TGFB-Induced Factor Homeobox 1 (TGIF) Expression in Breast Cancer

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Abstract

Background: Breast cancer (BC) is the most frequent female cancer its which preferentially metastasizes to bone. Mechanisms of bone tropism are poorly understood, however, the transcription factor TGFB-induced factor homeobox 1 (TGIF) is involved in bone metabolism and thus of potential interest.

Methods: TGIF expression was analyzed by immunohistochemistry in 1197 formalin-fixed, paraffin-embedded tissue from BC patients treated in the GAIN study with two adjuvant dose-dense schedules of chemotherapy with or without bisphosphonate ibandronate. TGIF expression was categorized into negative/low and moderate/strong staining.

Results: In 1197 samples, we found associations of higher TGIF protein expression with smaller tumor size ($p = 0.015$), well differentiated phenotype ($p < 0.001$) and estrogen receptor (ER)-positive BC ($p < 0.001$). Patients with higher TGIF expression levels showed a significantly better disease-free (log-rank p 0.019) and overall survival (log-rank $p = 0.018$), but no association with time to bone metastasis as first site of relapse. Stratified univariate analysis in molecular subgroups emphasized that elevated TGIF expression was prognostic for both DFS and OS in ER-positive BC patients (DFS: log-rank $p = 0.009$; OS: log-rank $p = 0.008$) and in the HER2-negative subgroup (DFS: log-rank $p = 0.004$; OS: log-rank $p = 0.002$).

Conclusions: Our findings suggest that a loss of TGIF expression is associated with BC progression, especially in luminal carcinomas.

Trial registration: This clinical trial has been registered with ClinicalTrials.gov; registration number: NCT00196872.

Background

Breast cancer (BC) is not only the leading cancer among women in every European country but is also the leading cause of death from cancer in women in Europe. Declines in BC mortality rates in most European countries have been reported, the favorable trends result from the combined effects of earlier detection (partly due to screening, partly due to increasing BC awareness), and a range of improvements in treatment [1]. However, the majority of deaths are not due to the primary tumor itself, but are the result of distant metastases to other organs in the body [2]. Metastatic BC remains an incurable disease with a median survival of approximately 20 months, and long-term survivors do exist but are very rare [3]. The most common metastatic site is bone with 60–70 % of all metastatic BC patients [4]. Bone metastases are largely incurable and associated with significant morbidity that negatively impacts the quality of life in metastatic BC patients. The development of BC bone metastases is a complex process involving crosstalk between disseminated BC cells and bone-derived molecules, leading to deregulation of signaling pathways critical for normal bone remodeling processes.

Bone formation is strongly activated by the canonical Wnt signaling pathway [5], and a TGFB-induced factor homeobox 1 (TGIF) was identified as a novel Wnt target gene and a crucial regulator of osteoblast

function [6]. TGIF is a member of the three-amino acid loop extension (TALE) superclass of atypical homeodomain proteins. The first two helices of the TALE superfamily are separated by a loop, which is likely to affect interactions with other proteins but not alter DNA binding properties [7, 8]. TALE homeobox proteins are highly conserved transcription regulators. The best-described function of TGIF is the repression of TGF-β signaling by recruiting mSin3A and histone deacetylases (HDACs) to the TGF-β-activated Smad complex or targeting Smad2 for degradation or sequestration [9, 10]. A number of studies have indicated a TGF-β/Smad-independent function of TGIF, which is mediated by its direct DNA binding ability [9–12]. Increasing evidence suggests that TGIF is a regulator in Wnt- and TGF-ß signaling and is associated with the initiation, development and progression of several tumor entities like gastric [13], colorectal [14] and lung cancer [15, 16].

Currently, intravenous bisphosphonates have been the mainstay of the prevention of local irreversible skeletal-related events (SRE) in patients with metastatic solid tumors. Bisphosphonates are pyrophosphate analogs which bind hydroxyl apatite in bone thus inhibiting osteoclast activity to restrict the progression of bone destruction and increase survival [17]. Studies evaluating the benefit of adjuvant bisphosphonate use revealed conflicting results [18] and therefore predictive/prognostic factors for the benefit of adjuvant bisphosphonate use are of high clinical relevance [19]. To address the potential prognostic value of TGIF in human BC, also in the context of bone metastases, we analyzed TGIF protein expression by immunohistochemistry in 1197 human BC samples using a tissue microarray prepared from tumors of BC patients treated with two different dose-dense schedules of chemotherapy with or without the bisphosphonate ibandronate.

Materials And Methods

Patients

The GAIN (German Adjuvant Intergroup Node-Positive) study was a multicenter, prospective, randomized, open-label phase III trial with a 2 x 2 factorial design. Women (aged ≥ 18 and biologically < 65 years) with involved axillary lymph nodes were randomly assigned to receive three courses each of epirubicin (E) 150 mg/m^2, paclitaxel (P) 225 mg/m^2 and cyclophosphamide (C) 2500 mg/m^2 (reduced to 2000 mg/m^2 after recruitment of 1200 patients) q2w intravenously (i.v.) (iddEPC-regimen) or ddEC (E 112.5 mg/m^2 + C 600 mg/m^2, i.v. q2w for 4 cycles) followed by paclitaxel weekly (Pw 67.5mg/m^2 i.v. q8d for 10 weeks) plus capecitabine (X 2000 mg/m^2 p.o. day 1–14, q22 for 4 cycles) (ddEC-PwX-regimen). Further randomization assigned patients to ibandronate for 2 years vs observation and to pegfilgrastim day 2 vs 4.

Ethical committee approval from all centers participating in the clinical study and from the Institutional Review Board of Charité University Hospital Berlin (Germany; Ethikvotum EA1/139/05) was obtained. All participants (if subjects were under 18, from a parent and/or legal guardian) had to sign a consent form. This study was conducted adhering to the REMARK (Reporting Recommendations for Tumor Marker

Prognostic Studies) criteria [20]. From June 2004 to August 2008, 2994 patients were randomized to either iddEPC (n = 1498) or ddEC-PwX (n = 1496) and started treatment.

Immunohistochemistry

The GAIN tissue microarray consists of formalin-fixed paraffin-embedded tissue samples including about 1380 BC tumor samples in 14 paraffin blocks. Freshly cut 4 µm tissue slides were used for immunohistochemistry (IHC). For the detection of TGIF, tissue slides were deparaffinized, and antigen retrieval was performed in citrate buffer solution (pH 6.1) in a steamer for 30 min. Tissue samples were then incubated overnight at 4 C with a rabbit TGIF antibody (Abcam plc, Cambridge, UK; dilution: 1:150) For detection, slides were incubated with biotinylated anti-rabbit secondary antibody and normal goat serum, then ABC Complex (Vectastain, Vector Laboratories) and DAB substrate kit (Vectastain, Vector Laboratories). All slides were counterstained with eosin/hematoxylin. As positive controls, paraffin sections of a BC sample which had been previously shown to stain positive for TGIF were treated in the same way. Omission of the primary antibody served as a negative control.

After TGIF immunohistochemistry, 183 (13.3%) tissue specimens were non-informative, due to the lack of tissue or absence of unequivocal cancer cells in the TMA spot. Thus, 1197 cases with evaluable TGIF expression were further analyzed (flow diagram Fig. 1). The staining results were evaluated independently in a blinded fashion by two individuals using the immunoreactive score (IRS) [21] which combines staining intensity and percentage of positive tumor cells resulting in a score of 0–12. For statistical analysis, the expression of TGIF was categorized into negative/low (IRS 0–2) and moderate/strong (IRS 3–12).

Statistical Methods And Analysis

Associations between TGIF expression as dichotomized variable with categorical clinical and histological parameters were assessed by Fisher’s exact (two classes) and Pearson Chi-square (three or more classes) test. Survival was analyzed by Kaplan-Meier product-limit method and compared between groups using the log-rank test. Median-follow-up time was estimated with the inverse Kaplan-Meier method. Cox proportional hazard model was performed to evaluate the potential prognostic value of TGIF for disease-free survival (DFS) and overall survival (OS). Hazard ratios (HR) with 95% confidence interval (CI) of the complete follow-up time were presented. The impact of TGIF on DFS or OS was assessed by univariate Cox regression in subgroups with regards to treatment arm (iddEPC vs. ddEC-PwX), ibadronate treatment (with vs. without), ER status (ER-pos. vs. ER-neg.), HER2 status (HER2-pos. vs. HER2-neg.) and TNBC vs. non-TNBC. The interaction between subgroups was assessed by bivariate Cox regression model. All reported p-values were two-sided, and $p < 0.05$ were considered statistically significant.

All time-to-event end points were defined as the time (in months) from random assignment to the event; patients without event were censored at the time of the last contact. Events for DFS were any loco-

regional (ipsilateral breast or local/regional lymph nodes) recurrence of disease, any contralateral breast cancer, any distant recurrence of disease, any secondary malignancy, or death as a result of any cause, whichever occurred first. OS was defined as the time since random assignment until death as a result of any cause. Event for time to primary bone metastasis (TTPBM) was any bone metastasis occurred as a first site of relapse; local recurrence, other distant metastases, contralateral BC, secondary malignancies or death were considered competing risks. TTPBM was analyzed using the Gray's competing risk model [22] and the hazard ratio of TTPBM was assessed using Fine-Gray's regression model [23].

All statistical analyses were performed using SPSS 22.0 (IBM SPSS Statistics 22) and SAS (version 9.4).

Results

Patient characteristics and clinicopathological data

After TGIF immunohistochemistry, 1197 cases with evaluable TGIF expression could be analyzed. Detailed patient characteristics and corresponding clinicopathological data are listed in Table 1. The median age was 49 years (range 23–71 years), the median follow-up was 74.1 months (range 0.0 to 113.7 months). 273 patients had a recurrence, and 159 patients died within the observation period. In 90 cases bone metastasis was the first site of relapse. Results of the GAIN study were published previously [24].

Table 1
Correlation of clinicopathological data of breast cancer patients with TGIF expression levels.

Parameter	Category	Overall		TGIF expression				p-value
				0–2		3–12		
		N	%	N	%	N	%	
All patients		1197		475	39.7%	722	60.3%	
Age (years)	< 40	185	15.5%	73	15.4%	112	15.5%	0.756
	40–50	489	40.9	196	41.3%	293	40.6%	
	51–65	496	41.4%	56	48.7%	440	40.7%	
	> 65	27	2.3%	8	1.7%	19	2.6%	
Tumor stage (pT)	pT1	394	33.0%	134	28.4%	260	36.1%	0.015
	pT2	665	55.7%	286	60.6%	379	52.6%	
	pT3/4	134	11.2%	52	11.0%	82	11.4%	
	total	1193		472		721		
	missing	4						
Lymph node status (pN)	pN1	493	41.2%	181	38.1%	312	43.2%	0.114
	pN2	398	33.2%	159	33.5%	239	33.1%	
	pN3	306	25.6%	135	28.4%	171	23.7%	
Tumor grading (G)	G1	39	3.3%	13	2.7%	26	3.6%	< 0.001
	G2	588	49.2%	196	41.4%	392	54.3%	
	G3	569	47.6%	265	55.9%	304	42.1%	
	missing	1						
Histological type	ductal	930	77.7%	389	81.9%	541	74.9%	0.009
	lobular	136	11.4%	39	8.2%	97	13.4%	
	others	131	10.9%	47	9.9%	84	11.6%	

Data are N (valid %) unless otherwise state;

mITT, modified intention-to treat; HER2, human epidermal growth factor receptor 2; ER, estrogen receptor; PR, progesterone receptor; iddEPC, intense dose-dense epirubicin plus paclitaxel plus cyclophosphamide; ddEC-PwX, dose-dense epirubicin plus cyclophosphamide plus paclitaxel weekly and capecitabine

Parameter	Category	Overall		TGIF expression				p-value
				0–2		3–12		
		N	%	N	%	N	%	
HER2	positive	248	22.0%	89	20.4%	159	23.0%	0.303
	negative	880	78.0%	348	79.6%	532	77.0%	
	missing	69						
ER	positive	886	74.0%	322	67.8%	564	78.1%	< 0.001
	negative	311	26.0%	153	32.2%	158	21.9%	
PR	positive	819	68.4%	285	60.0%	534	74.0%	< 0.001
	negative	378	31.6%	190	40.0%	188	26.0%	
Treatment in mITTset	iddEPC	597	49.9%	232	48.8%	365	50.6%	0.595
	ddEC-PwX	600	50.1%	243	51.2%	357	49.4%	
Ibandronate treatment	with Ibandronate	781	65.2%	307	64.6%	474	65.7%	0.756
	without Ibandronate	416	34.8%	168	35.4%	248	34.3%	

Data are N (valid %) unless otherwise state;

mITT, modified intention-to treat; HER2, human epidermal growth factor receptor 2; ER, estrogen receptor; PR, progesterone receptor; iddEPC, intense dose-dense epirubicin plus paclitaxel plus cyclophosphamide; ddEC-PwX, dose-dense epirubicin plus cyclophosphamide plus paclitaxel weekly and capecitabine

Tgif Expression In Tumor Tissue

TGIF immunoreactivity was located in the nucleus and in some cases also in the cytoplasm of normal luminal epithelial cells, if present within the slide. In 115 cases (9.6%), no TGIF staining was observed in tumor cells. Weak staining (IRS 1–2) was detected in 360 tumor tissues (30.1%), moderate staining (IRS 3–5) in 325 cases (27.2%) and strong staining (IRS 6–12) in 397 cases (33.2%). Representative images of these four groups are presented in Fig. 2. Analyzing TGIF expression as dichotomized variable demonstrated that 475 cases (39.7%) had TGIF-negative/low and 722 cases (60.3%) moderate/strong immunoreactivity.

We found that a moderate/strong TGIF protein expression compared to negative/low TGIF levels was significantly associated with smaller tumor size (36.1% vs 28.4%, respectively; $p = 0.015$) and a well differentiated phenotype (3.6% vs 2.7%; $p < 0.001$; Table 1). 564 cases (78.1%) with higher expression of TGIF were ER-positive, in contrast to 322 (67.8%) tumors with negative/low TGIF levels ($p < 0.001$). A similar association of TGIF expression and progesterone receptor (PR)-positive tumors was observed (74.0% vs 60.0%, respectively; $p < 0.001$). Furthermore, moderate/strong TGIF expression was less frequently detected in ductal carcinomas than negative/low TGIF expression (74.9% vs 81.9%, respectively; $p = 0.009$). No significant correlations with age, HER2 status or nodal involvement could be detected (Table 1).

Prognostic Impact Of Tgif Expression

Patients with moderate/strong TGIF expression showed a significantly longer DFS (HR 0.75 [95%CI 0.59–0.95]; log rank $p = 0.019$) and OS (HR 0.69 [95% CI 0.51–0.94]; log rank $p = 0.018$) compared to patients with negative/low TGIF expression. For TTPBM as first site of relapse no significant correlation with TGIF expression was found (HR 0.77 [95% CI 0.51–1.16]; $p = 0.213$) (Fig. 3 and Table 2). A subgroup analysis by treatment arm (iddEPC and ddEC-PwX) showed a significant improvement of DFS for patients with moderate/strong TGIF expression compared to those with negative/low TGIF staining in the iddEPC arm (HR 0.70 [95% CI 0.50–0.98]; log-rank $p = 0.036$), but not in the ddEC-PwX arm (HR 0.81 [95% CI 0.58–1.14]; log-rank $p = 0.228$; interaction p = 0.575). Regarding OS, the prognostic effect of TGIF expression was not significant in both treatment groups when analyzed separately (iddEPC: HR 0.69 [95% CI 0.45–1.07]; log-rank $p = 0.095$ and ddEC-PwX: HR 0.69 [95% CI 0.45–1.08]; log-rank $p = 0.105$) (Fig. 4, Table 2). Note that the TTPBM analysis in subgroups is not presented due to the small number of bone metastases occurred as first event.

Table 2
Analysis and prognostic value of TGIF expression overall and in patient subgroups.

Analysis	Parameter	DFS			OS		
		HR* (95%CI)	Log-rank p-value	Bivariate interaction p-value	HR* (95%CI)	Log-rank p-value	Bivariate interaction p-value
Overall							
	TGIF moderate/strong	0.75 (0.59–0.95)	0.019	-	0.69 (0.51–0.94)	0.018	-
Subgroups							
iddEPC	moderate/strong TGIF	0.70 (0.50–0.98)	0.036	0.575	0.69 (0.45–1.07)	0.095	0.978
ddEC-PwX	moderate/strong TGIF	0.81 (0.58–1.14)	0.228		0.69 (0.45–1.08)	0.105	
Ibandronate treatment	moderate/strong TGIF	0.79 (0.59–1.07)	0.131	0.620	0.72 (0.49–1.06)	0.094	0.729
without Ibandronate treatment	moderate/strong TGIF	0.68 (0.46–1.01)	0.057		0.64 (0.38–1.07)	0.087	
ER-negative	moderate/strong TGIF	1.03 (0.67–1.58)	0.893	0.130	1.02 (0.60–1.75)	0.933	0.107

*negative/low TGIF expression is reference

Prognostic value of TGIF for TTPBM, overall: HR = 0.77 (95%CI 0.51–1.16); p = 0.213. Of note, the TTPBM analysis in subgroups is not presented due to the small number of bone metastases occurred as first event.

HER2, human epidermal growth factor receptor 2; ER, estrogen receptor; PR, progesterone receptor; iddEPC, intense dose-dense epirubicin plus paclitaxel plus cyclophosphamide; ddEC-PwX, dose-dense epirubicin plus cyclophosphamide plus paclitaxel weekly and capecitabine; TNBC, triple-negative breast cancer; HR, hazard ratio; DFS, disease-free survival; OS, overall survival; CI, confidence interval; TTPBM, time to primary bone metastasis

Analysis	Parameter	DFS			OS		
		HR* (95%CI)	Log-rank p-value	Bivariate interaction p-value	HR* (95%CI)	Log-rank p-value	Bivariate interaction p-value
ER-positive	moderate/strong TGIF	0.68 (0.51–0.91)	0.009		0.60 (0.41–0.88)	0.008	
HER2-negative	moderate/strong TGIF	0.67 (0.50–0.88)	0.004	0.034	0.57 (0.40–0.82)	0.002	0.015
HER2-positive	moderate/strong TGIF	1.27 (0.75–2.18)	0.375		1.79 (0.80-4.00)	0.151	
TNBC	moderate/strong TGIF	1.00 (0.57–1.75)	0.995	0.427	1.05 (0.56–1.96)	0.876	0.291
non-TNBC	moderate/strong TGIF	0.76 (0.58-1.00)	0.049		0.70 (0.48–1.01)	0.055	
*negative/low TGIF expression is reference							
Prognostic value of TGIF for TTPBM, overall: HR = 0.77 (95%CI 0.51–1.16); p = 0.213. Of note, the TTPBM analysis in subgroups is not presented due to the small number of bone metastases occurred as first event.							
HER2, human epidermal growth factor receptor 2; ER, estrogen receptor; PR, progesterone receptor; iddEPC, intense dose-dense epirubicin plus paclitaxel plus cyclophosphamide; ddEC-PwX, dose-dense epirubicin plus cyclophosphamide plus paclitaxel weekly and capecitabine; TNBC, triple-negative breast cancer; HR, hazard ratio; DFS, disease-free survival; OS, overall survival; CI, confidence interval; TTPBM, time to primary bone metastasis							

Considering treatment with ibandronate, the prognostic value of moderate/strong TGIF expression did not reach statistical significance for DFS and OS but showed a tendency towards improved survival in the individual groups with or without ibandronate treatment: In patients with ibandronate the HR for DFS was 0.79 (95%CI 0.59–1.07; log-rank $p = 0.131$) and for OS 0.72 (95%CI 0.49–1.06; log-rank $p = 0.094$). Patients without ibandronate treatment showed a HR for DFS of 0.68 (95%CI 0.46 1.01; log-rank $p = 0.057$), as well as a HR for OS of 0.64 (95%CI 0.38–1.07; log rank $p = 0.087$) (Table 2).

With regards to ER status, the subgroup analysis showed that moderate/strong TGIF expression was significantly prognostic in patients with ER-positive tumors for both DFS (HR 0.68 [95%CI 0.51–0.91]; log-

rank $p = 0.009$; interaction p = 0.130) and OS (HR 0.60; 95%CI 0.41–0.88; log-rank $p = 0.008$; interaction p = 0.107; Fig. 5A-B), in contrast to patients with ER-negative tumors but the interaction between TGIF expression and ER status was not statistically significant (Table 2). Regarding the HER2 status, in the HER2-negative subgroup, elevated TGIF expression was significantly associated with better DFS (HR 0.67 [95%CI 0.50–0.88]; log-rank $p = 0.004$; interaction p = 0.034) and OS (HR 0.57 [95%CI 0.40–0.82]; log-rank $p = 0.002$; interaction p = 0.015; Fig. 5C-D) compared to HER2-positive subgroup with a significant interaction between TGIF expression and HER2 status (Table 2). Comparing triple-negative breast cancer (TNBC) patients with non-TNBC patients, we found that only in non-TNBC, moderate/strong TGIF expression was significantly associated with a longer DFS (HR 0.76 [95%CI 0.58-1.00]; log-rank $p = 0.049$; interaction p = 0.427) and we could observe a trend towards a better OS (HR 0.70 [95%CI 0.48–1.01]; log-rank $p = 0.055$; interaction p = 0.291) (Table 2 and Fig. 5E-F). However, no significant interaction was observed between TGIF expression and triple-negative status.

Discussion

Cancer related formation of metastasis is a highly complex process and bone is the most frequent site of metastatic relapse in BC. Bisphosphonates are established in the treatment of manifest bone metastases and evidence suggests also the ability of these compounds to prevent bone metastases. However, most studies examining the adjuvant use of bisphosphonates did not reach their endpoint despite a positive trend and a positive meta-analysis [25]. Therefore, our aim was to examine a potential marker for metastases formation that is also potentially prognostic for an adjuvant bisphosphonate effect. Due to its reported role in cancer, TGIF seemed a promising candidate in this respect.

The rationale for our analysis was the observation of TGIF as a novel Wnt target gene and a crucial regulator of osteoblast function: Absence of TGIF impairs osteoblast differentiation *in vitro* and osteoblast activity and bone formation *in vivo* [6] and might therefore influence bone metastasis in cancer patients.

Canonical Wnt signaling is among the most prominent pathways promoting osteoblast differentiation, function, and bone formation [5]. This is also true for TGF-ß signaling, as members of this super family also play a central role in bone metabolism [26]. TGIF, as a possible actuator, may be of particular importance in both signaling [11, 12]. Loss of TGIF expression might consequently lead to deregulation of these pathways promoting tumor progression and metastasis in BC. In this context, the role of TGIF in Wnt signaling might also connect estrogen and TGIF signaling in osteoblast [27] with possible effects on BC metastasis.

The GAIN study examined the use of adjuvant ibandronate [19] and is to our knowledge so far the only study on adjuvant bisphosphonate use with tumor tissue available. Here, we retrospectively investigated 1197 BC samples prospectively collected from this trial for TGIF immunostaining. Our results suggest that moderate to high TGIF expression is a common feature of breast cancer cells and that its loss of expression is linked to tumor progression.

A moderate/strong expression of TGIF correlated with favorable prognostic parameters like smaller tumor size, a well-differentiated phenotype and a HR-positive status. Moreover, we found a significant association between loss of TGIF expression and an adverse clinical outcome with shorter DFS and OS. Stratifying into molecular subgroups, we showed a significant favorable prognostic effect of TGIF expression on DFS in ER-positive and HER2-negative tumors and, consequently, in non-TNBC cases in univariate analysis with significant interaction only for HER2-negative BC subgroup. Regarding the treatment with ibandronate, the prognostic value of moderate/strong TGIF expression for both DFS and OS did not reach statistical significance in the individual groups with or without ibandronate treatment but showed a similar tendency towards improvement in both examined groups.

However, regarding bone metastases that occurred as first site of relapse, we were not able to demonstrate a prognostic influence of TGIF in the total cohort.

Currently, the most exciting potential of new biomarkers is the prediction of response to targeted therapy. In case of TGIF and its role as regulator in osteoblast function, it could be hypothesized that patients with elevated TGIF expression respond to treatment with bisphosphonate, such as ibandronate. However, we could not observe a significant predictive effect of TGIF here either (data not shown).

Until now, there are only few data regarding the role of TGIF in breast cancer cells in clinical studies. Recently, Zhang *et al* reported that in TNBC, elevated levels of TGIF correlate with high Wnt signaling and poor survival of BC patients, which is opposite to our results [28]. However, this study was conducted in only 173 BC patients which were subjected to various treatment regimens [28]. Interestingly, the BC subgroups (ER-positive, HER2-negative) which exhibit a strong favorable prognostic impact of TGIF expression are also reported to preferentially metastasize to the bone [29]. Yet, regarding time to bone metastasis, our results did not demonstrate an impact of TGIF expression on the development of skeletal metastases, but only on DFS and OS in non-TNBC patients.

One potential drawback of our study is the small number of cases with bone metastasis documented as first site of relapse. This could explain the lack of correlation between TGIF and bone metastases in general or with the use of adjuvant bisphosphonates. However, data from von Minckwitz et al. concluded that, two years of adjuvant treatment with ibandronate after dose-dense chemotherapy in the GAIN trial had acceptable adverse effects but did not improve survival in patients with high-risk breast cancer. Post hoc subgroup analyses support the hypothesis that adjuvant bisphosphonate activity is restricted to patients with low estrogen levels, either because of medical ovarian suppression or definite menopause. Future meta-analyses on an individual patient data level may reliably reveal subgroups in which this approach has the best efficacy [19]. Patient follow-up in the GAIN trial was based on clinical routine which does not recommend routine bone scans or other radiology assessments. This approach is in line with clinical guidelines and also common practice in most adjuvant BC trials. Therefore, the endpoint of bone disease as first site of metastatic spread might be difficult to assess in current clinical trials since patients could also develop other metastases before they show symptoms of their disease. However, a major strength of this work is the large cohort of this study with over 2994 patients, of whom 1380

samples were used for the TMA and 1197 cases was evaluable for TGIF expression. The uniform treatment of the patients with dose-dense chemotherapy which is still considered as standard in the adjuvant setting, and our findings support a biologic role of TGIF in BC patients treated in this context.

Previous studies suggest that TGIF might be associated with the initiation and progression of lung [15, 16], gastric [13] or colorectal cancer [14], which is opposite to our present results on breast cancer. Yet, those studies were largely performed by *in vitro* experiments, and only small numbers of human tumor tissue samples were analyzed, mostly without follow-up data. Our data generated on a high number of well-characterized breast cancer patients clearly show that, although TGIF is expressed in most tumor samples, loss of this protein is significantly associated with a less differentiated phenotype and poor outcome. This suggests differences in TGIF function in various tumor entities. Among breast cancer patients, differences were also found according to molecular subtypes: while negative/low TGIF expression correlates with shorter DFS and OS in ER-positive BC, it lacks any prognostic significance in TNBC, suggesting an interference of TGIF and ER signaling, which was already described in osteoblasts [27]. Furthermore, a significant interaction was observed only between high TGIF expression and HER2-negative BC. The nature of this interaction in BC cells should be further analyzed in experimental systems to elucidate the exact role and underlying mechanism of TGIF.

Conclusions

In this study we have demonstrated that loss of TGIF expression is significantly associated with BC progression, especially in luminal carcinomas. However, comprehensive analysis of *in vivo* models is necessary, particularly to the mechanistic regulation of TGIF.

List Of Abbreviations

BC Breast cancer

DFS Disease-free survival

HR Hormone receptor

OS Overall survival

TGIF TGFB-induced factor homeobox 1

TNBC Triple-negative breast cancer

TTBM Time to bone metastasis

SRE skeletal-related events

Declarations

Consent for publication

Not applicable

Ethics approval and consent to participate

Ethical committee approval from all centers participating in the clinical study and from the Institutional Review Board of Charité University Hospital Berlin (Germany; Ethikvotum EA1/139/05) was obtained. Written consent was obtained from each participant or their proxy. This study was conducted adhering to the REMARK (Reporting Recommendations for Tumor Marker Prognostic Studies) criteria [20].

Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Funding

Not applicable

Conflict of interest

V.M: Dr. Möbus received speaker honoraria from Amgen, Astra Zeneca, Celgene, Roche, Teva, and consultancy honoraria from Roche, Amgen, Tesaro and Myelo Therapeutics GmbH.

V.M: Dr. Müller received speaker honoraria from Amgen, Astra Zeneca, Celgene, Daiichi-Sankyo, Eisai, Pfizer, Novartis, Roche, Teva, and consultancy honoraria from Genomic Health, Hexal, Roche, Pierre Fabre, Amgen, Novartis, MSD, Daiichi-Sankyo and Eisai, Lilly, Tesaro and Nektar. Research support from Novartis, Roche, Seattle Genetics, Genentech.

PAF: Dr. Fasching reports grants from Novartis, grants from Biontech, personal fees from Novartis, personal fees from Roche, personal fees from Pfizer, personal fees from Celgene, personal fees from Daiichi-Sankyo, personal fees from Astra Zeneca, personal fees from Macrogenics, personal fees from Eisai, personal fees from Merck Sharp & Dohme, grants from Cepheid, personal fees from Lilly, during the conduct of the study.

ES: Dr. Stickeler reports personal fees from Roche Pharma, personal fees from Novartis, personal fees from Pfizer, personal fees from Tesaro, personal fees from Astra Zeneca, outside the submitted work.

CD: Dr. Denkert received honoraria from Novartis and Roche; Research funding from Myriad Genetics, reports consulting or advisory role for MSD Oncology and Daiichi Sankyo and stock and other ownership interest with Sividon Diagnostics (now Myriad and travel expenses from Roche. In addition, Dr. Denkert has patents, royalities and intellectual property with VMscopedigital pathology software: Patent

application: EP18209672 - cancer immunotherapy; Patent application EP20150702464 - therapy response; Patent application EP20150702464 - therapy response.

LH: Dr. Hanker received speaker honoraria from Astra Zeneca, Clovis, GSK/Tesaro Novartis, Roche, and consultancy honoraria from Astra Zeneca, Clovis, GSK/Tesaro and Roche.

FM: Dr. Marmé reports personal fees from Roche, personal fees from AstraZeneca, personal fees from Pfizer, personal fees from Tesaro, personal fees from Novartis, personal fees from Amgen, personal fees from PharmaMar, personal fees from GenomicHealth, personal fees from CureVac, personal fees from EISAI, outside the submitted work.

SL: Dr. Loibl reports grants and other from Abbvie , grants and other from Amgen, grants and other from Astra Zeneca, grants and other from Celgene, grants and other from Novartis, grants and other from Pfizer, grants and other from Roche, other from Seattle Genetics, other from PriME/ Medscape, personal fees from Chugai, grants from Teva, grants from Vifor, grants and other from Daiichi-Sankyo, other from Lilly, other from Samsung, other from Eirgenix, other from BMS, other from Puma, other from MSD, grants from Immunomedics, outside the submitted work; In addition, Dr. Loibl has a patent EP14153692.0 pending.

The other authors (CS; KML; SS; JR; RPH; TK; CS; CHK; LH; US; VV; VN) declare that they have no competing interests.

Author contributions

All authors (CS, VM, KML, SS, PF, JR, ES, RPH, CD, LH, CS, VV, TK, VN, CHK, FM, US, SL, VM) contributed to the study conception and design. VM,SS,PF,JR,ES,RPH,CD,LH,CS,VV,TK,VN,CHK,FM,SL,VM provide patient data for the trial described in the article. Material preparation, data collection and analysis were performed by Karin Milde-Langosch (KML), Christine Stürken (CS), Valentina Vladimirova (VV) and Volkmar Müller (VM). The first draft of the manuscript was written by Christine Stürken (CS) and Karin Milde-Langosch (KML) and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Figures

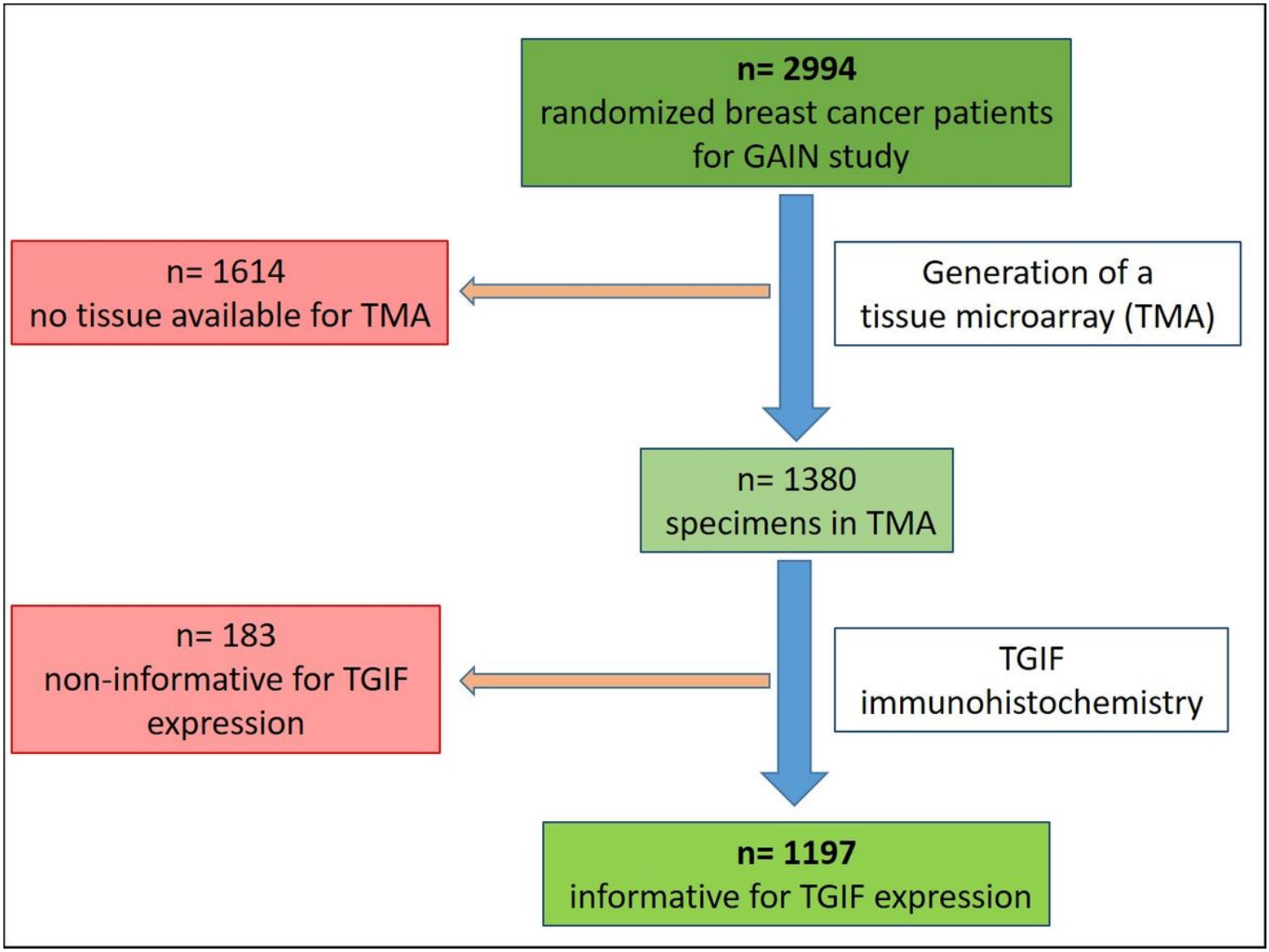

Figure 1

Flow diagram of the technical proceeding.

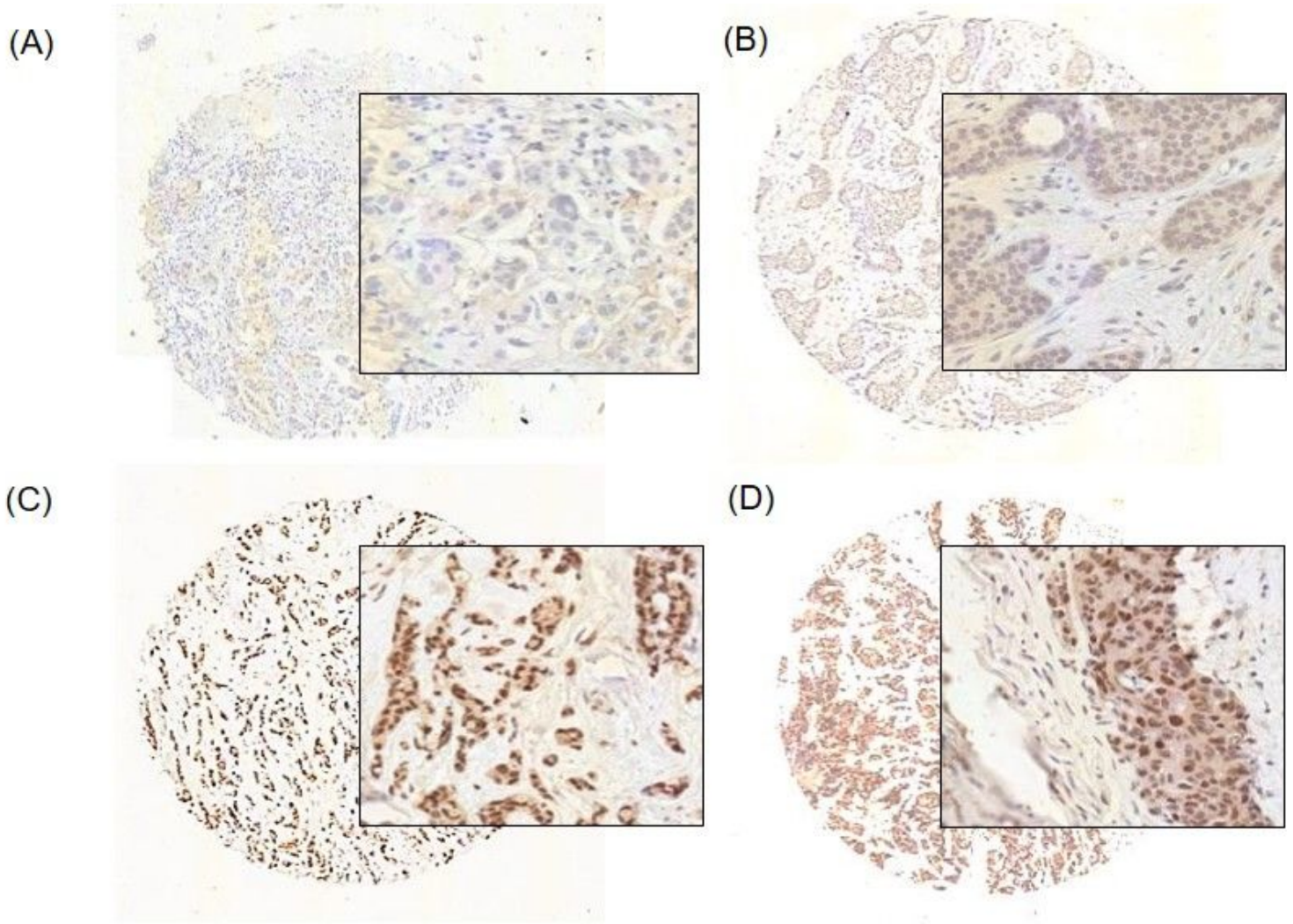

Figure 2

Representative pictures of TGIF immunostaining; (A) negative; (B) low; (C) moderate and (D) strong TGIF expression in breast cancer samples. Scale bar 200 μm.

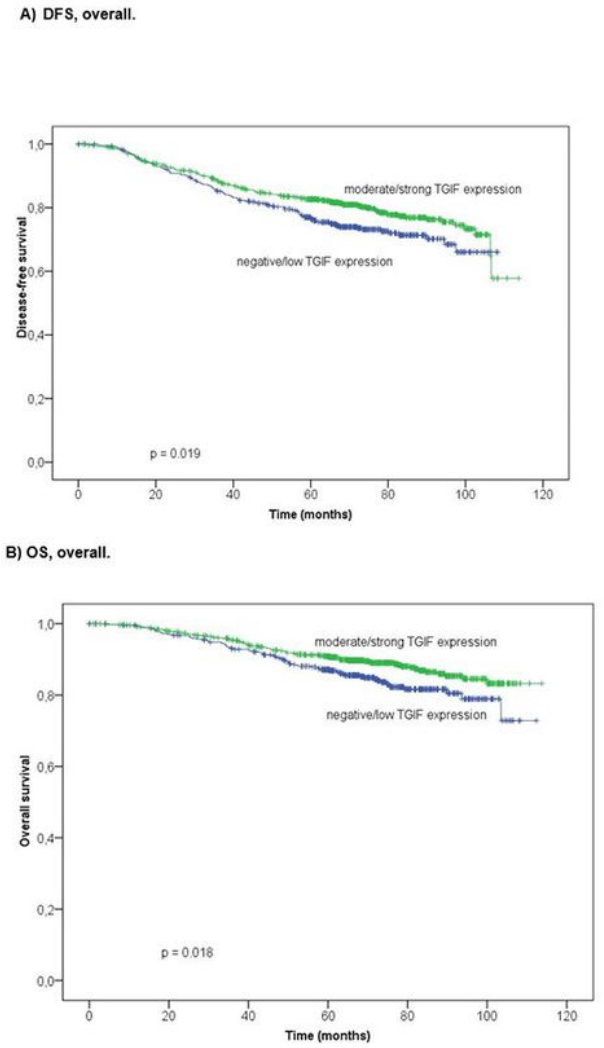

Figure 3

Kaplan-Meier analysis for DFS (A) (HR 0.75 [95%CI 0.59-0.95]; log rank p=0.019), OS (B) HR 0.69 [95%CI 0.51-0.94]; log rank p=0.018) and TTPBM (C) (HR 0.77 [95%CI 0.51-1.16]; p=0.213) according to the overall TGIF immunostaining (moderate/strong vs negative/low). DFS, disease-free survival, OS, overall survival; TTPBM, time to primary bone metastasis

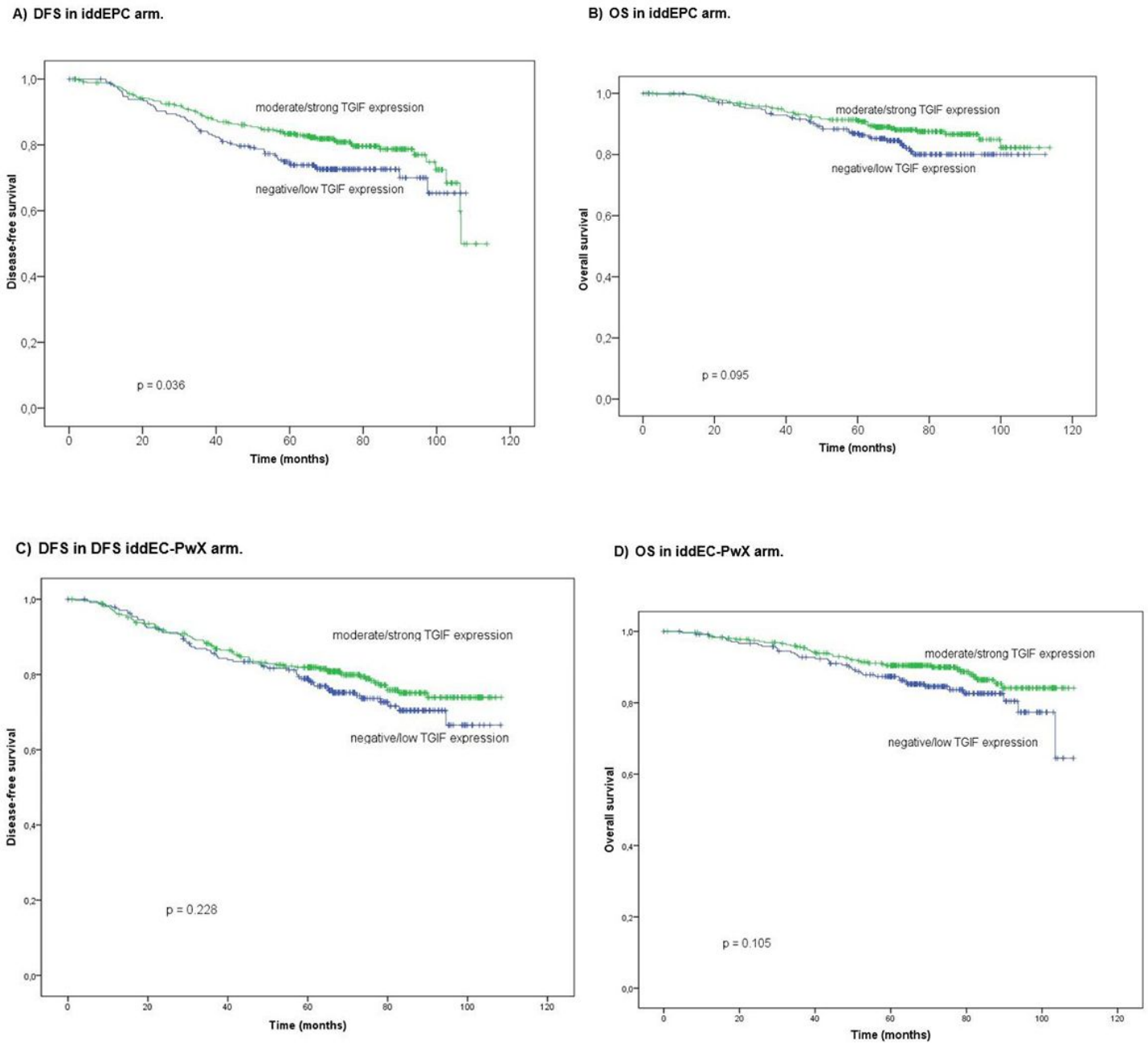

Figure 4

Subgroup analysis for DFS and OS according to the TGIF immunostaining (moderate/strong vs negative/low) with regards to treatment arm (iddEPC and ddEC-PwX): A) DFS iddEPC treatment (HR 0.70 [95% CI 0.50-0.98]; log-rank p=0.036) B) OS iddEPC treatment (HR 0.69 [95% CI 0.45-1.07]; log-rank p=0.095) C) DFS iddEC-PwX treatment (HR 0.81 [95% CI 0.58-1.14]; log-rank p=0.228) D) OS iddEC-PwX treatment HR 0.69 [95% CI 0.45-1.08]; log-rank p=0.105.

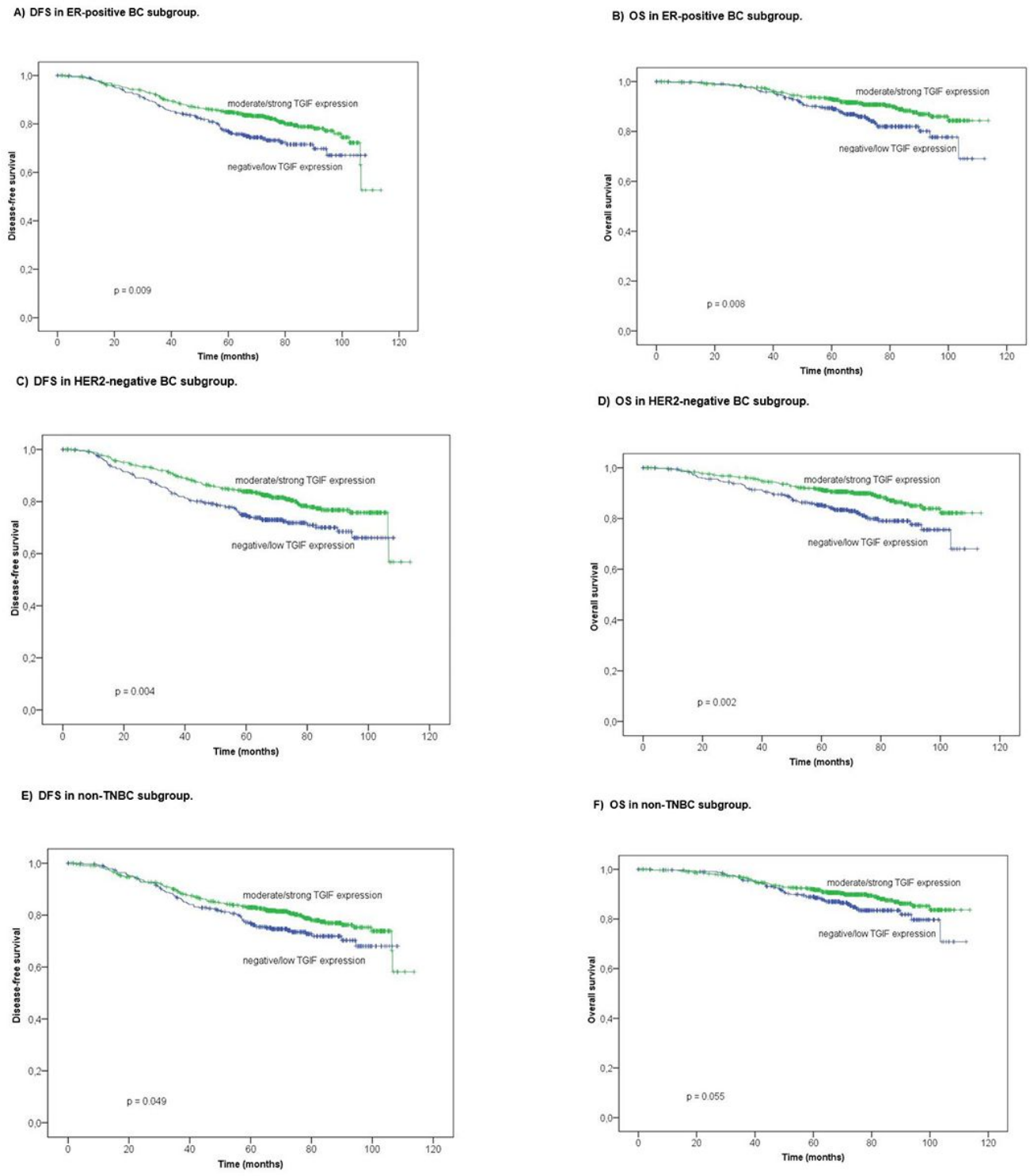

Figure 5

Stratified Kaplan-Meier analysis for DFS and OS by estrogen receptor (ER) status, by HER2 status, and by triple-negative status: A) DFS in ER-positive BC subgroup (HR 0.68; 95%CI 0.51-0.91; log-rank p=0.009) B) OS in ER-positive BC subgroup (HR 0.60; 95%CI 0.41-0.88; log-rank p=0.008); C) DFS in HER2-negative BC subgroup (HR 0.67; 95%CI 0.50-0.88; log-rank p=0.004) D) OS in HER2-negative BC subgroup (HR 0.57; 95%CI 0.40-0.82; log-rank p=0.002) E) DFS in non-TNBC subgroup (HR 0.76; 95%CI 0.58-1.00; log-rank p=0.049) F) OS in non-TNBC subgroup (HR 0.70; 95%CI 0.48-1.01; log-rank p=0.055).