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CLINICAL TRIAL

Effects of conventional neoadjuvant chemotherapy for breast cancer on tumor angiogenesis

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Abstract The effects of breast cancer conventional chemotherapy on tumor angiogenesis need to be further characterized. Neoadjuvant chemotherapy is an ideal model to evaluate the results of chemotherapy, allowing intra-patient direct comparison of antitumor and antiangiogenic effects. We sought to analyze the effect of neoadjuvant chemotherapy on tumor angiogenesis and its clinical significance in breast cancer. Breast cancer patients ($n = 108$) treated with neoadjuvant sequential anthracyclines and taxanes were studied. Pre- and post-chemotherapy microvessel density (MVD) and mean vessel size (MVS) were analyzed after CD34 immunohistochemistry and correlated with tumor expression of pro- and antiangiogenic factors (VEGFA, THBS1, HIF1A, CTGF, and PDGFA) by qRT-PCR. Angiogenic measures at diagnosis varied among breast cancer subtypes. Pre-treatment higher MVS was associated with triple-negative subtype and more advanced disease. Higher MVS was correlated with higher VEGFA ($p = 0.003$), while higher MVD was correlated with lower antiangiogenic factors expression (THBS1, $p < 0.0001$; CTGF, $p = 0.001$). Increased angiogenesis at diagnosis (high MVS and glomeruloid microvascular proliferation) and higher VEGFA expression were associated with tumor recurrence ($p = 0.048$ and 0.009, respectively). Chemotherapy-induced angiogenic response (defined as decreased MVD) was present in 35.2 % of patients. This response correlated with an increase in antiangiogenic factors (THBS1) without changes in VEGFA expression, and it was associated with tumor downstaging, but not with clinical response, pathologic complete response, or prognosis. Global effects of chemotherapy mainly consisted in an increased expression of antiangiogenic factors (THBS1, CTGF), with significant changes neither of tumor VEGFA nor of MVS. Conventionally scheduled neoadjuvant chemotherapy exerts antiangiogenic effects, through an increase in antiangiogenic factors, THBS1 and CTGF, but the expression of VEGFA is maintained after treatment. Better markers of angiogenic response and a better understanding of the cooperation of chemotherapy and antiangiogenic therapy in the neoadjuvant clinical scenario are needed.

Keywords Breast cancer · Angiogenesis · VEGF · THBS1 · Neoadjuvant chemotherapy · MVD

Abbreviations

CT	Chemotherapy
MVD	Microvessel density
MVS	Mean vessel size
AR	Angiogenic response
pCR	Pathologic complete response
HR	Hormone receptor

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Introduction

Tumor angiogenesis, the process by which new vessels are created, has been a relevant therapeutic target in breast cancer during the last decade. Multiple experimental data and in vivo models support the role of angiogenesis in cancer progression and metastases development. Histological markers of breast cancer angiogenesis activation, such as microvessel density (MVD) and expression of some proangiogenic factors, such as vascular endothelial growth factor (VEGF), have also shown prognostic value in different settings [1]. In contrast with these strong preclinical and translational data, practical results have been disappointing, with only small gains in response and progression-free survival in breast cancer patients treated with anti-VEGF therapies [2]. The development of resistance to antiangiogenic agents, mediated both by tumor- and stroma-related mechanisms, is also a potential caveat of anti-VEGF drugs. Even more recent approaches with multikinase inhibitors targeting angiogenesis have not rendered better results, and the real value of the whole antiangiogenic strategy for breast cancer has been recently questioned [3].

The development of antiangiogenic treatments has been limited by the lack of reliable predictive markers of therapeutic benefit. Considering that most clinical studies are based on the combination of antiangiogenic drugs, such as bevacizumab, with a backbone chemotherapy regimen [4, 5], a more complete knowledge of the angiogenic effects of chemotherapy would be relevant for the rational development of therapeutic combinations. Conventional chemotherapy exerts its own antiangiogenic effects, especially when administered with metronomic or weekly schedules [6, 7]. Even with conventional three-weekly schedules, some drugs, such as taxanes, seem to have antiangiogenic effects [8]. Since both pre- and post-treated biopsies are usually available, the neoadjuvant or preoperatory setting is especially interesting for determining the vascular effects of chemotherapy. A few studies in this therapeutic context have shown mixed effects of neoadjuvant chemotherapy for breast cancer, with an inconsistent relationship between angiogenic response (defined as decreased MVD) and pathologic response or prognosis. However, most clinical studies focusing on vascular effects of chemotherapy either are small [9–12] or include a combination of chemotherapy and antiangiogenic agents [13, 14] or were published before the wide incorporation of combinations of anthracyclines and taxanes to breast cancer treatment [15, 16]. As far as we know, only two of the neoadjuvant series addressing this problem have included a taxane (docetaxel) [13, 17], and none of them showed a significant change of MVD after chemotherapy. The work by Miller, so far the only study including a regimen similar to the current standard (although with weekly docetaxel), also evaluated some circulating angiogenic markers (VCAM-1, bFGF, MMP-2, MMP-9), of which only bFGF showed an increase with treatment, but again without correlation with MVD.

The aim of our work was to determine the angiogenesis-related effects (defined both by the vascular characteristics and the angiogenic biomarkers profile in the tumor) of sequential neoadjuvant anthracyclines and taxanes for breast cancer, and to evaluate the clinical relevance of those changes.

Patients and methods

Patients and treatment

A group of 108 consecutive patients treated with neoadjuvant chemotherapy for breast cancer in the Department of Hematology and Medical Oncology, University Hospital Morales Meseguer, Spain, were retrospectively studied. Neoadjuvant chemotherapy included anthracyclines and taxanes. Pre-treatment study included mammography and breast MRI, core biopsy, and either axillary node fine-needle aspiration or sentinel lymph node biopsy. After neoadjuvant chemotherapy, clinical response was evaluated with MRI and definitive surgery was performed. Adjuvant hormone therapy, radiotherapy, and trastuzumab (when appropriate) were administered according to current practice. Pathological complete response (pCR) was defined as the absence of invasive tumor both in the breast and in the axilla (ypT0/Tis ypN0 M0). Tumor downstaging was defined as any decrease of pT category considering cT as a reference. Informed consent was obtained from all patients, and the study was approved by the hospital Institutional Review Board.

Pathology and vascular assessment

Vascular assessment was performed in full 4-μm sections of pre-chemotherapy (core) and post-chemotherapy (surgical) biopsies. In those cases with pCR, sections were obtained from tumor bed, after careful evaluation by a pathologist. An anti-CD34 antibody (Clone QBEnd-10; #M7165; DAKO, Glostrup, Denmark) was used for immunohistochemistry with an automatized stainer (Autostainer Link 48, DAKO, Carpinteria, CA, USA), according to manufacturer's instructions. Staining was performed simultaneously for pre- and post-chemotherapy sections of each patient to avoid analytic variability. We used standard DAKO Envision systems for secondary

antibody and visualization procedures. Each slide was digitally scanned using an automated scanning system (Leica SCN400F). About three to five 100× digital pictures (for a total area of 2.40–4.00 mm^2) were obtained by two independent observers (GLG, FAP). Slides with inadequate staining or less than 3 evaluable fields were excluded. Automatic image analysis to determine MDV (normalized to a 0.20-mm^2 field) and mean vessel size (MVS; vessel area per vessel; μm^2) was performed with macros developed for ImageJ software (NIH) in collaboration with the Scientific Image Department of the University of Murcia. The presence of glomerular vascular proliferation (GMP), as defined by Akslen et al. [18], was determined by one of the authors (FAP) in the whole section; cases were considered GMP+ if at least one GMP structure was observed. Since no standardized criteria are available for vascular changes, angiogenic response was arbitrarily defined as any decrease in MVD after neoadjuvant chemotherapy.

RNA purification and qRT-PCR

We obtained RNA from pre- and post-treatment formalin-fixed paraffin-embedded biopsies. After deparaffinization with xylene and ethanol washes, the RNeasy FFPE kit (QIAGEN, Germantown, MD, USA) was used for RNA extraction. For real-time PCR for VEGFA, thrombospondin 1 (THBS1), connective tissue growth factor (CTGF), platelet-derived growth factor A (PDGFA), and hypoxia-induced factor 1-α (HIF1A), we used Taqman Gene Expression Assays (Applied Biosystems, Carlsbad, CA, USA) and a LightCycler 480 System (Roche Diagnostics, Basel, Switzerland). A pre-amplification step was performed for paraffinated samples with Master-cycler nexus (Eppendorf, Hamburg, Germany). The 2-DDCt method with ACTB as an endogenous control was used for calculation of the relative expression levels of each marker [19].

Statistical analyses

Statistical analysis was performed with software SPSS 20.0 (SPSS, Inc., Chicago, IL, USA). For comparison of continuous variables between groups defined by clinical and pathological characteristics in each time-point (pre- and post-chemotherapy), non-parametric tests (*U* Mann–Whitney or Kruskal–Wallis) were used. Comparison of proportions was performed with the Chi squared test and association between continuous variables by the Spearman rank-correlation coefficient. Continuous vascular and biomarker parameters were compared between the two different time-points by paired Wilcoxon signed-rank test. The Holm–Bonferroni procedure was used to adjust *p* values for multiple comparisons [20]. Overall survival (OS) and disease-free survival (DFS) were analyzed with the Kaplan–Meier method, and comparisons between groups were performed by log-rank test. For multivariate survival analysis, Cox models were constructed. All tests were two-sided, and statistical significance was defined as a *p* value of ≤ 0.05.

Results

Patient characteristics and treatment outcomes

We included 108 patients, with a median age of 56 years, with locally advanced tumors or tumor sizes that precluded breast-preserving surgery. Patient and tumor characteristics are shown in Table 1. All patients were treated with neoadjuvant chemotherapy, including anthracyclines and taxanes in 97.2 % of cases. Clinical response rate was 87 %, while primary tumor downstaging was observed in 56.9 % of patients. After treatment, 47.2 % underwent conservative surgery, and the pCR was 19.4 %. DFS and OS has not been reached after a median follow-up of 5 years.

Pre-chemotherapy vascular and angiogenic markers profile

Pre-treatment vascular assessment was possible in 86 cases (79.6 %) with enough tissue to evaluate CD34 + vessels (Fig. 1a). Median value of MVS was 102.5 (range: 51.7–273). A higher (over the median) MVS was more frequent in the group of patients with extensive nodal involvement (cN2-3; $p = 0.025$) and with triple-negative breast cancer ($p = 0.05$) (Fig. 1b, c). No association with survival or with other clinical variables was observed (Online Resource: Suppl. Table 1). GMP was found in 32.5 % of the cases, and its presence was also associated with a higher vascular size ($p = 0.012$) (Fig. 1d) (Online Resource: Suppl. Table 2). While GMP + tumors did not show a worse prognosis, the simultaneous presence of high vascular size and GMP + (24.4 % of cases) was more frequent in hormone receptor (HR)-negative tumors (45.8 vs. 15.1 %; $p = 0.01$) and defined a high-risk group with lower DFS (log-rank; $p = 0.048$; HR: 2.78, 95 %CI 0.96–8.03) (Fig. 1e). Median MVD was 26.5 (range: 5.7–97.2) for the whole group and was not associated with tumor stage, nodal involvement, breast cancer subtype, histologic grade, or other clinical and pathological variables (Online Resource: Suppl. Table 1). A higher MVD (over the median) was associated neither with DFS nor with OS. Vascular area was also determined, but results were highly concordant with MVD (ρ: 0.88; $p < 10^{-6}$),

Table 1 Patient characteristics

Characteristic	*n*	%
N	108	100 %
Age (median; range)	56.5 (21–79)	
Family history of breast cancer		
No	85	78.7
Yes	22	20.4
Unknown	1	0.9
Menopausal status		
Post-menopausal	54	50.0
Pre-menopausal	54	50.0
Clinical stage		
IIA	19	17.6
IIB	32	29.6
IIIA	35	32.4
IIIB	4	3.7
IIIC	18	16.7
Clinical stage of primary tumor		
cT1-2	50	46.3
cT3-4	58	53.7
Lymph node clinical stage		
cN0-1	71	65.7
cN2-3	37	34.3
Histological type		
Ductal	102	94.4
Lobular	4	3.7
Others	2	1.9
Histological tumor grade		
GI-II	41	38.0
GIII	56	51.9
Not available	11	10.1
Lymphovascular invasion		
No	85	78.7
Yes	17	15.7
Not available	6	5.6
Pre-chemotherapy IHC subtypes		
Hormone-sensitive	53	49.1
Hormone-sensitive HER2+	14	13.0
HER2NEU+	13	12.0
Triple negative	24	22.2
Not available	4	3.7
Treatment		
ACx4–Docetaxelx4	90	83.3
Anthracyclines & weekly paclitaxel	9	8.3
Scheme without anthracyclines	2	1.9
Scheme with anthracyclines without taxanes	1	0.9
Scheme with anthracyclines & concomitant taxanes	6	5.6
Treatment with trastuzumab		
No	86	79.6
Neoadjuvant and adjuvant	17	15.7
Only adjuvant	5	4.7
Surgery		
Mastectomy	57	52.8
Breast-conserving	51	47.2
Clinical response		
Yes	94	87.0
No	10	9.3
Non evaluable	4	3.7
Pathological complete response (pCR)		
ypT0/Tis + ypN0	21	19.4
ypT0/Tis	23	21.3
ypN0	42	38.9
ypN+		
Negative	59	54.6
Positive	49	45.4

and the correlation with clinical variables was similar; therefore, only data for MVD are reported below.

The expression of proangiogenic (VEGFA, PDGFA, HIF1A) and antiangiogenic (THBS1, CTGF) markers was analyzed in pre-chemotherapy tissue samples, showing a predominant expression of THBS1, CTGF, and VEGFA, and lower expression of HIF1A and PDGFA (Table 2). The expression of VEGFA correlated with that of HIF1A (ρ: 0.362; $p = 0.001$), while a stronger correlation was observed for THBS1 and CTGF (ρ: 0.489; $p < 0.00001$). The biomarker profile was clearly different between the different breast cancer subtypes: While VEGFA showed a trend to lower expression ($p = 0.09$) in HR-positive (HR+) tumors, triple-negative tumors showed higher VEGFA ($p = 0.04$) and lower THBS1 expression ($p = 0.009$). HER2+ carcinomas (with or without HR positivity) were characterized by low expression of both VEGFA and THBS1 ($p = 0.003$). No differences were found for expression of CTGF ($p = 0.48$) and PDGFA (0.45) among the diverse phenotypes (Fig. 2a). The proportion of tumor cells in each core biopsy was not correlated with the expression of any of the angiogenic biomarkers.

The vascular pattern was clearly associated with the angiogenic biomarker profile in the tumor: THBS1 (ρ: −0.40; $p < 0.0001$) and CTGF (ρ: −0.37; $p = 0.001$) expression were inversely correlated with MVD (Fig. 2b), while a higher MVS was correlated with higher VEGFA (ρ: 0.35; $p = 0.003$) and THBS1 expression (ρ: 0.27; $p = 0.02$) (Fig. 2c). No similar associations were observed for HIF1A or PDGFA. GMP + carcinomas also showed a higher VEGFA expression level ($p = 0.015$; U Mann–Whitney). Tumors with both high MVS and GMP + showed the highest VEGFA expression ($p = 0.03$) (Fig. 2d). Not surprisingly, the expression of VEGFA in tumor tissue by itself was also

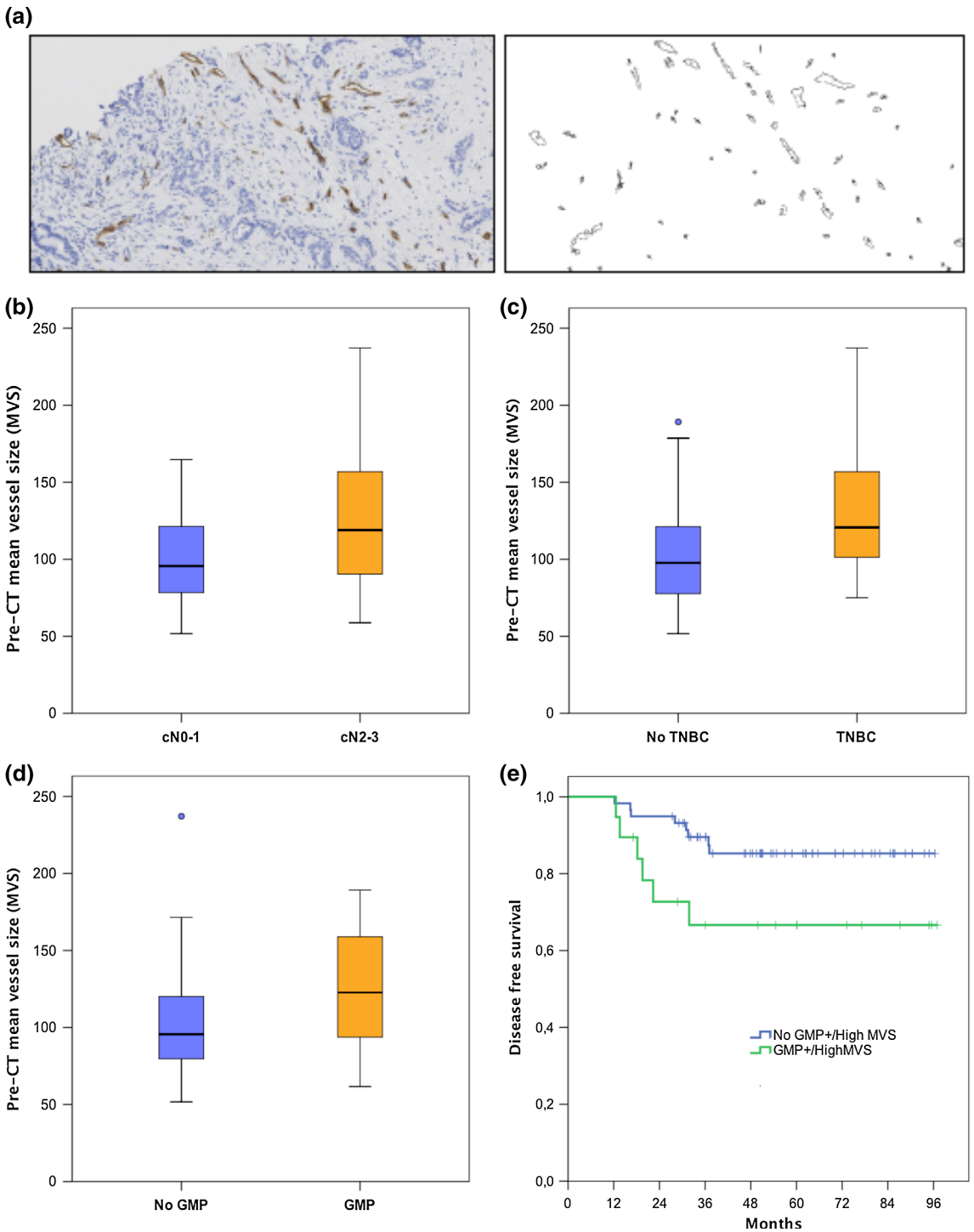

Fig. 1 Pre-chemotherapy vessel profile of breast cancer. **a** Representative pictures of CD34 immunohistochemistry in breast cancer biopsies and digital processing for MVD and MVS determination. **b** Association of pre-treatment mean vessel size (MVS) with clinical nodal stage ($p = 0.025$). **c** Association of pre-treatment MVS with triple-negative breast cancer subtype ($p = 0.05$). **d** Higher pre-chemotherapy MVS in tumors with glomeruloid microvascular proliferation (GMP) ($p = 0.012$). **e** Kaplan–Meier DFS according to the vascular phenotype: high-risk group defined as GMP + tumors with MVS over the median value ($p = 0.048$, log-rank test)

prognostically relevant, with lower DFS ($p = 0.009$) in those patients showing a high baseline VEGFA expression. This impact on DFS was even maintained (HR: 3.55; 95 %CI 11.14–11.07; $p = 0.03$) when VEGFA was included in a Cox model together with cN2-3 and pCR, which were the only significant covariates in a Cox model without angiogenic

Table 2 Pre-chemotherapy and post-chemotherapy expression of angiogenic biomarkers

	$N = 75$	Mean; SD	Median	Range	p*
HIF1A	Pre-CT	0.028765; 0.020617	0.023577	0.001400–0.167000	0.00003
	Post-CT	0.039672; 0.020511	0.037077	0.008315–0.113178	
PDGFA	Pre-CT	0.00093; 0.00123	0.00051	0.00003–0.00778	0.002
	Post-CT	0.001585; 0.001600	0.001035	0.000003–0.009334	
THBS1	Pre-CT	0.544393; 0.411610	0.450636	0.093428–2.116480	0.000004
	Post-CT	0.949927; 0.666970	0.813868	0.066370–4.189176	
VEGFA	Pre-CT	0.177445; 0.160186	0.115959	0.032918–1.162046	0.14
	Post-CT	0.209239; 0.154481	0.166093	0.030607–1.099362	
CTGF	Pre-CT	0.163969; 0.176307	0.115000	0.000192–0.993000	0.0000005
	Post-CT	0.851420; 0.705635	0.655000	0.000014–3.540000	

* Wilcoxon signed-rank test (Holm–Bonferroni adjustment of p values for multiple comparisons)

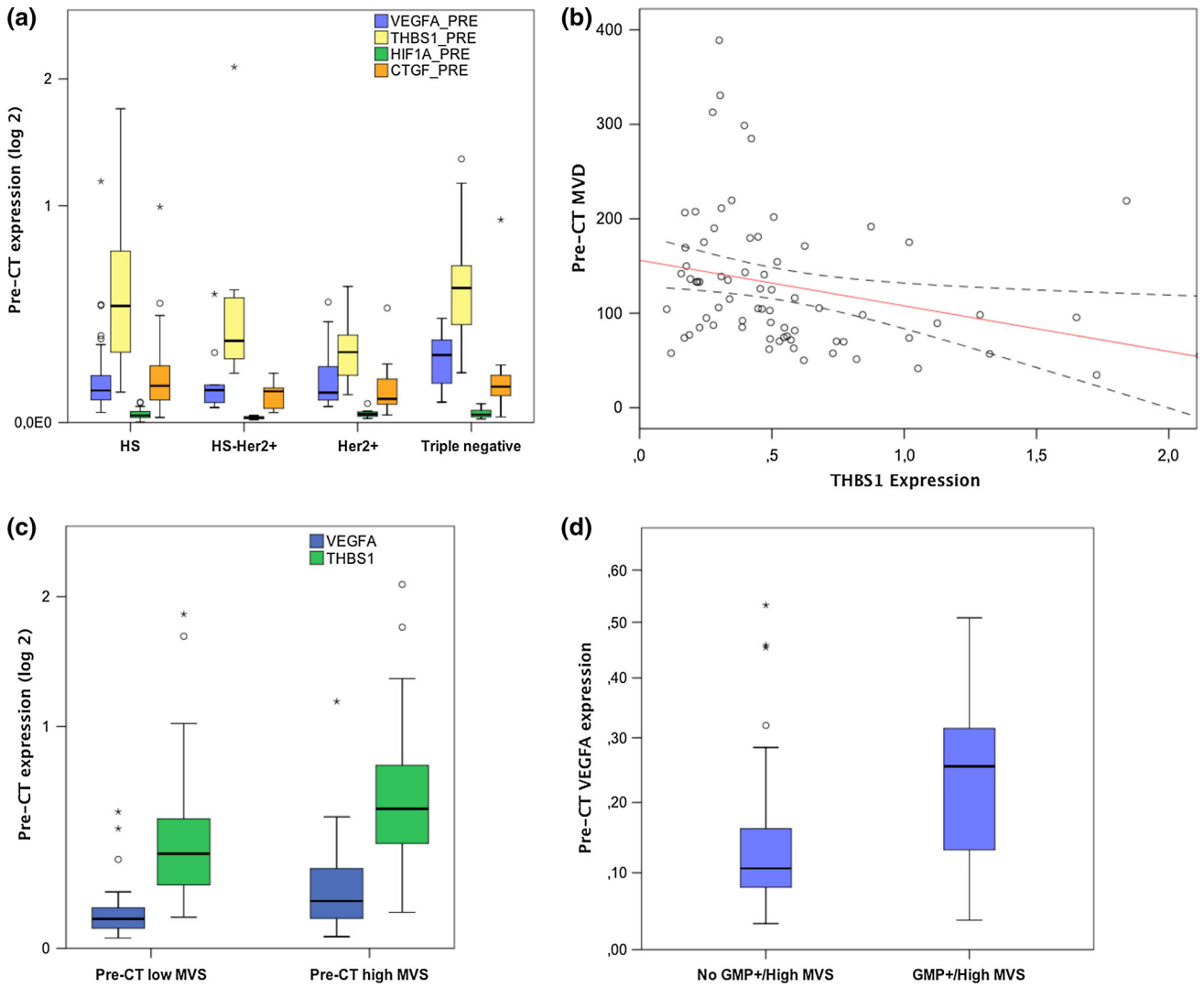

Fig. 2 Pre-chemotherapy expression of angiogenic markers and association with vascular pattern of breast carcinomas. **a** Pre-chemotherapy expression of main angiogenic markers according to breast cancer subtype. **b** Inverse correlation of thrombospondin 1 (THBS1) expression with MVD in pre-treated breast cancer tumors. (Spearman coefficient: −0.40; $p = 0.0004$). **c** Association of higher (over the median) MVS with higher baseline VEGFA ($p = 0.005$) and THBS1 ($p = 0.04$) tumor expression. **d** Higher expression of VEGFA in tumors with a high-risk vascular profile (GMP +/high MVS) ($p = 0.03$)

markers. To further discard the potential confusion between high VEGFA expression and triple-negative subtype, we also tested a multivariate model including triple-negative classification, cN2-3, and pCR as covariates: high VEGFA expression kept its prognostic relevance for DFS (HR: 3.42; 95 %CI 1.08–10.82; $p = 0.04$). OS was modified neither by VEGF nor by other angiogenic markers expression.

Chemotherapy-induced changes in vascular pattern and angiogenic biomarkers

We performed a comparison of the vascular morphology and the angiogenic markers profile before and after chemotherapy with anthracyclines and taxanes in the group of patients in which both pre- and post-treatment matched samples were evaluable ($n = 54$). For the whole group, we observed a post-chemotherapy increase in MVD ($p = 0.0003$) (Fig. 3a) (Online Resource: Suppl. Table 3). Post-treatment MVD was not associated with clinical or pathological variables of residual tumor. Since no standard definition of angiogenic response (AR) is currently available, we defined it as any decrease of MVD. A chemotherapy-induced angiogenic response appeared only in 35.2 % of the patients (Fig. 3b), with no response or even with increased MVD after treatment in the rest of the cases. AR was more frequent in those patients with higher pre-treatment MVD ($p = 0.015$) (Fig. 3c), but no association was found for other clinical or pathological variables (Online Resource: Supp. Table 4). When we explored the biological and clinical meaning of AR, chemotherapy-related AR was associated with tumor downstaging defined as any decrease in pT category (73.7 vs. 35.3 %; $p = 0.028$). However, an association of AR was found neither for clinical ($p = 0.29$) or pCR ($p = 0.87$) nor for survival (DFS, $p = 0.65$; OS, $p = 0.49$).

Neoadjuvant chemotherapy did not significantly change the size of vessels (Fig. 3d), with MVS consistently maintained in the whole group ($p = 0.41$) (Online Resource: Suppl. Table 3). While the lack of change was mainly derived from those cases without pCR ($p = 0.82$), in the group of patients achieving a pCR ($n = 8$) a non-significant trend for a lower MVS was found after chemotherapy (MVS: 106.4 vs. 80.6; $p = 0.15$). Similarly to the pre-treatment situation, a higher post-CT MVS was significantly associated with adverse pathological characteristics such as lack of HR expression (40.7 vs. 7.4 %; $p = 0.012$) and higher residual disease both at the axillary lymph nodes (70 % ypN + vs. 37.8 %; $p = 0.018$) and at the breast (36.7 % ypT3-4 vs. 10.8 %; $p = 0.03$), even when the analysis was restricted only to those patients without pCR (ypT3-4, $p = 0.08$; ypN +, $p = 0.04$; HR-, $p = 0.012$).

Changes in angiogenic biomarkers mainly consisted of an increased expression after chemotherapy; only VEGFA levels did not change significantly, while higher levels were found for both antiangiogenic (THBS1, CTGF) and proangiogenic (HIF1A, PDGF) markers (Table 2). CTGF expression changes were remarkable, with around ten-fold increase after chemotherapy. These results were not modified when pCR cases were excluded from the analysis. In those patients with chemotherapy-induced angiogenic response, the only significant change was a three-fold increase of THBS1 ($p = 0.05$), which was consistent with an angiogenic balance leading to a lower MVD, and significantly different of that observed in patients without AR (Online Resource: Suppl. Table 5). Significant differences of chemotherapy-related angiogenic markers change were observed neither between cases with or without clinical response nor between cases with or without pathological response, except for a higher increase of PDGFA (Online Resource: Suppl. Table 6).

Discussion

We here describe the baseline angiogenic profile of breast carcinoma, as defined by vessel characteristics and by the expression of the main angiogenic factors, and its change after sequential anthracyclines and taxanes neoadjuvant chemotherapy. Our results show that the angiogenic pattern of breast cancer, although not relevant for prediction of clinical or pathological response, shows prognostic value when vascular morphology and VEGFA expression are considered. Chemotherapy-related angiogenic changes are characterized by the increase of MVD, the stability of MVS, and variable changes in the balance of angiogenic biomarkers. The angiogenic response, defined as any decrease of MVD, is associated with primary tumor downstaging, but not associated with any other relevant prognostic or pathological characteristic of residual breast carcinoma, thereby pointing to the difficult integration of antiangiogenic strategies with conventional chemotherapy in the neoadjuvant setting.

While the prognostic impact of MVD in breast cancer has been previously shown by several groups in large adjuvant series [21], only a few works have addressed the relevance of breast cancer vascular morphology. In our series, a higher MVS was consistently associated with unfavorable tumor characteristics, triple-negative subtype, and with a lower DFS. Similar results using two parameters related to vessel size and complexity were recently reported in a large series of breast cancer [22]. As far as we know, no previous clinical data are available concerning the relationship between breast cancer vascular morphology and tumor expression of angiogenesis-related markers. In our patients, different vessel patterns were associated with different profiles of tumor expression of

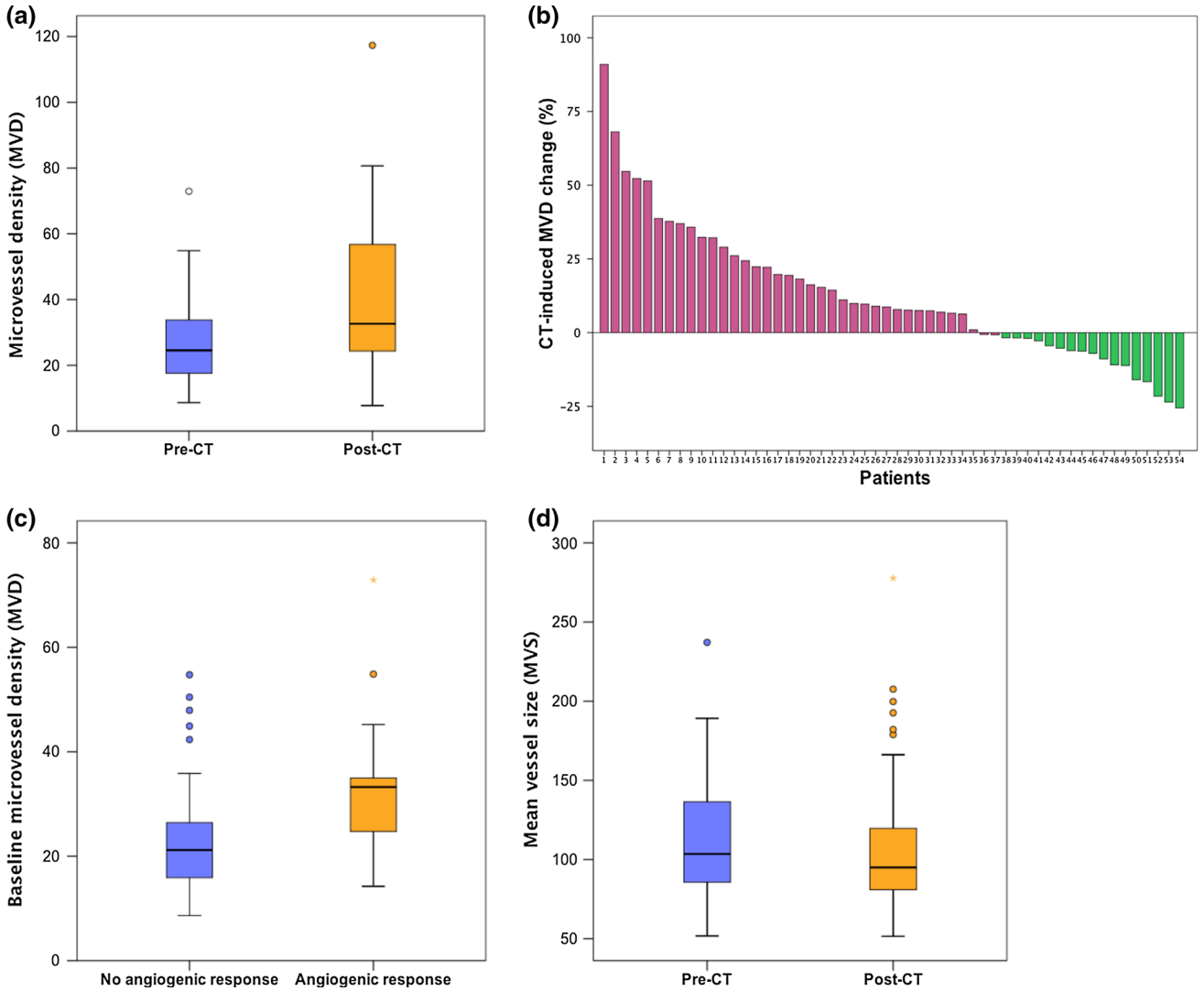

Fig. 3 Chemotherapy-induced changes on breast cancer angiogenic characteristics. **a** Higher mean MVD values after chemotherapy ($p = 0.0003$; Wilcoxon signed-rank test). **b** Water-flow graphic showing angiogenic response (defined as any decrease of MVD) in around 35 % of patients. **c** Association of angiogenic response with higher pre-treatment MVD ($p = 0.015$; X^2 test). **d** Lack of significant MVS change after neoadjuvant chemotherapy ($p = 0.41$)

angiogenic markers, and this association was consistent with a model in which vascular density, as determined by MVD, was mainly related to the expression of antiangiogenic factors, such as CTGF and THBS1, while the morphology of tumor vessels (MVS and GMP), which had a higher prognostic impact, was associated both to the expression of VEGFA and THBS1. These results are in accordance with previous experimental data showing that larger size vessels are present in breast cancer in the TSP-1-null mouse model [23] and that sustained VEGFA expression leads to denser and larger size tumor vascular networks [24].

We did not find a prognostic impact for MVD, which differs from other reports in the adjuvant setting, although the sample size and the predominantly node-positive population might justify this result [21, 25]. However, VEGFA expression was itself a marker of worse overall survival in this set of patients. Besides the potential angiogenesis-independent tumor-promoting effects of VEGFA [26], high expression of VEGFA was associated with the presence of an anomalous vessel pattern characterized by GMP and higher MVS, thereby suggesting the implication of VEGF, the major proliferative stimulus for endothelial cells [27], in its generation. Our results suggest that this angiogenic profile might confer a worse prognosis, and similar data have been reported for GMP [18] and for high VEGF expression [1, 28, 29] by other groups, both in breast cancer and in other tumors. Similarly, a higher expression of VEGFA has also been observed in many triple-negative breast carcinomas, also conferring a lower survival [30].

Chemotherapy-induced angiogenic response, arbitrarily defined as any decrease of MVD, was related to tumor downstaging in our patients. A similar correlation of angiogenic response with tumor response has been also observed in other settings using functional imaging changes as surrogate markers of tumor MVD modifications [31]. However, the impact of angiogenic changes on survival has been variable depending on the drug and the tumor context, and we did not find any clear impact of MVD changes in the primary tumor on survival or pathological response, which suggest a limited role of MVD-based angiogenic response as a surrogate endpoint in the neoadjuvant chemotherapy setting. The use of MVD changes as an endpoint in the context of neoadjuvant treatment is hampered by the double, and potentially divergent, effect of chemotherapy causing both tumor mass shrinkage and vascular effects; different combinations of them might lead to apparent increases or decreases of MVD independently of the real antiangiogenic action of chemotherapy [31, 32]. Recent experimental data also suggest that the reasons for this discordance might derive from differences between the effects of treatment on metastases and in the primary tumor, together with differences in the type of angiogenic response, which may differ between chemotherapy and antibody-based inhibition of VEGF [33, 34].

Independently of the questionable value of MVD as a surrogate endpoint, both the 35 % rate of angiogenic response and the pattern of changes in angiogenic factors strongly support the notion of an antiangiogenic effect of current neoadjuvant chemotherapy regimens. Our results show, in the clinical setting, that the increase of THBS1 and other antiangiogenic factors, such as CTGF, is not restricted to metronomic schedules of chemotherapy [7], but also observed after conventional sequential anthracyclines and taxanes. Conversely, we did not observe a significant chemotherapy-related decrease of VEGFA, a frequent effect of hormonal therapy and metronomic chemotherapy [35] but not of conventional chemotherapy [36]. The almost absent impact of neoadjuvant chemotherapy on VEGF levels and MVS, the main angiogenesis-related prognostic factors in our series, might explain the limited prognostic relevance of its antiangiogenic effects. In fact, a trend for a lower vessel size MVS was only found in those patients achieving a pCR after chemotherapy. These data might be relevant for designing combinations of antiangiogenic drugs and conventional chemotherapy in the neoadjuvant setting. Two possibilities are raised by our results: First, the generation by chemotherapy of a tumor microenvironment with a predominant anti-angiogenic (and anti-VEGF) imbalance, as suggested both by the increased thrombospondin and CTGF levels and by the higher increase of THBS1 and CTGF when compared with mildly increased or stable HIF1A and PDGFA. In this setting, the sole incorporation of additional anti-VEGF drugs would be of limited utility, and the combination of thrombospondin analogs might be a more powerful strategy of cooperation with chemotherapy [37]. Second, the lack of a decrease in VEGFA expression after chemotherapy, also observed in other series treated with neoadjuvant docetaxel [38], might support the value of VEGFA as an anticancer drug target, particularly in the more angiogenic triple-negative tumors. Further insights are clearly needed into the biological meaning of the pattern of increased expression of both antiangiogenic and proangiogenic markers after chemotherapy, but defining the precise role and the vascular effects of each of them is beyond the scope of this work.

Our work has some limitations. First, the sample size precluded a more detailed analysis of angiogenesis patterns in the different breast cancer subtypes and of the meaning of MVS changes in the group of patients with pCR. Second, the correlation of MVD measures between core needle biopsies and tumor has been previously questioned, although some data point to a better correlation when larger tumors and larger areas are evaluated [39, 40]. Besides the unavailability of other types of samples in the neoadjuvant setting, the evaluation of a total area of 2.4–4 mm^2 and the tumor size of over 2 cm in most of our cases might have compensated this methodological limitation. Third, we did not perform functional imaging or immunohistochemical evaluations of vascular normalization [41], which might have provided further data on potential antiangiogenic mechanisms of chemotherapy or on potential cooperation between chemotherapy direct antitumor and antiangiogenic effects. Our finding of the association of post-chemotherapy decreased vessel size with pCR might be related to this model of vascular normalization, but this hypothesis should be confirmed with other experimental designs and larger studies. Finally, we did not evaluate other angiogenesis-related markers, such as NRP1 or ANGPT2, potentially involved in antiangiogenic evasive resistance. However, the matched comparison of pre- and post-CT full-section biopsies and the correlation between clinical and vascular parameters and the tumor expression of angiogenic factors strengthen our conclusions and provide new clinically relevant data on the usually neglected antiangiogenic effects of neoadjuvant chemotherapy.

In conclusion, larger size of tumor vessels and higher VEGFA expression were associated with adverse clinical and pathologic tumor characteristics and lower DFS. Although decreased MVD was observed only in one-third of the patients, conventionally scheduled neoadjuvant chemotherapy exerted antiangiogenic effects, with a marked increase of antiangiogenic factors such as THBS1 and CTGF, and stable or only mildly increased proangiogenic factors such as VEGFA. These chemotherapy-

mediated changes in angiogenesis concurred with tumor downstaging, but were associated neither to pathologic or clinical response nor to prognosis. Given the limited results of the antiangiogenic therapeutic strategies in the neoadjuvant setting, with only small improvements in response rates [4, 5], and the shortcomings of current experimental models [42], better markers of angiogenic response together with a better understanding of the cooperation of chemotherapy and antiangiogenic therapy both in the clinical setting and in relevant experimental models are urgently needed.

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