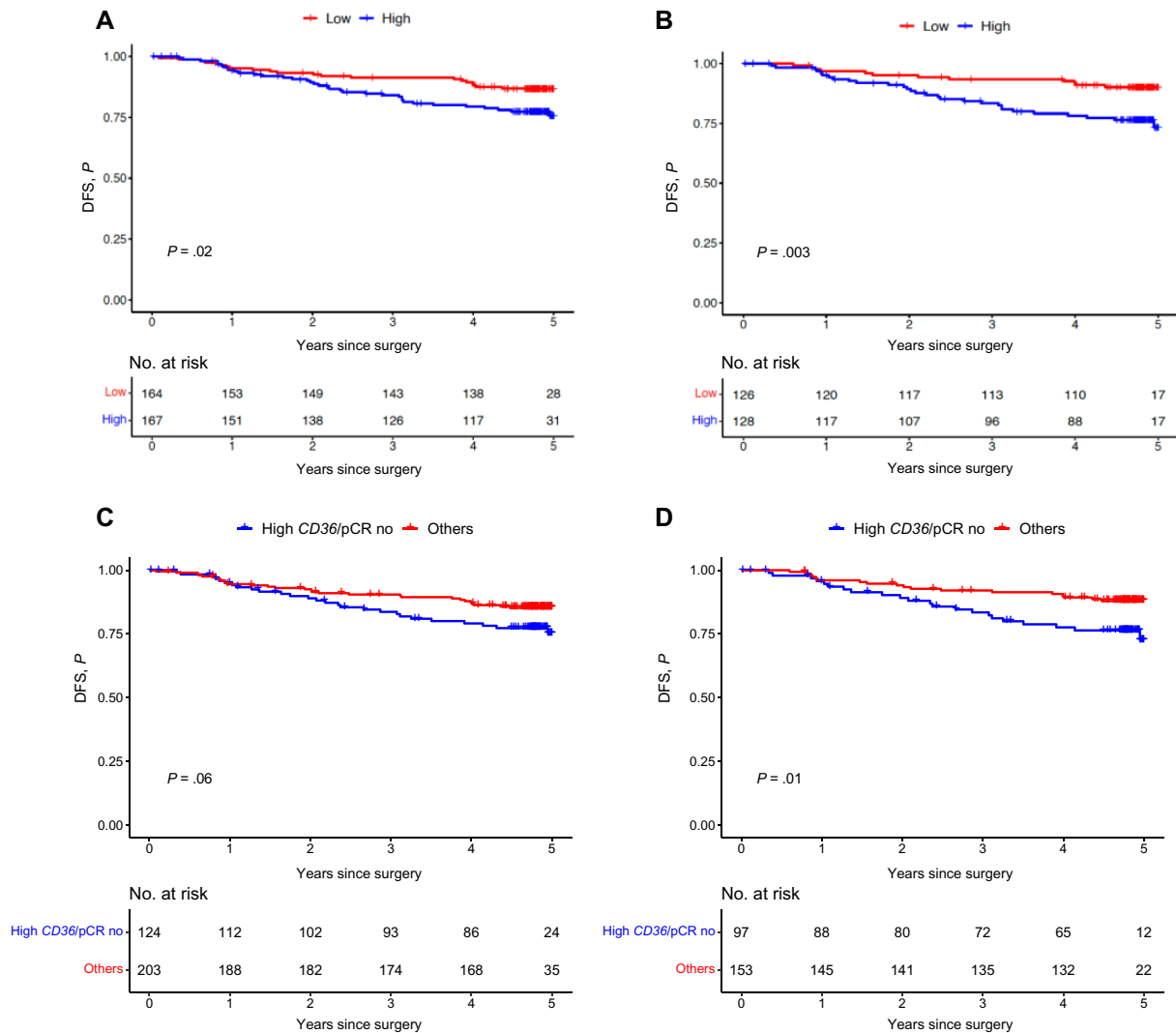


Figure 4. Disease-free survival (DFS) probability in NeoSphere patients according to baseline CD36 expression. Kaplan-Meier curves representing patient DFS according to CD36 expression, considered as a dichotomous variable, in the whole study cohort (A) and in patients in the jointed trastuzumab cohorts (B). Kaplan-Meier curves representing patient DFS according to both CD36 expression, considered as dichotomous variable, and pathologic complete response (pCR) status in whole study cohort (C) and in patients in the jointed trastuzumab cohort (D). In this analysis, patients were grouped according to both pCR status and CD36 expression in the following 2 cohorts: high CD36/no pCR; others: low CD36/any pCR status (yes/no) or high CD36/pCR. The P value of the log-rank test for the indicated comparisons is reported. Median CD36 levels were used as a threshold for the definition of CD36 categories (low and high).

overexpression in mediating HER2+ BC resistance to anti-HER2 therapies, our findings point to CD36 as a new and promising target in HER2+ BC treatment.

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Data Availability

Restrictions apply to the availability of these data, which were used under license for this study, and so are not publicly available. Data are however available from the authors upon reasonable request and with permission of the TransALTTO committee and the Fondazione Michelangelo.

References

- Baselga J, Bradbury I, Eidtmann H, et al.; NeoALTTO Study Team. Lapatinib with trastuzumab for HER2-positive early breast cancer (NeoALTTO): a randomised, open-label, multicentre, phase 3 trial. *Lancet*. 2012;379(9816):633–640.
- Gianni L, Pienkowski T, Im YH, et al. Efficacy and safety of neoadjuvant pertuzumab and trastuzumab in women with locally advanced, inflammatory, or early HER2-positive breast cancer (NeoSphere): a randomised multicentre, open-label, phase 2 trial. *Lancet Oncol*. 2012;13(1):25–32.
- Loibl S, Jackisch C, Schneeweiss A, et al. Dual HER2-blockade with pertuzumab and trastuzumab in HER2-positive early breast cancer: a subanalysis of data from the randomized phase III GeparSepto trial. *Ann Oncol*. 2017;28(3):497–504.
- van Ramshorst MS, van der Voort A, van Werkhoven ED, et al. Neoadjuvant chemotherapy with or without anthracyclines in the presence of dual HER2 blockade for HER2-positive breast cancer (TRAIN-2): a multicentre, open-label, randomised, phase 3 trial. *Lancet Oncol*. 2018;19(12):1630–1640.
- Schneeweiss A, Chia S, Hickish T, et al. Long-term efficacy analysis of the randomised, phase II TRYPHAENA cardiac safety study: evaluating pertuzumab and trastuzumab plus standard neoadjuvant anthracycline-containing and anthracycline-free chemotherapy regimens in patients with HER2-positive early breast cancer. *Eur J Cancer*. 2018;89:27–35.
- National Comprehensive Cancer Network. *Breast Cancer (Version 2.2022)*. https://www.nccn.org/professionals/physician_gls/pdf/breast.pdf. Accessed December 20, 2021.
- Gianni L, Pienkowski T, Im Y-H, et al. 5-Year analysis of neoadjuvant pertuzumab and trastuzumab in patients with locally advanced, inflammatory, or early-stage HER2-positive breast cancer (NeoSphere): a multicentre, open-label, phase 2 randomised trial. *Lancet Oncol*. 2016;17(6):791–800.
- de Azambuja E, Holmes AP, Piccart-Gebhart M, et al. Lapatinib with trastuzumab for HER2-positive early breast cancer (NeoALTTO): survival outcomes of a randomised, open-label, multicentre, phase 3 trial and their association with pathological complete response. *Lancet Oncol*. 2014;15(10):1137–1146.
- Piccart-Gebhart M, Holmes E, Baselga J, et al. Adjuvant lapatinib and trastuzumab for early human epidermal growth factor receptor 2-positive breast cancer: results from the randomized phase III adjuvant lapatinib and/or trastuzumab treatment optimization trial. *J Clin Oncol*. 2016;34(10):1034–1042.
- von Minckwitz G, Procter M, de Azambuja E, et al.; APHINITY Steering Committee and Investigators. Adjuvant pertuzumab and trastuzumab in early HER2-positive breast cancer. *N Engl J Med*. 2017;377(2):122–131.
- Fumagalli D, Venet D, Ignatiadis M, et al. RNA sequencing to predict response to neoadjuvant anti-HER2 therapy: a secondary analysis of the NeoALTTO randomized clinical trial. *JAMA Oncol*. 2017;3(2):227–234.
- Bianchini G, Kiermaier A, Bianchi GV, et al. Biomarker analysis of the NeoSphere study: pertuzumab, trastuzumab, and docetaxel versus trastuzumab plus docetaxel, pertuzumab plus trastuzumab, or pertuzumab plus docetaxel for the neoadjuvant treatment of HER2-positive breast cancer. *Breast Cancer Res*. 2017;19(1):16.
- Salgado RD, Campbell C, Savas P, et al. Tumor-infiltrating lymphocytes and associations with pathological complete response and event-free survival in HER2-positive early-stage breast cancer treated with lapatinib and trastuzumab: a secondary analysis of the NeoALTTO trial. *JAMA Oncol*. 2015;3(4):448–454.
- Denkert C, von Minckwitz G, Brase JC, et al. Tumor-infiltrating lymphocytes and response to neoadjuvant chemotherapy with or without carboplatin in human epidermal growth factor receptor 2-positive and triple-negative primary breast cancers. *J Clin Oncol*. 2015;33(9):983–991.
- Ingold Heppner B, Untch M, Denkert C, et al. Tumor-infiltrating lymphocytes: a predictive and prognostic biomarker in neoadjuvant-treated HER2-positive breast cancer. *Clin Cancer Res*. 2016;22(23):5747–5754.
- Di Cosimo S, Triulzi T, Pizzamiglio S, et al. The 41-gene classifier TRAR predicts response of HER2 positive breast cancer patients in the NeoALTTO study. *Eur J Cancer*. 2019;118:1–9.
- Menendez JA, Lupu R. Fatty acid synthase and the lipogenic phenotype in cancer pathogenesis. *Nat Rev Cancer*. 2007;7(10):763–777.
- Röhrlig F, Schulze A. The multifaceted roles of fatty acid synthesis in cancer. *Nat Rev Cancer*. 2016;16(11):732–749.
- Ligorio FP, Castagnoli L, Vingiani A, et al. Targeting lipid metabolism is an emerging strategy to enhance the efficacy of anti-HER2 therapies in HER2-positive breast cancer. *Cancer Lett*. 2021;511:77–87.
- Feng WW, Wilkins O, Bang S, et al. CD36-mediated metabolic rewiring of breast cancer cells promotes resistance to HER2-targeted therapies. *Cell Rep*. 2019;29(11):3405–3420.e5.
- Prat A, Bianchini G, Thomas M, et al. Research-based PAM50 subtype predictor identifies higher responses and improved survival outcomes in HER2-positive breast cancer in the NOAH study. *Clin Cancer Res*. 2014;20(2):511–521.
- Spring LM, Fell G, Arfe A, et al. Pathologic complete response after neoadjuvant chemotherapy and impact on breast cancer recurrence and survival: a comprehensive meta-analysis. *Clin Cancer Res*. 2020;26(12):2838–2848.
- Ma X, Xiao L, Liu L, et al. CD36-mediated ferroptosis dampens intratumoral CD8+ T cell effector function and impairs their antitumor ability. *Cell Metab*. 2021;33(5):1001–1012.e5. <https://doi.org/10.1016/j.cmet.2021.02.015>.
- Barok M, Isola J, Pályi-Krek Z, et al. Trastuzumab causes antibody-dependent cellular cytotoxicity-mediated growth inhibition of submacroscopic JIMT-1 breast cancer xenografts despite intrinsic drug resistance. *Mol Cancer Ther*. 2007;6(7):2065–2072.
- Musolino A, Naldi N, Bortesi B, et al. Immunoglobulin G fragment C receptor polymorphisms and clinical efficacy of trastuzumab-based therapy in patients with HER-2/neu-positive metastatic breast cancer. *J Clin Oncol*. 2008;26(11):1789–1796.
- Curigliano G, Burstein HJ, Winer EP, et al.; St. Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2017. De-escalating and escalating treatments for early-stage breast cancer: The St. Gallen International Expert Consensus Conference on the Primary Therapy of Early Breast Cancer 2017. *Ann Oncol*. 2017;28(8):1700–1712.