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Epidermal growth factor receptor and vascular endothelial growth factor receptor 2 are specific biomarkers in triple-negative breast cancer. Results from a controlled randomized trial with long-term follow-up

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Abstract Triple-negative breast cancer (TNB) has poor prognosis and moreover patients with TNB do not benefit from established targeted drugs with endocrine therapy or trastuzumab. The aim of the study was to analyze the prevalence of candidate biomarkers in tumors from patients with TNB. Tissue microarrays were prepared from primary tumors from premenopausal breast cancer patients (500/564) randomized to adjuvant tamoxifen or no adjuvant treatment. Immunohistochemical (IHC) staining included ER, PR, HER2, epidermal receptor growth factor (EGFR), vascular endothelial growth factor A (VEGF-A), and vascular endothelial growth factor receptor 2 (VEGFR2). EGFR and HER2 gene copy number was defined by fluorescence in situ hybridization (FISH). All patients were included in the descriptive analysis, but only untreated

patients in the survival analysis. TNB was diagnosed in 96 patients and correlated significantly to low age, Nottingham histological grade (NHG) III, high Ki67-index, T2 tumors, node negativity, EGFR positivity, increased EGFR gene copy number and high VEGFR2 expression. TNB was an independent prognostic factor for decreased 5-year breast cancer specific survival (BCSS) (HR 2.0 (95% CI 1.1–3.6), $P = 0.01$), but not for 10-year BCSS. High VEGFR2 expression was significantly correlated to decreased BCSS in TNB patients. TNB was associated with decreased BCSS and clinicopathological characteristics of an aggressive tumor type. High VEGFR2 expression, EGFR expression, and EGFR gene copy number were significantly correlated to TNB, supporting their role as putative candidate biomarkers for selection of targeted therapy in TNB.

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Keywords Triple-negative breast cancer ·
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Vascular endothelial growth factor receptor 2 · Prognosis

Introduction

Breast cancer is a heterogeneous disease and the classification into distinctive subgroups as proposed by Sorlie et al. [1] (luminal A, luminal B, normal-like, human epidermal growth factor 2 (HER2) expressing, and basal-like tumors) gives important clinical information about prognosis and potential therapeutic biomarkers. Basal-like breast cancer can be diagnosed by gene expression profiles, whereas no validated immunohistochemical test is available for the diagnosis [2, 3]. Since basal-like breast cancer is mainly composed of triple-negative breast cancer (TNB) defined by lack of estrogen (ER) and progesterone (PR)

immunoreactivity and lack of HER2 overexpression, the term TNB is often used in clinical practice [4, 5].

TNB comprises around 15% of all breast cancer and is characterized by its aggressive clinical behavior and insensitivity toward available targeted treatment strategies with endocrine and anti-HER2 therapies [6, 7]. Although TNB is sensitive to chemotherapy, early relapse with metastatic disease is common and the prognosis is poor [8]. Development of novel treatment strategies is, therefore, needed and the study of other putative targets in TNB, like angiogenic markers and tyrosine kinase receptors, is a topic of interest to find biomarkers, which can serve as therapeutic targets in this subset of breast cancer [9, 10].

Specific clinical characteristics have been linked to TNB such as young age at diagnosis, a higher incidence in African-American women compared to non African-American as well as histopathological features like medullary and ductal carcinomas, node negativity, and high histological grade [11, 12]. Breast cancer in younger women with BRCA1 mutations almost exclusively exhibit the TNB phenotype. Much effort has been put into further tumor biological characterization of TNB showing an increased rate of *TP53*-mutations, increased expression of epidermal growth factor (EGFR), cyclin E, c-KIT, and basal cytokeratins (5/14/17) [2, 5].

Angiogenic markers, like vascular endothelial growth factor A (VEGF-A) and its receptors, are of special interest in TNB since they also represent putative therapeutic targets for novel anti-angiogenic therapies [10]. Immunotherapy with bevacizumab directed toward VEGF-A seem to be a promising treatment strategy, although data up until now are restricted to retrospective subset analysis of metastatic breast cancer and results from trials of neoadjuvant treatment [13, 14]. Inhibition of receptor tyrosine kinases as vascular endothelial growth factor receptor 2 (VEGFR2) are currently being tested in clinical trials for metastatic TNB and can be a therapeutic option in TNB patients [15] as well as blocking of (epidermal growth factor receptor) EGFR by cetuximab [16]. Selection of TNB patients before inclusion into a study protocol according to potential predictive tumor markers has so far not been performed.

The aim of the present study was to study candidate biomarkers of possible relevance to select targeted therapy in TNB using a defined cohort of premenopausal patients included in a randomized clinical trial with a systemically untreated arm enabling excellent opportunities to explore the prevalence of the biomarkers and their association with standard prognostic factors. The biomarkers chosen were EGFR protein expression and gene copy number, as well as VEGF-A and VEGFR2 as they are today targets for available systemic therapies. The TNB group of patients had a worse prognosis and was characterized by increased expression of EGFR protein, increased number of EGFR

gene copy number, and VEGFR2 protein expression. Whereas EGFR protein expression and gene copy number were not associated with prognosis, high expression of VEGFR2 identified TNB with decreased breast cancer specific survival (BCSS).

Clinical trial

Premenopausal patients with stage II primary breast cancer ($n = 564$) were enrolled between January 1984 and September 1991 in a randomized multicenter trial of 2 years of adjuvant tamoxifen versus no adjuvant systemic treatment. Patients were included irrespective of hormone receptor status. The trial design and clinical outcome in relation to treatment arm have been described in detail before and information on clinical patient and tumor-related factors were already available [17]. These factors include information on age, tumor size, lymph node status, Nottingham Histological Grade (NHG), Ki67 labelling index, ER and PR status, HER2 status defined by immunohistochemistry (IHC), and fluorescence in situ hybridization (FISH) [18]. The clinicopathological characteristics were balanced between the arms and less than 2% of the patients received additional adjuvant therapy [17]. The flow-chart of the present study is given in Fig. 1.

The median follow-up time was 13.9 years for patients without any event. The study was approved by the Ethics Committees at Lund and Linköping Universities.

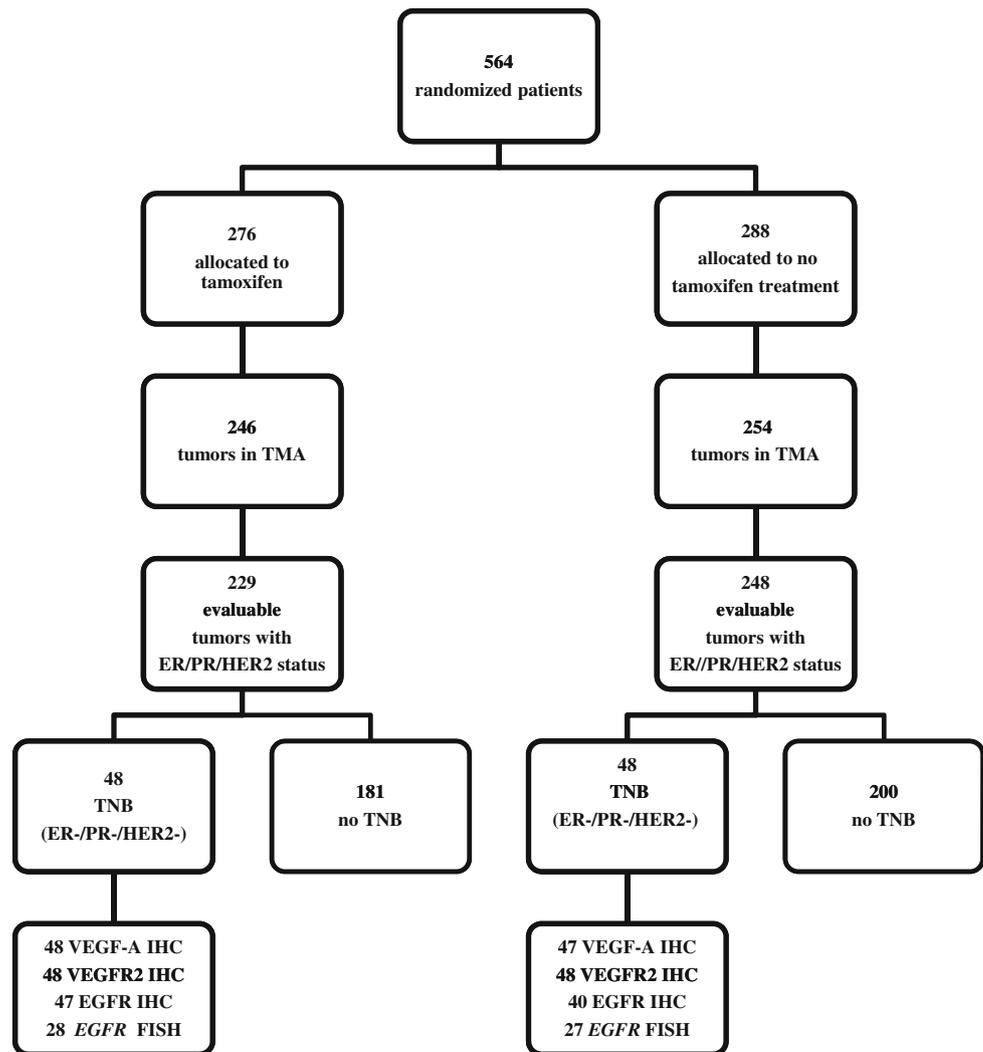
Tumor tissue microarray

Paraffin-embedded blocks from the primary tumors were retrieved in 500 of 564 patients and a tumor tissue microarray (TMA) was constructed. Two biopsies, 0.6 mm in diameter, were obtained from each donor block, corresponding to a previously marked area on a slide of invasive tumor, and mounted in a recipient block using a tissue array machine according to the manufacturer's instructions (Beecher Instruments, MD, USA). Staining with hematoxylin and eosin was carried out for morphological overview and evaluation of the quality of the biopsies.

Immunohistochemical analysis of VEGF-A, VEGFR2, and EGFR

Four micrometer section from the TMA were mounted onto capillary microscope slides (DAKO, Glostrup, Denmark). The sections were dried, deparaffinized, rehydrated, and microwave treated for antigen retrieval as previously described [13]. The immunostaining was performed in an

Fig. 1 Flow-chart of the study. *TMA* tissue microarray, *TNB* triple-negative breast cancer, *VEGF-A* vascular endothelial growth factor A, *IHC* immunohistochemistry, *VEGFR2* vascular endothelial growth factor receptor 2, *EGFR* epidermal growth factor receptor, *EGFR* EGFR gene copy number, *FISH* fluorescence in situ hybridization



automatic immunohistochemistry staining machine (TechMate™ 500 Plus, DAKO, Glostrup, Denmark) according to standard procedure using a polyclonal VEGF antibody (A-20) diluted 1:400 (Santa Cruz, Ca, USA) for detection of VEGF-A and a monoclonal KDR/VEGFR2 antibody (A-3) diluted 1:1,000 (Santa Cruz, Ca, USA). For detection of EGFR, the Dako EGFR Pharm DX kit (DAKO, Glostrup, Denmark) was used according to the manufacturer's instruction. The immunoreactivity was evaluated by two investigators independently (LR, KJ) and divergent results were re-examined followed by a conclusive decision. For VEGF-A and VEGFR2, only invasive tumor cells were considered and the cytoplasmic staining intensity was scored using a semi-quantitative scale (0–3) as previously described [13]. In statistical analysis, strong immunoreactivity (3) was compared to weak or absent staining (0–2). EGFR was scored according to a validated protocol used for colorectal cancer according to the manufacturer's instruction considering membranous staining in invasive

tumor cells [19]. Any staining above background in >1% of the cells were considered positive for EGFR immunoreactivity and in statistical analysis positive immunoreactivity was compared to no immunoreactivity according to the written protocol.

EGFR gene copy number by fluorescence in situ hybridization

A standard protocol for evaluating EGFR gene copy number was followed using the dual color Spectrum Orange/LSI EGFR/Spectrum Green CEP 7 probe set (Vysis/ABBOTT, IL, USA) [20]. The TMA slides were deparaffinized in xylene and ethanol, incubated with a pretreatment reagent, art 2J06-30 (Vysis/ABBOTT, IL, USA), at 80°C for 10 min, washed and digested with protease, art 6J93-01 (Vysis/ABBOTT, IL, USA), at 37°C for 40 min. The slides were then washed and dehydrated in ethanol. The probes

were applied to the slides with TMA biopsies, which were covered with glass coverslips and sealed with glue. DNA denaturation was performed at 85°C for 1 min and the slides were then incubated at 37°C for 20 h using Hybridizer ThermoBrite™ (Abbott Molecular). Post hybridization washes were performed with 2XSSC/0.3% NP-40 at +72°C for 2 min. The slides were thereafter washed and chromatin counterstaining with DAPI (0.3 µg ml⁻¹ in Vectashield, Vector Laboratories) was performed. Analysis was performed using an epifluorescence microscope (Nikon Eclipse E80i) using single interference filter sets for green (FITC) and red (Texas red). For each core, only non-overlapping, intact nuclei were evaluated and at least 20 intact nuclei were evaluated in each sample. FISH positivity, was defined as either amplification EGFR/CEP7 ratio $U \geq U 2.0$ or high polysomy with $U \geq U 4.0$ copies per cell as previously described [21].

Statistical methods

The statistical calculations were performed using SPSS Version 16.0 (SPSS, Chicago, IL). Breast cancer specific survival (BCSS) was chosen as endpoint in the present study and all analysis was done according to the intention to treat rule. Kaplan–Meier estimate was used to illustrate the survival in specified cohorts and the log-rank test to test for equality of survival curves. Due to long-term follow-up, 5- and 10-year BCSS was used, i.e., patients experiencing breast cancer-related death after 5 and 10 year, respectively, were censored. Hazard ratios were estimated using Cox regression for 5- and 10-year BCSS in uni- and multivariate analysis. Comparisons of tumor characteristics were made by χ^2 -test for categorized variables and by χ^2 -test for trends for variables with more than two categories. All *P* values corresponded to two-sided tests and values less than 0.05 were considered significant.

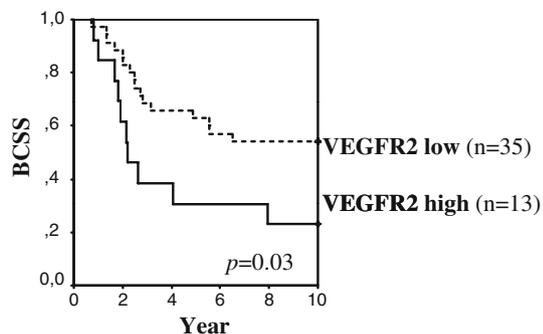


Fig. 2 Kaplan–Meier estimate of 10-year Breast cancer specific survival (BCSS) for patients with triple-negative tumors allocated to no systemic adjuvant therapy according to VEGFR2 expression. The *P* value was calculated using the log-rank test

Results

The triple-negative phenotype and relation to patient and tumor characteristics

Tumors were classified as TNB when they were ER- and PR-negative as well as HER2-negative. HER2 status was defined by the combination of IHC and FISH (HER2-positive when either 3+ and/or HER2 amplified) according to the Swedish guidelines for HER2-status. 477 of 500 biopsies could be evaluated according to the above criteria and 96 (20%) of evaluable tumors were classified as having a TNB phenotype. The distribution according to the trial arm is given in Fig. 1.

For descriptive analysis, all evaluable tumors were included and TNB was significantly associated with age below median (*P* = 0.002), T2 tumors (*P* = 0.002) and lymph node negativity (*P* = 0.001), NHG 3 (*P* < 0.001), high Ki67 labelling index (*P* < 0.001), and medullary histological classification (*P* < 0.001) (Table 1).

Cytoplasmic VEGF-A expression was not correlated to the TNB phenotype, whereas VEGFR2 expression was more commonly encountered in TNB than in non-TNB (*P* = 0.03) (22% highly expressing tumors in TNB vs. 13% in non-TNB) (Table 1). Furthermore, EGFR protein expression was more frequently diagnosed in TNB than in non-TNB (*P* < 0.001), (41% positive cases in TNB vs. 11% positive cases in non-TNB). High EGFR gene copy number was found in 18% of the patients with TNB cancers compared to 10% of patients without TNB (*P* = 0.003). In line with reports from others, EGFR protein expression and EGFR gene copy number were not correlated (*P* = 0.6). VEGFR2 protein expression correlated to VEGF-A protein expression (*P* < 0.001), but not to EGFR protein expression or EGFR gene copy number (*P* = 0.2 and *P* = 0.7, respectively).

TNB and clinical outcome in systemically untreated patients

For survival analysis according to the TNB phenotype, we selected patients allocated to no systemic treatment and TNB status was evaluable in 248 of 254 tumors included in the TMA (Fig. 1). Patients with TNB phenotype had a significantly decreased 5-year BCSS (HR 3.4 (95% CI 2.0–5.9), *P* < 0.001) by univariate analysis, Table 2. When adjusting for other clinicopathological variables being significant by univariate analysis (node status and NHG), TNB was still a significant indicator of bad prognosis (HR 2.0 (95% CI 1.1–3.6), *P* = 0.01), Table 2. Ten-year BCSS was significantly decreased for patients with TNB by univariate analysis HR 2.1 (95% CI 1.4–3.3) *P* = < 0.01, but when adjusting for node status and NHG, TNB was not an

Table 1 Clinicopathological and biomarker characteristics in relation to triple-negative breast cancer

Characteristics	TNB N (%)	No TNB N (%)	<i>P</i> value
Age			
<45 years	61 (64)	176 (46)	0.002
≥45 years	35 (36)	205 (54)	
Tumor size			
T1	22 (23)	151 (40)	0.002
T2	74 (77)	229 (60)	
Nodal status			
N0	40 (42)	94 (25)	0.001
N+	56 (58)	285 (75)	
NHG			
NHG 1–2	8 (9)	245 (67)	<0.001
NHG 3	83 (91)	123 (33)	
EGFR IHC			
EGFR neg	51 (59)	265 (89)	<0.001
EGFR pos	36 (41)	34 (11)	
EGFR			
EGFR FISH–	45 (82)	208 (94)	0.003
EGFR FISH+	10 (18)	13 (6)	
VEGF-A IHC			
VEGF-A low	46 (48)	145 (41)	ns
VEGF-A high	49 (52)	209 (59)	
VEGFR2 IHC			
VEGFR2 low	75 (78)	317 (87)	0.03
VEGFR2 high	21 (22)	48 (13)	
Ki67			
Ki67 0–10%	16 (19)	156 (52)	<0.001
Ki67 11–25%	12 (14)	81 (27)	
Ki67 > 25%	58 (67)	60 (20)	
Histopathological tumor type			<0.001
Ductal CA	73 (77)	323 (85)	
Lobular CA	2 (2)	37 (10)	
Medullary CA	19 (20)	5 (1)	
Others	1 (1)	9 (4)	

NHG Nottingham histological grade, EGFR epidermal growth factor receptor, IHC immunohistochemistry, VEGFR2 vascular endothelial growth factor receptor 2, VEGF-A vascular endothelial growth factor A, EGFR EGFR gene copy number, FISH fluorescence in situ hybridization, TNB triple-negative breast cancer, *P* value χ^2 -test for categorised variables and χ^2 -test for trends for categories with more than two variables

independent prognostic factor (HR 1.5 (95% CI 0.9–2.6) *P* = 0.1), Table 2.

In addition, we used the whole cohort including 477 patients with TNB status for survival analysis and TNB was still a significant prognostic factor for decreased 5-year BCSS by univariate analysis (HR 2.8 (95% CI 1.9–4.1), *P* < 0.001). In multivariate analysis adjusting for tamoxifen treatment, node status and NHG it remained an

Table 2 5- and 10-year breast cancer specific survival by Cox uni- and multivariate analysis in patients not allocated to systemic adjuvant treatment (*n* = 248)

Variable	Univariate analysis			Multivariate analysis		
	HR	95%CI	<i>P</i> value	HR	95%CI	<i>P</i> value
5-year breast cancer specific survival						
TNB						
Yes vs. no	3.4	2.0–5.9	<0.001	2.0	1.1–3.6	0.03
Age						
<45 vs. ≥45 years	1.4	0.9–2.3	0.2			
Tumor size						
T2 vs. T1	1.6	1.0–2.7	0.06			
Node status						
Pos vs. neg	2.5	1.2–5.1	0.01	5.2	2.3–12.3	<0.001
NHG						
3 vs. 1–2	5.7	3.1–10.3	<0.001	4.9	2.4–10.2	<0.001
10-year breast cancer specific survival						
TNB						
Yes vs. no	2.1	1.4–3.3	<0.01	1.5	0.9–2.6	0.1
Age						
<45 vs. ≥45 years	1.3	0.9–1.9	0.1			
Tumor size						
T2 vs. T1	1.4	0.9–2.1	0.08			
Node status						
Pos vs. neg	2.1	1.3–3.5	<0.01	2.9	1.7–5.1	<0.001
NHG						
3 vs. 1–2	2.7	1.8–4.1	<0.001	2.9	1.8–4.7	<0.001

TNB triple-negative breast cancer, NHG Nottingham histological grade, HR hazard ratio, CI confidence interval

Uni- and multivariate analysis were performed using Cox regression and factors being significant in the univariate analyses were included in the multivariate analysis

independent prognostic factor (HR 1.8 (95% CI 1.2–2.8), *P* < 0.01).

Clinical outcome in relation to biomarkers

When analyzing the clinical outcome in patients with TNB not allocated to adjuvant treatment (*n* = 48), we used 5-year BCSS as end-point as TNB was an independent prognostic factor only at 5-year follow-up and not at 10-year follow-up. Five-year BCSS was further explored in the TNB cohort by stratifying for clinicopathological variables as well as the analyzed biomarkers by uni- and multivariate analysis, Table 3. High VEGFR2 expression was significantly correlated to decreased BCSS in TNB at 5-year BCSS (HR 2.6 (95% CI 1.1–6.3) *P* = 0.03) as was node positivity, whereas no other of the analyzed variables influenced outcome, Table 3. When adjusting for node status in a multivariate analysis, VEGFR2 expression was

Table 3 5-year breast cancer specific survival in patients with triple-negative breast cancer allocated to no systemic adjuvant treatment ($n = 48$)

Variable	Univariate analysis			Multivariate analysis		
	HR	95%CI	P-value	HR	95%CI	P value
5-year breast cancer specific survival						
Age (years)						
<45 vs. \geq 45	0.7	0.5–2.7	0.7			
Tumor size						
T2 vs. T1	0.8	0.3–1.9	0.6			
Node status						
Pos vs. neg	9.5	2.2–40.2	0.002	8.5	2.0–37.0	0.004
NHG						
3 vs. 1–2	0.8	0.1–5.6	0.8			
EGFR IHC						
Pos vs. neg	0.6	0.2–1.8	0.4			
EGFR FISH						
Pos vs. neg	0.8	0.2–3.6	0.8			
VEGF-A IHC						
High vs. low	2.3	0.9–6.0	0.08			
VEGFR2 IHC						
High vs. low	2.6	1.1–6.3	0.03	1.9	0.8–4.5	0.1

TNB triple-negative breast cancer, NHG Nottingham histological grade, HR hazard ratio, CI confidence interval

Uni- and multivariate analysis were performed using Cox regression and factors being significant in the univariate analyses were included in the multivariate analysis

not correlated to 5-year BCSS as given in Table 3. At 10-year follow-up, BCSS was decreased in patients with high VEGFR2 expression as illustrated in Fig. 2 by Kaplan–Meier estimate (HR 2.6 (95% CI 1.1–5.5), $P = 0.03$). When including all 96 patients with TNB from both study arms in the survival analysis, VEGFR2 was still a negative prognostic factor for 5-year BCSS (HR 2.0 (95% CI 1.1–3.8) $P = 0.02$) but not for 10-year BCSS (HR 1.8 (95% CI 0.9–3.5) $P = 0.09$).

In patients with ER+ tumors, expression of VEGF-A, VEGFRs, and EGFR did not influence outcome, whereas increased *EGFR* gene copy number was a significant prognostic factor at 5-year BCSS (HR 4.8 (95% CI 1.9–11.6) $P < 0.001$).

Discussion

The present study of premenopausal breast cancer patients included in a controlled randomized trial confirms the negative prognostic information yielded by the TNB phenotype. We were able to evaluate TNB status in 477 patients and basic clinicopathological characteristics in the

TNB subgroup compared to non-TNB cases showed results in line with other publications making the included cohort suitable for further biomarker analysis [11]. TNB was an independent negative prognostic factor for 5-year BCSS, which emphasizes the aggressive clinical course of the disease characterized by early relapses, whereas at 10-year follow-up TNB was not an independent prognostic factor for outcome. The TNB subgroup constituted 20% of the patients, indicative of the young age of the patients included in our cohort, which was restricted to premenopausal patients [17].

EGFR protein expression is a positive characteristic of TNB and a putative therapeutic target in this group of patients [22–24]. Moreover, Viale et al. recently found the protein expression score to be a prognostic factor among TNB supporting EGFR-targeted therapy to be clinically relevant [24]. The EGFR PharmDX kit is today an approved testing for colorectal cancers suitable for EGFR-directed therapy with the monoclonal antibody cetuximab [19]. However, in patients with metastatic lung and colorectal cancer, EGFR gene copy number is reported to be a superior predictive marker for EGFR-inhibiting treatment with cetuximab compared to EGFR protein expression [25, 26]. We used both methods for determining EGFR status and in the present study EGFR protein expression defined by the EGFR PharmDX kit was found in >40% of TNB and increased EGFR gene copy number by FISH in 18% of them. EGFR immunoreactivity and EGFR-increased gene copy number were both significantly more often diagnosed in TNB than in non-TNB. However, none of them added prognostic information in the current cohort of TNB patients, which does not exclude EGFR protein expression or gene copy number to be a predictive marker for EGFR-targeting therapy in TNB. In contrast, EGFR gene copy number was linked to poorer outcome in ER-positive disease. Of note is that the substantial tissue loss experienced by us (55/96 tumors with TNB phenotype evaluable) when performing FISH analysis makes the results preliminary for both ER-negative and ER-positive patients. Clinical data obtained from a study on metastatic TNB using cetuximab is discouraging, although the efficacy of the drug should ideally be examined when patients are selected prospectively according to EGFR status by IHC or FISH [16]. Since EGFR protein expression in the study was diagnosed in >40% of patients with TNB, EGFR seems to be a candidate biomarker for selection of targeted therapy in these patients.

Vascular endothelial growth factor A (VEGF-A)-blocking therapy with bevacizumab represents an attractive treatment option for patients with TNB in addition to polychemotherapy [10]. Promising data have been presented in the neoadjuvant setting for TNB, although the selection of patients has not been performed according to

any putative target for bevacizumab [14]. In the metastatic setting, the drug is already established in breast cancer treatment, whereas ongoing studies in the adjuvant setting will elucidate its future role in defending relapses in TNB [13]. Although bevacizumab has shown clinical efficacy in treatment of several tumor types, no treatment predictive marker has been identified so far in the clinics. Genomic activation of VEGF-A has been identified to be higher in TNB compared to other subgroups of breast cancer as an indication of a specific role for bevacizumab treatment in this subgroup of breast cancer. We used a semiquantitative method for immunohistochemical evaluation of VEGF-A and by this method we were not able to find VEGF-A to be a positive marker for the TNB subtype, although the prognostic information indicated that VEGF-A expression can be of some importance (HR 2.3 $P = 0.08$). However, determination of VEGF-A by cytosolic methods seems to be a more accurate method for evaluating prognosis in breast cancer measuring additional amount of VEGF-A secreted by the tumor cells and in other cellular compartments.

Sunitinib is a multityrosine kinase inhibitor directed against VEGFR2, PDGFRA, and c-KIT, and the drug has been used in trials of metastatic breast cancer and seems to have some effect in patients with TNB or HER2-positive tumors [15]. C-KIT is a tyrosine kinase receptor commonly identified in TNB, whereas VEGFR2 has to our knowledge not been examined in TNB before. In the current study, we were able to evaluate all TNB tumors for VEGFR2 expression and high expression of VEGFR2 was a significant characteristic of the TNB subtype. Moreover, patients with TNB tumor and high expression of VEGFR2 had decreased 5-year BCSS, supporting that VEGFR2 is an interesting candidate biomarker of possible relevance for targeted therapies in TNB patients. In gliomas, tumor-specific protein expression and gene amplification of EGFR, c-KIT, and VEGFR2 are commonly observed in the same tumor and seem to be a molecular characteristics of both primary and recurrent gliomas [27]. C-KIT and VEGFR2 are located at 4q12 and in gliomas a common genomic aberration has been suggested at this specific locus [27].

In summary, the present study has confirmed TNB to be a subgroup with independent prognostic information for early death in breast cancer in premenopausal breast cancer. TNB showed increased protein expression of EGFR and VEGFR2, whereas expression of VEGF-A was not a specific biomarker of TNB. Increased EGFR gene copy number was also more frequently encountered in TNB, but neither EGFR protein expression nor gene copy number added prognostic information. Patients with TNB and high expression of VEGFR2 had decreased 5- and 10-year BCSS by univariate analysis making VEGFR2 an interesting biomarker for further studies in this cohort of patients. The number of patients with TNB in the current

study was limited and consequently no definitive conclusions can be drawn. The data presented should rather stimulate to conduct further studies on the role of tyrosine kinase receptors as EGFR and VEGFR2 in TNB.

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