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Evaluation of soluble carbonic anhydrase IX as predictive marker for efficacy of bevacizumab: A biomarker analysis from the geparquinto phase III neoadjuvant breast cancer trial

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Key words: breast cancer, carbonic anhydrase IX, predictive biomarker, bevacizumab, neoadjuvant treatment

Abbreviations: B: bevacizumab; CAIX: carbonic anhydrase IX; CI: confidence interval; CR: complete remission; DFS: disease-free survival; ELISA: enzyme-linked immunosorbent assay; ER: estrogen receptor; FDA: Food and Drug Administration; G: Grading; G5: GeparQuinto; HER2: Human epidermal growth factor receptor 2; HR: hormone receptor; NCT: neoadjuvant chemotherapy; NCT-B: neoadjuvant chemotherapy plus bevacizumab; NSABP: National Surgical Adjuvant Breast and Bowel Project; pCR: pathological complete response; PD: progressive disease; PFS: progression-free survival; PR: progesterone receptor; REMARK: reporting recommendations for tumor marker prognostic studies; OR: odds ratio; OS: overall survival; SD: stable disease; TNBC: triple negative breast cancer; VEGF: vascular endothelial growth factor

Additional Supporting Information may be found in the online version of this article.

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We analyzed the predictive potential of pretreatment soluble carbonic anhydrase IX levels (sCAIX) for the efficacy of bevacizumab in the phase III neoadjuvant GeparQuinto trial. sCAIX was determined by enzyme-linked immunosorbent assay (ELISA). Correlations between sCAIX and pathological complete response (pCR), disease-free and overall survival (DFS, OS) were assessed with logistic and Cox proportional hazard regression models using bootstrapping for robust estimates and internal validation. 1,160 HER2-negative patient sera were analyzed, of whom 577 received bevacizumab. Patients with low pretreatment sCAIX had decreased pCR rates (12.1 *vs.* 20.1%, $p = 0.012$) and poorer DFS (adjusted 5-year DFS 71.4 *vs.* 80.5 months, $p = 0.010$) compared to patients with high sCAIX when treated with neoadjuvant chemotherapy (NCT). For patients with low sCAIX, pCR rates significantly improved upon addition of bevacizumab to NCT (12.1 *vs.* 20.4%; $p = 0.017$), which was not the case in patients with high sCAIX (20.1% for NCT *vs.* 17.0% for NCT-B, $p = 0.913$). When analyzing DFS we found that bevacizumab improved 5-year DFS for patients with low sCAIX numerically but not significantly (71.4 *vs.* 78.5 months; log rank 0.234). In contrast, addition of bevacizumab worsened 5-year DFS for patients with high sCAIX (81 *vs.* 73.6 months, log-rank 0.025). By assessing sCAIX levels we identified a patient cohort in breast cancer that is potentially undertreated with NCT alone. Bevacizumab improved pCR rates in this group, suggesting sCAIX is a predictive biomarker for bevacizumab with regards to treatment response. Our data also show that bevacizumab is not beneficial in patients with high sCAIX.

What's new?

While the addition of bevacizumab to neoadjuvant therapy can improve rates of pathological complete response (pCR) in different malignancies, a biomarker to identify patients likely to benefit from the combined therapy is lacking. In this study, serum soluble carbonic anhydrase IX (sCAIX) was identified as a marker for the selection of patients with early breast cancer responding to combined bevacizumab and neoadjuvant chemotherapy. Significant improvements in pCR rates were observed in patients with low sCAIX levels. The addition of bevacizumab further improved 5-year disease-free survival in low sCAIX patients, while having detrimental effects in patients with high sCAIX levels.

Introduction

Bevacizumab is a humanized monoclonal antibody targeting vascular endothelial growth factor A (VEGF-A), which has been approved for the treatment of various malignancies. Yet only a fraction of patients respond to anti-angiogenic treatment and, even in responding patients, responses are limited to a few months in most cases.[1] In breast cancer bevacizumab shows modest efficacy in the metastatic setting.[2–5] The Food and Drug Administration (FDA) withdrew approval in 2011 due to inconsistent long-term survival data and bevacizumab-related toxicities, while the European Medicines Agency maintained the approval status.[6]

Recent trials in locally advanced breast cancer, including the randomized, phase III GeparQuinto (G5) trial, reported modestly improved rates of pathological complete response (pCR) upon addition of bevacizumab to neoadjuvant chemotherapy (NCT).[7–11] The achievement of pCR is a well-known surrogate marker for long-term outcome and is recommended by the FDA as a primary end point in neoadjuvant trials.[12] However, bevacizumab is currently not approved for the treatment of early-stage breast cancer because long-term results were not consistent between different trials.[13,14]

To date, very few promising biomarker candidates for the efficacy of bevacizumab, that are ready for prospective evaluation, have emerged. They include short VEGF isoforms, modified expression of neuropilin-1, genetic variants and modified expression of VEGF receptor 1.[15–19] In the first prospective biomarker trial of short VEGF isoforms (MERiDiAN), pretreatment levels of the short isoforms of VEGF-A were not able to predict progression-free survival (PFS) upon treatment with bevacizumab in patients with HER2-negative, metastatic breast cancer.[20] Further studies analyzing biomarkers in large, randomized cohorts based on scientific rationales are therefore warranted.

Anti-VEGF therapies increase carbonic anhydrase IX (CAIX) expression in tumor tissue,[21,22] but little is known about the role of CAIX as a predictor of response to anti-VEGF therapies in cancer patients. CAIX is upregulated in hypoxic conditions and catalyzes the hydration of CO2 to HCO3- and H+. HCO3- is used to neutralize intracellular pH, which supports the survival of tumor cells, whilst H+ acidifies the extracellular environment, leading to increased invasion and metastasis.[23,24] Therefore, intratumoral CAIX expression has been associated with poor prognosis and poor response to therapy in many cancers, including breast cancer.[25–28] In clear cell renal cell cancer however, most studies suggest an association of low CAIX expression with worse outcomes.[29,30]

A soluble form of CAIX (sCAIX) is actively released from the cell surface by proteolytic cleavage of the extracellular domain from the transmembrane protein and can be measured in serum of patients.[31,32]

The primary aim of our study was to evaluate the potential of sCAIX to predict pCR in patients with locally advanced, HER2-negative breast cancer. Therefore, we analyzed serum from 1,160 patients treated with neoadjuvant chemotherapy (NCT) alone or NCT plus bevacizumab (NCT-B) in the G5 study. This study demonstrated improved pCR rates from 14.9 to 18.4% [Odds ratio (OR), 1.29; 95% confidence interval (CI), 1.02–1.65; $p = 0.04$] upon the addition of bevacizumab to NCT.[11] We also analyzed the predictive potential of sCAIX levels for disease free survival (DFS) and overall survival (OS).

Materials and Methods

Patients and study design

The G5 phase III trial (ClinicalTrials.gov identifier: NCT00567554) recruited HER2-positive and HER2-negative patients into two different treatment settings. Patients with HER2-positive breast cancer were randomly assigned to an anti-HER2 related study arm.[33] HER2-negative patients were randomized to receive NCT alone *vs.* NCT-B. Notably, in our study we exclusively analyzed sera from patients included in the HER2-negative study population.

The trial design and the results of the HER2-negative arm have been described in detail previously.[11,14] Briefly, 1948 patients with untreated, histologically confirmed HER2-negative, invasive, nonmetastatic breast cancer were recruited between November 2007 and June 2010. Patients were eligible for this study if they were considered candidates for adjuvant chemotherapy. This included patients with large tumors (cT3 or cT4), patients with hormone-receptor (HR)-negative disease (HER2-negative, estrogen-receptor (ER) - and progesterone-receptor (PR) - negative = triple negative breast cancer, TNBC), and patients with HR-positive disease (HER2-negative, ER- and/or PR-positive) with palpable nodes (cN+ for cT2) or positive findings on sentinel-node-biopsy (pNSLN+ for cT1).

In the HER2-negative study arm, patients were randomly assigned to receive four cycles of epirubicin and cyclophosphamide followed by four cycles of docetaxel, with either eight concomitant cycles of bevacizumab or no additional treatment (974 patients were randomized in each group, Fig. 1). Randomization into the HER2-negative study arm was stratified by participating center, HR-status (HR-positive = HER2-negative, ER- and/or PR-positive *vs.* TNBC) and extent of disease (cT4 or cN3 *vs.* cT1-3 and cN0-2).

For patients who did not have a confirmed response to four cycles of epirubicin and cyclophosphamide, the study treatment was discontinued and they were randomly assigned to receive weekly paclitaxel with or without everolimus.[34] Patients with tumor progression on this treatment were counted as not having a pCR and further local or systemic treatment was permitted at the discretion of the study site.

Serum samples for our study were collected from patients participating in a preplanned, nonmandatory translational research program within the HER2-negative arm of the G5 study. There was no randomization or stratification according to participation in the biomarker program. The clinical and translational studies were approved by competent ethics committees.

Sample collection and biomarker assessment

Serum samples from patients who voluntarily participated in the translational biomarker program were collected locally at the study sites. Samples were kept on site at −20°C, subsequently shipped on dry ice and stored at −80°C at a central facility. sCAIX levels in the samples were analyzed by investigators blinded for treatment and clinicopathological subgroups. sCAIX protein levels were quantified using a commercially available enzyme-linked immunosorbent assay (ELISA) (WILEX Inc., Oncogene Science, Cambridge, MA, USA). The ELISA was performed according to the manufacturer as previously described.[35,36] sCAIX concentrations were calculated from a standard curve. Each sample, standard and control was analyzed in duplicate. Internal control samples were provided by the manufacturer (CAIX ELISA Controls, Wilex Inc. Oncogene Science, Cambridge, MA, USA). The intra- and inter-assay variability of the assay is 10 and 12%, respectively (information provided by the manufacturer). Cross reactivity with related proteins CAII and CAXII is less than 5% (information provided by the manufacturer).

Statistical analysis

Depending on scale, means, medians and ranges or counts and percentages were compared using Student's t-test, chi-squared tests or univariate ANOVA as indicated.

Appropriate logarithmic transformation was used to address the skewed distribution of sCAIX levels. Supporting Information Figure S1 shows the distribution of sCAIX before and after logarithmic transformation.

The predictive effect of sCAIX was assessed using all available pretreatment serum samples (583 samples from patients treated with NCT alone and 577 samples from patients treated with NCT-B, highlighted in the CONSORT diagram in gray; Fig. 1).

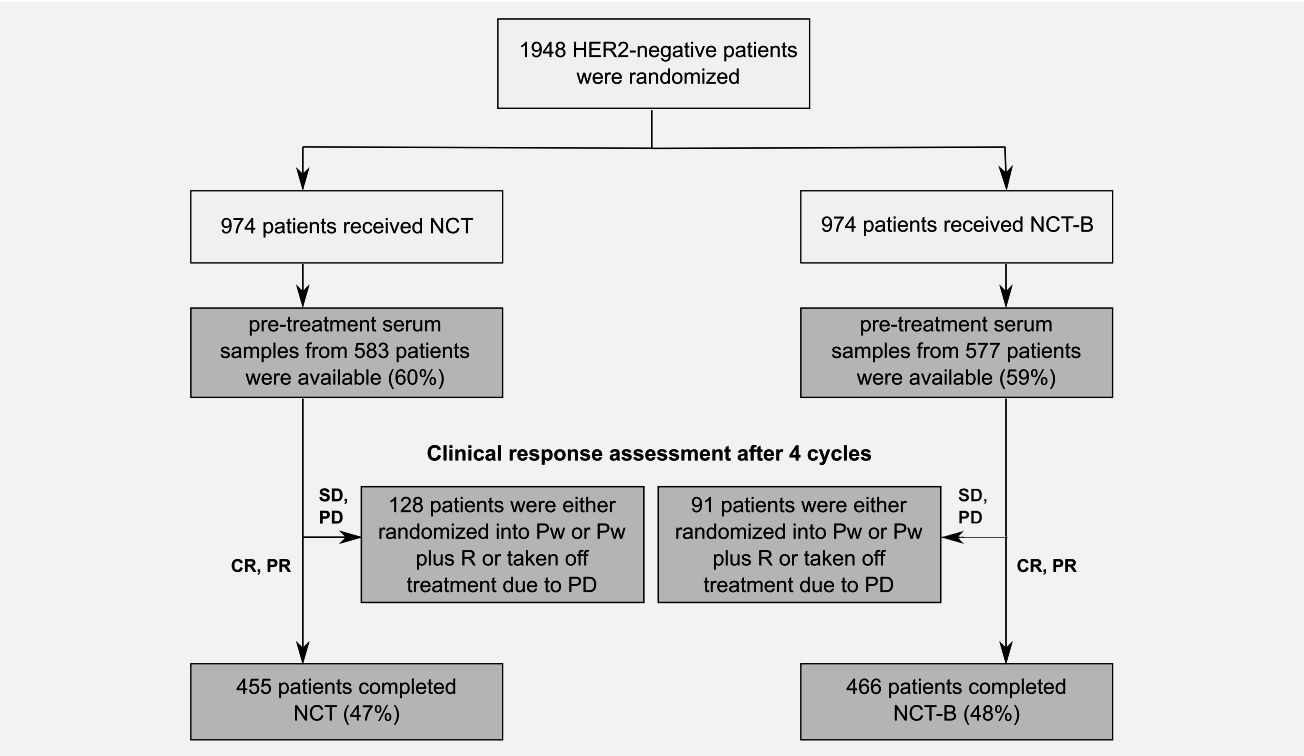

Figure 1. CONSORT flow diagram. Gray boxes indicate flow of patients included in the present biomarker study. CR, complete remission; PD, progressive disease; PR, partial remission; Pw, paclitaxel weekly; R, everolimus; SD, stable disease.

The aim of our study was to assess the predictive effect of sCAIX for the achievement of pCR upon addition of bevacizumab to NCT. pCR was also the primary endpoint of the G5 trial. Consistent with previously published data of the G5 study,[11] pCR was defined as the absence of invasive and intraductal disease in breast and nodes (pT0, pN0) after neoadjuvant treatment.

DFS was calculated from the time of randomization to the time of invasive disease recurrence, metastasis or death irrespective of any cause or lost to follow-up. OS was calculated from the date of randomization to the date of death, irrespective of any cause or lost to follow-up. DFS and OS were secondary endpoints of the G5 trial and we also assessed the predictive effect of sCAIX levels on these endpoints in our study.

First, we determined whether sCAIX levels were differentially associated with pCR rates in patients treated with NCT alone *vs.* in patients treated with NCT-B. Therefore, we conducted a multivariable logistic regression analysis with bias-reduced estimators, including a logarithmic transformation of continuous sCAIX levels and treatment and HR-status, as well as all interactions between these terms. The analysis was adjusted for age, t-stage and nodal status at study entry. Information about the molecular subgroups was not available to further separate HER2-negative, HR-positive patients into luminal A and luminal B subgroups. We therefore separated the HER2-negative, HR-positive patients into luminal A-like (HER2-negative, HR-positive, G1-2) and luminal B-like (HER2-negative, HR-positive, G3) subtypes.

In compliance with the REMARK criteria, bootstrap resampling techniques with 10,000 replicates were used as internal validation for all analyses.[37,38] Multivariable Cox proportional hazard regression models with the same predictors as for the primary endpoint were used to test for a different effect of sCAIX level (continuous) on DFS and OS in patients treated with and without bevacizumab.

Next, we aimed to determine a cut-off for sCAIX, which separates patients benefitting from addition of bevacizumab to NCT from those that benefit more from NCT alone. Instead of taking the median or any other percentiles, we conducted an unbiased exploratory analysis, based on the idea of creating splits in decision trees (CHAID = CHi-squared Automatic Interaction Detector, CART = Classification And Regression Tree).[39] Among all possible splits for sCAIX, we selected the one which maximizes the test statistic $|z|$ for the interaction term of treatment and sCAIX split by that specific cut-off. This cut-off was subsequently tested using a logistic regression model and Cox proportional hazard regression models adjusted for age, t-stage, nodal status and HR-status for prediction of pCR. 5-year DFS was adjusted for age, t-stage, nodal status and HR-status.

Statistical significance was defined as $p < 0.05$. Statistical analysis was performed using IBM SPSS Statistics 20.0 software (IBM, Armonk, NY, USA) and R software (version 3.1.2, R Foundation for Statistical Computing, Vienna, Austria).

Table 1. Clinico-pathological characteristics of patients

Characteristics		NCT (*N* = 583) no. (% / range)	NCT - B (*N* = 577) no. (% / range)	*p*-value
Age	Mean	49	49	0.40
	Median	48 (24–78)	49 (22–75)	
	Missing	0	0	
T-stage	cT1-cT3	519 (89.0)	515 (89.3)	0.98
	cT1	101 (17.3)	103 (17.9)	
	cT2	329 (56.4)	329 (57.0)	
	cT3	83 (14.4)	89 (15.3)	
	cT4a-c	32 (5.5)	29 (5.0)	
	cT4d	31 (5.3)	32 (5.5)	
	Missing	1 (0.2)	1 (0.2)	
Lymph node	cN0	298 (51.1)	306 (53.0)	0.76
	cN1-3	274 (47.0)	262 (45.4)	
	Missing	11 (1.9)	9 (1.6)	
Histological type	Ductal invasive	462 (79.2)	466 (80.7)	0.73
	Lobular invasive	55 (9.4)	57 (9.9)	
	Other	34 (5.8)	29 (5.0)	
	Missing	32 (5.5)	25 (4.3)	
Tumor grade	G1	25 (4.3)	19 (3.3)	0.47
	G2	300 (51.5)	308 (53.4)	
	G3	252 (43.2)	247 (42.8)	
	Missing	6 (1.0)	3 (0.5)	
ER-Status	Negative	231 (36.9)	202 (35.0)	0.10
	Positive	352 (60.4)	375 (65.0)	
	Missing	2 (0.3)	0	
PR-Status	Negative	271 (46.5)	244 (42.3)	0.14
	Positive	310 (53.2)	333 (57.7)	
	Missing	1 (0.2)	0	
Subtype	HER2-negative, HR-positive, G1-2	270 (46.3)	281 (48.7)	0.42
	HER2-negative, HR-positive, G3	97 (16.6)	104 (18.0)	
	TNBC	215 (36.9)	192 (33.3)	
	Missing	1 (0.2)	0	

Differences between treatment groups were analyzed with independent t-test (Welch-test) for the subgroup age. A Chi-Square test was used for analyzes of the subgroups t-stage, nodal stage, histology, grading and HR subgroups. Percentages may not sum up to 100% because of rounding.

Results

Patient population

We analyzed all available pretreatment serum samples from patients treated in the HER2-negative part of the G5 study (1,160 samples, 60% of entire patient cohort). 583 of these patients were treated with NCT alone and 577 patients were treated with NCT-B (indicated in gray in Fig. 1). Patient characteristics of this biomarker cohort are shown in Table 1. They were similar to the overall, HER2-negative study cohort and did not significantly differ between treatment groups with respect to age, t-stage, HR-status, histology and grading (Supporting Information Table S1).

Determination of sCAIX level

In a first step, we determined sCAIX protein levels by ELISA in the available serum samples. They ranged from 2 to 4,230 pg/mL (mean 150 pg/mL) and were not correlated with HR-status, age, t-stage, grading and histologic subtype of tumors (Supporting Information Table S2). Among patients receiving NCT-B, sCAIX levels were higher in node-positive patients (Supporting Information Table S2). In order to correct for this and other potentially confounding factors, we adjusted all multivariable calculations for clinicopathological parameters, including nodal status.

Evaluation of association of sCAIX (continuous levels) with pCR in patients treated with and without bevacizumab

Next, we wished to determine whether sCAIX levels were correlated with the responses of patients to treatment. Therefore, we analyzed the association of continuous pretreatment sCAIX levels with pCR rates using a multivariable logistic regression

Table 2. Association of continuous sCAIX level with pCR, DFS and OS in patients treated with and without bevacizumab

		Treatment	No. of patients	Odds ratio, (95% CI)	*p*-Value	Test for interaction[1]	3-Way interaction[2]
pCR	all patients	NCT	583	**1.58 (1.21–2.12)**	**<0.001**	**0.007**	
		NCT-B	577	0.92 (0.71–1.23)	0.543		
	HER2-negative, HR-positive	NCT	366	1.10 (0.78–1.65)	0.627	0.943	**0.033**
		NCT-B	385	1.13 (0.75–1.76)	0.578		
	TNBC	NCT	215	**1.86 (1.26–2.86)**	0.001	**0.002**	
		NCT-B	192	0.83 (0.59–1.16)	0.267		
		Treatment	**No. of patients**	**Hazard ratio, (95% CI)**	***p*-Value**	**Test for interaction[1]**	**3-Way interaction[2]**
DFS	all patients	NCT	583	**0.81 (0.67–0.99)**	**0.045**	**0.033**	
		NCT-B	577	1.15 (0.90–1.50)	0.280		
	HER2-negative, HR-positive	NCT	366	**0.72 (0.57–0.93)**	0.017	**0.028**	0.669
		NCT-B	385	1.14 (0.82–1.62)	0.455		
	TNBC	NCT	215	0.93 (0.67–1.31)	0.627	0.401	
		NCT-B	192	1.15 (0.80–1.68)	0.480		
OS	all patients	NCT	583	0.86 (0.64–1.15)	0.285	0.293	
		NCT-B	577	1.07 (0.80–1.47)	0.657		
	HER2-negative, HR-positive	NCT	366	0.70 (0.41–1.22)	0.180	0.529	0.883
		NCT-B	385	0.87 (0.57–1.42)	0.526		
	TNBC	NCT	215	0.98 (0.70–1.40)	0.861	0.443	
		NCT-B	192	1.19 (0.82–1.77)	0.370		

pCR as primary and DFS and OS as secondary endpoints were analyzed using logistic regression (pCR) and Cox proportional hazard models (DFS, OS) including interaction between sCAIX (continuous level) and treatment (NCT-B *vs.* NCT). All calculations were adjusted for age, t-stage, HR-status und nodal status. Bootstrap with 10.000 replicates.
[1]Test for interaction between sCAIX and treatment.
[2]Test for the 3-way interaction of sCAIX, treatment and HR-status.

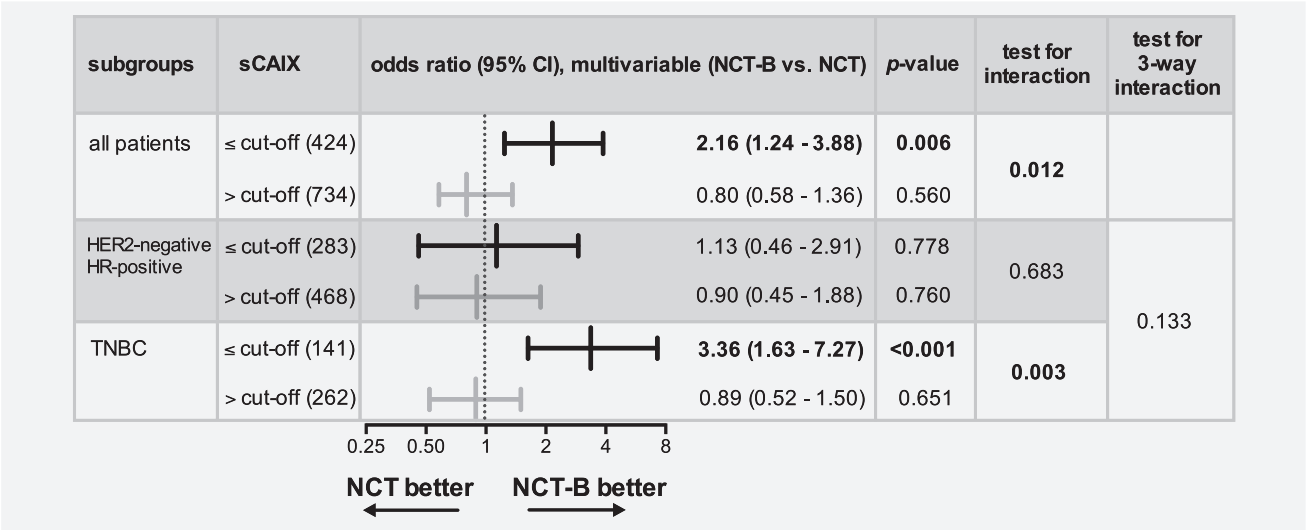

Figure 2. Likelihood for pCR for patients with high and low pretreatment sCAIX level. Forest plot of Odds ratios for pCR in patient cohorts with pretreatment sCAIX levels above and below a cut-off of 122 pg/mL comparing NCT-B *vs.* NCT-treated patients. This multiple logistic regression analysis was adjusted for HR-status, age, nodal status, t-stage. Bootstrap with 10.000 replicates.

model. Interestingly, the data indicated that increasing sCAIX level had a different effect on the likelihood of achieving a pCR in patients treated with and without addition of bevacizumab (Table 2). Increasing sCAIX level were associated with a significantly augmented likelihood for pCR in NCT-treated patients, while in NCT-B-treated patients sCAIX concentrations did not have an effect on pCR (OR: 1.58, 95%CI 1.21–2.12, $p < 0.001$ and OR: 0.92, 95% CI 0.71–1.23, $p = 0.543$ for NCT and NCT-B-treated patients, respectively).

The test for interaction revealed that the correlation of sCAIX levels with pCR was significantly different in NCT *vs.* NCT-B-treated patients ($p = 0.007$, Table 2). This different effect in the two treatment groups suggests that sCAIX may have predictive potential.

This effect was mostly present in the TNBC subgroup ($p = 0.002$), while there was no differential association of sCAIX levels and pCR with regards to treatment in HR-positive patients ($p = 0.94$) (Table 2). Additionally, the 3-way interaction (0.033) indicated a significantly different effect of sCAIX level on pCR in both treatment groups in HR-positive compared to TNBC patients, thereby indicating that the effect of sCAIX on pCR might be exclusively present in the TNBC subgroup.

Evaluation of association of sCAIX (continuous levels) with DFS and OS in patients treated with and without bevacizumab

In concordance with our observations when analyzing pCR, the effects of sCAIX levels on DFS significantly differed in both treatment arms (Table 2, test for interaction = 0.033). Of note, the odds ratio <1 indicates a lower chance for pCR with increasing sCAIX level while in the context of DFS a hazard ratio <1 indicates a lower risk to achieve a DFS event with increasing sCAIX level. Therefore, sCAIX levels have a similar predictive potential for pCR and DFS: lower sCAIX levels predict a detrimental effect on DFS in NCT-treated patients (HR: 0.81, 95%CI 0.67–0.99, $p = 0.045$, Table 2).

The different correlations of sCAIX level with pCR and DFS in patients treated with and without bevacizumab suggest that sCAIX might be a novel predictor of patient outcome in response to the addition of bevacizumab (Table 2). In contrast to our observations when analyzing pCR, the association of sCAIX level with DFS was mostly present in the HER2-negative, HR-positive subgroup and not in the TNBC subgroup (Table 2). However, the test for 3-way interaction for the association of sCAIX levels, treatment group and HR-status with DFS was not significant (0.669, Table 2). This finding suggests that the overall association of sCAIX level with DFS in the entire biomarker cohort was not only based on the HER2-negative, HR-positive subgroup, but also on effects observed in TNBC patients, although they were not significant for DFS ($p = 0.627$ and $p = 0.480$ for NCT- and NCT-B-treated patients, respectively, test for interaction = 0.401).

Interestingly, further separation of the HER2-negative, HR-positive patients into luminal A-like and luminal B-like subgroups revealed that the effects of sCAIX as a predictor for DFS are mostly based on the luminal B-like subgroup (Supporting Information Table S3).

We did not observe a predictive effect of sCAIX levels on OS (Table 2).

Cut-off analysis for pretreatment sCAIX

For potential clinical application and prospective testing of the predictive potential of sCAIX levels, a cut-off needs to be identified. This cut-off is necessary to divide patients in a group benefitting from the addition of bevacizumab to NCT *vs.* a group not benefitting from this treatment intensification.

Table 3. pCR rates and estimated median 5-year DFS in the total biomarker cohort and according to sCAIX cut-off

		Total biomarker cohort no. of pCR (%)	Patients with low sCAIX no. of pCR (%)	Patients with high sCAIX no. of pCR (%)	
pCR	*All patients*				
	NCT	102 (17.1)	27 (12.1)	75 (20.1)	**$p = 0.012$**
	NCT-B	108 (18.3)	46 (20.4)	62 (17.0)	$p = 0.232$
		$p = 0.573$	**$p = 0.017$**	$p = 0.913$	
	HER2-negative, HR-positive				
	NCT	29 (7.7)	11 (7.3)	18 (7.9)	$p = 0.832$
	NCT-B	30 (7.6)	12 (7.8)	18 (7.5)	$p = 0.906$
		$p = 0.959$	$p = 0.880$	$p = 0.852$	
	TNBC				
	NCT	71 (32.4)	16 (21.6)	55 (37.9)	**$p = 0.015$**
	NCT-B	78 (40.0)	34 (47.2)	44 (35.8)	$p = 0.115$
		$p = 0.109$	**$p = 0.001$**	$p = 0.715$	
		Total biomarker cohort 5-year DFS in % (95% CI)	**Patients with low sCAIX 5-year DFS in % (95% CI)**	**Patients with high sCAIX 5-year DFS in % (95% CI)**	
DFS	*All patients*				
	NCT	76.5 (72.7–80.4)	71.4 (64.7–78.9)	81.0 (76.4–85.9)	**$p = 0.010$**
	NCT-B	74.0 (69.9–78.3)	78.5 (72.7–84.7)	73.6 (68.2–79.3)	$p = 0.541$
		$p = 0.300$	$p = 0.234$	**$p = 0.025$**	
	HER2-negative, HR-positive				
	NCT	77.5 (72.8–82.6)	69.8 (61.3–79.4)	83.9 (78.4–89.6)	**$p = 0.008$**
	NCT-B	75.4 (70.3–80.8)	78.8 (71.6–86.6)	74.0 (63.1–85.1)	$p = 0.598$
		$p = 0.399$	$p = 0.273$	**$p = 0.034$**	
	TNBC				
	NCT	74.6 (68.8–80.9)	71.2 (61.0–83.1)	75.5 (67.3–81.3)	$p = 0.327$
	NCT-B	71.0 (64.2–78.5)	73.3 (63.1–85.1)	71.2 (62.6–80.9)	$p = 0.908$
		$p = 0.410$	$p = 0.713$	$p = 0.295$	

Low sCAIX, patients with sCAIX levels ≤122 pg/mL; high sCAIX, patients with sCAIX level >122 pg/mL. Chi-squared test for comparison of pCR rates and log ranks test for comparison of adjusted, estimated mean 5-year DFS survival. 5-year DFS was adjusted for age, t-stage and nodal status.

Therefore, we determined possible cut-off levels of sCAIX based on creating splits in decision trees using the test statistic |z| in an exploratory approach.[39] By using the interaction between sCAIX level and treatment, the best cut-off was identified at sCAIX levels of 114 and 101 pg/mL for prediction of pCR and DFS, respectively (Supporting Information Figs. S2A and S2B). For both endpoints, an optimal cut-off was determined at 122 pg/mL corresponding to the 39th percentile (Supporting Information Fig. S2C). We used this combined cut-off for further analyses because pCR and DFS are both highly relevant clinical endpoints.

Analysis of pCR rates by sCAIX cut-off

Next, we analyzed the association of the different treatments with pCR rates in patients with sCAIX below (sCAIX low) and above the cut-off (sCAIX high). In concordance with the logistic regression model for continuous sCAIX, patients with low sCAIX levels had a significantly higher likelihood of achieving a pCR when treated with NCT-B compared to patients treated with NCT (OR: 2.16, 95%CI 1.24–3.88; $p = 0.006$; Fig. 2). However, there was no difference with regards to treatment prediction for patients with sCAIX above the cut-off ($p = 0.59$; Fig. 2). The test for interaction revealed that treatment with NCT and NCT-B had significantly different effects on pCR in patients with sCAIX above *vs.* below the cut-off ($p = 0.012$, Fig. 2).

Consistently, pCR rates were lower in NCT-treated patients with sCAIX level below the cut-off compared to patients with sCAIX levels above the cut-off (12.1% below the cut-off *vs.* 20.1 above the cut-off, $p = 0.012$). Furthermore, in patients with low sCAIX levels, pCR rates were improved in NCT-B-treated patients (20.4%) compared to NCT-treated patients

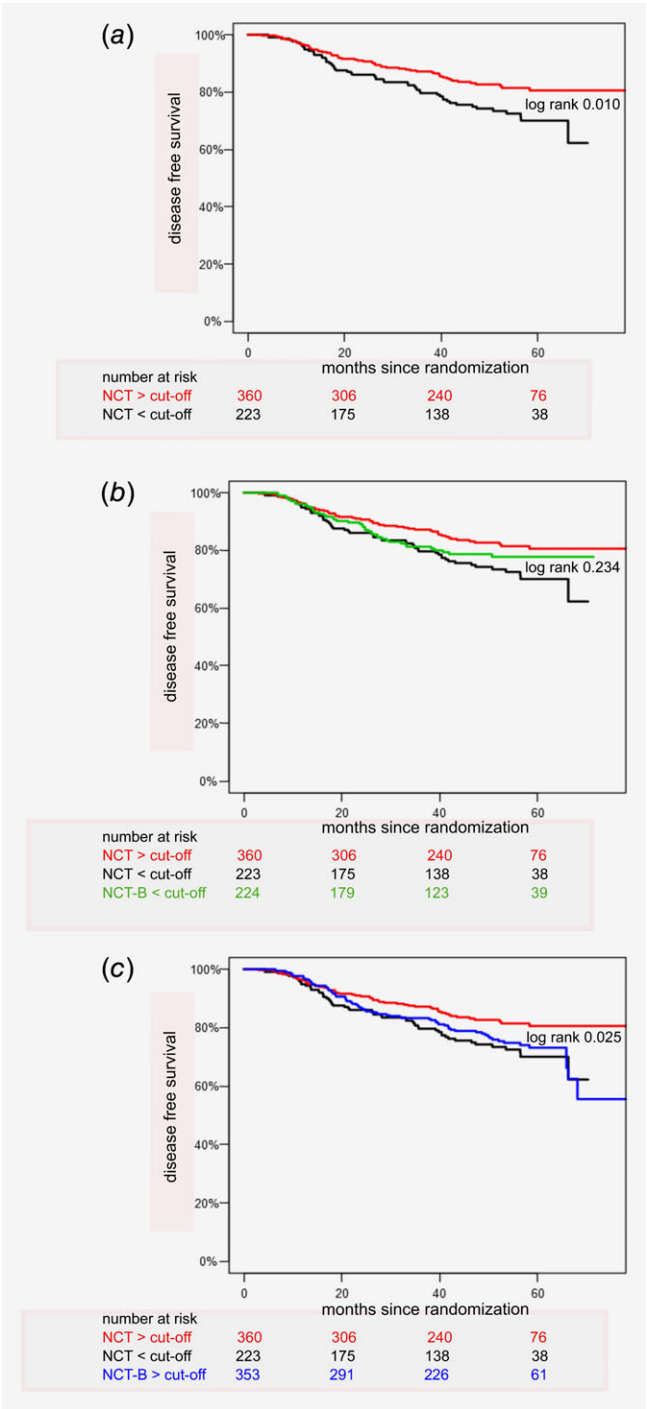

Figure 3. Disease-free survival for patients with high and low pretreatment sCAIX level. Kaplan–Meier analyses depicting DFS separated by sCAIX for NCT-treated patients in (*a*) and with addition of bevacizumab in (*b* and *c*). Pairwise log rank test compares NCT ‹ cut-off with NCT-B ‹ cut-off in (*b*) (log rank 0.234) and NCT and NCT-B › cut-off in (*c*) (log rank 0.025).

(12.1%, $p = 0.017$, Table 3). In patients with sCAIX levels above the cut-off, pCR rates were 20.1% in the NCT group *vs.* 17.0% for patients in the NCT-B group ($p = 0.91$, Table 3).

As in the continuous model (Table 2), the effect was mostly observed in TNBC patients (test for 3-way interaction = 0.133, Fig. 2). In this group, pCR rates were 21.6% in NCT-treated *vs.* 47.2% in NCT-B-treated patients with low sCAIX levels ($p = 0.001$ Table 3; OR: 3.36, 95%CI 1.63–7.27, $p = 0.002$, Fig. 2). For HR-positive patients no treatment prediction could be made with the determined cut-off (test for interaction = 0.68, Fig. 2).

Analysis of DFS by sCAIX cut-off in patients treated with NCT alone

Patients exhibiting sCAIX levels below the cut-off had a worse DFS compared to patients with sCAIX levels above the cut-off when treated with NCT alone (Fig. 3*a*, log rank 0.010). In this group, the adjusted 5-year DFS was 71.4% for patients with low sCAIX levels compared to 81.0% for patients with high sCAIX levels ($p = 0.010$, Table 3). These data are consistent with the negative predictive effect of low sCAIX levels on pCR rates in patients treated with NCT alone, as described above (Fig. 2, Table 3). Hence, patients exhibiting low sCAIX levels respond worse to NCT alone compared to patients with high sCAIX levels. However, unlike the predictive effect of sCAIX levels on pCR, which is mainly present in TNBC patients, the effect of sCAIX levels on DFS mainly occurs in HR-positive patients (Tables 2 and 3, Fig. 2).

Analysis of DFS in patients with low sCAIX levels treated with NCT alone *vs.* NCT-B

In patients with sCAIX levels below the cut-off, the 5-year DFS of 71.4% could be numerically improved by the addition of bevacizumab to NCT (5-year DFS 78.5% for NCT-B below the cut-off, log rank 0.234, Table 3, Fig. 3*b*). The adjusted hazard ratio indicated a mildly improved DFS when bevacizumab was added to NCT in this group. However, this effect was not significant [Hazard ratio (HR): 0.80, 95%CI 0.52–1.22, $p = 0.30$].

Analysis of DFS in patients with high sCAIX levels treated with NCT alone *vs.* NCT-B

Patients with sCAIX levels above the cut-off had a significantly shorter DFS when treated with NCT-B compared to patients treated with NCT alone (HR: 1.47, 95%CI 1.04–2.08, $p = 0.028$, Fig. 3*c*). The adjusted 5-year DFS was 81.5% in patients treated with NCT *vs.* 73.6% in patients treated with NCT-B (Table 3, log rank 0.025). Thus in this patient group, the addition of bevacizumab appeared to have detrimental effect on DFS.

Discussion

We evaluated the association of sCAIX levels with pCR, DFS and OS in samples from 1,160 HER2-negative patients treated with NCT alone *vs.* NCT-B in the G5 trial.[11,14]

The main finding of this paper is that patients with low pretreatment sCAIX levels treated with standard NCT were less likely to achieve a pCR and had a worse DFS compared to those

with high sCAIX levels. This unfavorable outcome in patients with low pretreatment sCAIX levels was improved by the addition of bevacizumab. However, this effect was only statistically significant with respect to pCR but not to DFS. One important conclusion from our study is that treatment intensification warrants exploration in early breast cancer patients with low sCAIX levels.

The unfavorable effect of low sCAIX level is surprising at first sight because increased intratumoral CAIX expression is associated with adverse prognosis in early- and late stage breast cancer patients.[27,28] However, membrane-bound intratumoral CAIX is different from sCAIX, which represents the extracellular domain of CAIX that is actively shed into the circulation by proteolytic cleavage.[31,40] Therefore, membrane-bound intratumoral CAIX and sCAIX most likely represent different biomarkers. Corroborating this theory, no correlation between sCAIX and intratumoral CAIX mRNA levels was detected in early breast cancer patients before surgery.[21] This finding shows that sCAIX levels do not necessarily reflect intratumoral CAIX expression. Similarly, presurgery sCAIX levels in early-stage primary nonsmall cell lung cancer patients did not correlate with immunohistochemical quantification of intratumoral CAIX.[41]

We observed a divergent effect of baseline sCAIX level in the NCT arm compared to the NCT-B arm. The mechanisms underlying our finding have yet to be elucidated. Until today, only little is known about the regulation of the shedding process of sCAIX.[40] Interestingly, recent *in vitro* studies showed that extracellular domain shedding of CAIX is increased after chemotherapy-induced apoptosis.[42] Considering that intratumoral, membrane-bound CAIX protects the tumor cells from hypoxia and acidosis, a reduction of CAIX by extracellular domain shedding might render tumor cells more sensitive to chemotherapy and environmental stress factors.[42] This could explain why in our patient cohort low sCAIX levels predict worse outcome in patients treated with NCT alone, since they might be less prone to chemotherapy-induced apoptosis. In line with this hypothesis, one study reported that an increase in sCAIX level during neoadjuvant chemotherapy in locally advanced colorectal cancer predicted outcome, as shown by improved 5-year PFS in patients with a sCAIX increase (94% 5-year PFS for patients with an sCAIX increase *vs.* 56% 5-year PFS in patients with no increase, 66 patients).[43]

One study including 472 patients reported an association of high sCAIX levels with significantly shorter PFS in HER2-positive metastatic breast cancer patients.[44] At first sight, this finding seems to contradict our data showing that low sCAIX level are associated with lower pCR rates and DFS. Of note, we were investigating sCAIX in early-stage breast cancer patients, which substantially differs from the metastatic setting due to lower tumor burden and aggressiveness. Another possible explanation could lie in the fact that we were investigating a HER2-negative patient population. The association of sCAIX levels with outcome might be different in patients treated with chemotherapy, with and without bevacizumab, *vs.* HER2-positive patients treated with HER2-targeted approaches.

However it is not yet clear why bevacizumab improved the pCR rates in patients with low sCAIX levels. One reason could be the association of increased sCAIX shedding in hypoxic conditions.[42] It was demonstrated that hypoxia leads to expression of a plethora of different pro-angiogenic factors besides the VEGF axis, thus hypoxic tumors become independent of VEGF to maintain angiogenesis.[45,46] This could explain why the addition of bevacizumab to NCT only improves pCR rates in patients expressing low levels of sCAIX, which could indicate less hypoxia and resistance to bevacizumab. Along these lines high sCAIX levels could be a surrogate for increased hypoxia and consecutive resistance to anti-VEGF therapy.

Another aspect to consider is that it is currently unknown whether sCAIX is tumor or host-derived. In order to better understand the regulation and roles of tumor- and host-derived sCAIX, and its relationship to efficacy of anti-angiogenic and chemotherapy, functional studies are needed.

The predictive potential of sCAIX was previously assessed in patients with metastatic renal cancer treated with sorafenib *vs.* placebo in a phase III trial. In this setting, sCAIX levels were not predictive for efficacy of sorafenib.[47] The reasons for the lack of predictive effect of sCAIX in this context are presently unclear. However, only samples from 128 patients corresponding to 14% of the study cohort were included in the analysis.[47] Thus, the sample size might be too small to draw reliable conclusions. Furthermore, it has been reported that small molecule VEGF receptor inhibitors, with their ability to inhibit multiple kinases, differ from bevacizumab with regards to their mechanisms of action, responsive tumor types and efficacy in combination with chemotherapy.[48,49] Thus, class-specific biomarkers may exist in the context of anti-angiogenic therapies.

Another study reported an increase of mean sCAIX level in 57 patients with locally advanced breast cancer upon treatment with paclitaxel and the multi-kinase tyrosine inhibitor sunitinib.[50] Patients with sCAIX level below the median had higher pCR rate compared to low sCAIX in the sunitinib-treated patients (44.8 *vs.* 11.5%, respectively). However, in this study the patient cohort was very small and no control arm was treated without sunitinib for comparison.

Few studies reported an adverse prognostic effect of high sCAIX levels, while others indicated no association of sCAIX levels with prognosis.[36,41,51–53] In early-stage breast cancer, sCAIX levels did not have an adverse prognostic effect.[35] In line with these data, we did not detect a correlation of sCAIX levels with tumor stage in the present study. However, the prognostic effect of sCAIX might vary between different cancers because sCAIX was correlated with increased tumor stage in a cohort of 209 patients with non-small cell lung cancer.[41] Altogether, these data suggest that the effects of sCAIX on clinical parameters may be influenced by the tumor type, tumor burden and possibly by different ELISA assays. More

work is necessary to dissect the prognostic *vs.* predictive effect of sCAIX in cancer.

In our study, the most prominent improvement of pCR rates was observed in TNBC patients. Here, NCT-B was clearly favored over NCT in patients with sCAIX levels below the determined cut-off. Of note, the lack of effect of sCAIX in HR-positive patients might not only be due to the different biology of these two subgroups but also due to low pCR rates in HR-positive tumors.[12,54]

One limitation of our study is the lack of external validation. However, the large number of patients from a randomized phase III trial and the internal validation suggest that sCAIX is a promising novel predictive biomarker for bevacizumab and pCR in breast cancer, and thus warrants prospective validation. Our findings have possible implications beyond early breast cancer and therefore it is of interest to determine the predictive potential of sCAIX for the efficacy of bevacizumab in other cancer types and in the adjuvant and metastatic settings.

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Authors' contributions

SoL, GvM, SiL and VM participated in design of this translational study. MeJ, MC-C, VG and IB-B did the experiments. MeJ, EV, CE, and SoL analyzed data. SiL, VM, CS, PF, BS, TKa, TF, MaJ, TKü, FH, FO, PK, BR, CD, MU, HT, MR, KK and GvM are part of the German Breast Group, treated patients and provided biomaterial. All authors were involved in data interpretation and in revision and finalizing the manuscript.

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