Pathol. Oncol. Res. (2012) 18:33–41
DOI 10.1007/s12253-011-9413-8

RESEARCH

Immunohistochemical Study of the Angiogenetic Network of VEGF, HIF1α, VEGFR-2 and Endothelial Nitric Oxide Synthase (eNOS) in Human Breast Cancer

Maria Kafousi · Thomas Vrekoussis · Eleftheria Tsentelierou · Kitty Pavlakis · Iordanis Navrozoglou · Vassilios Dousias · Elias Sanidas · Dimitrios Tsiftsis · Vassilios Georgoulias · Efstathios N. Stathopoulos

Received: 13 October 2010 / Accepted: 11 May 2011 / Published online: 14 June 2011
© Arányi Lajos Foundation 2011

Abstract
Background The role of Nitric Oxide (NO) in angiogenesis has not been fully clarified yet. A dual role for NO, either inductive or inhibitory, has been proposed on the basis of different effects that high or low concentrations of NO may exert on the angiogenic process. Additionally, it has been referred that NO may induce VEGF production, while VEGF may induce NO production via up-regulation of the endothelial nitric oxide synthase (eNOS), the two pathways being reverse. The aim of the current study was to investigate the expression of key molecules involved in these opposite pathways in primary breast cancer.
Methods Representative tumor samples from 242 patients with early-stage breast cancer (invasive ductal breast carcinomas) were investigated for the expression of VEGF, VEGFR-2, HIF1α, iNOS, and eNOS using immunohistochemistry.
Results Endothelial NOS was found in 159 cases, VEGF in 131 cases, HIF-1α in 139 cases, VEGFR2 in 185 cases and inducible NOS (iNOS) in 22 cases. There was a significant correlation between the expression of VEGF and VEGFR-2, eNOS and VEGF, eNOS and VEGFR-2, eNOS and HIF1α. No statistically significant correlation was found between iNOS and the rest of the studied molecules.
Conclusions In breast cancer cases, the major molecules regulating NO and VEGF production can be co-expressed in the individual carcinomas implying a possibility for the relevant pathways to be active; however appropriate functional experiments remain to be conducted to prove such a hypothesis

Keywords Angiogenesis · Breast Cancer · eNOS · VEGF

Vassilios Georgoulias and Efstathios N. Stathopoulos share senior authorship

M. Kafousi · T. Vrekoussis · E. Tsentelierou · E. N. Stathopoulos (✉)
Department of Pathology, Medical School of the University of Crete,
Voutes, 70003 Heraklion, Crete, Greece
e-mail: stath@med.uoc.gr

T. Vrekoussis · I. Navrozoglou · V. Dousias
Breast Unit, Department of Obstetrics and Gynecology, Medical School, University of Ioannina,
Ioannina, Epirus, Greece

K. Pavlakis
Department of Pathology, Medical School, University of Athens,
Athens, Greece

E. Sanidas · D. Tsiftsis
Department of Surgical Oncology, Medical School, University of Crete,
Heraklion, Crete, Greece

V. Georgoulias
Department of Medical Oncology, Medical School, University of Crete,
Heraklion, Crete, Greece

Background

Angiogenesis is essential for a tumor to grow and disseminate [1]. Several factors have been identified as inducers or inhibitors of angiogenesis. Among them, nitric oxide (NO) seems to be enrolled in modulating either positively or negatively the angiogenic process [2].

NO is produced by nitric oxide synthases (NOS), enzymes that catalyze the transformation of L-arginine to L-citruline. To date two categories of NOS have been recognized: a) the calcium-dependent constitutive isoforms that are found in neural tissue (neuronal NOS or nNOS) and in endothelial cells (endothelial NOS or eNOS) and b) the calcium-independent inducible NOS or iNOS, found primarily in mesenchymal or parenchymal tissues of several organs [2].

The role of NO in tumor angiogenesis has not been entirely clarified yet. Low NO concentrations seem to induce tumor angiogenesis, while high NO concentrations are inhibitory. Several studies so far have revealed that NO may induce VEGF production via the PI3K/Akt/PKB—HIF-1α pathway [3–6]; however reports claim that NO might also act as an inhibitor of VEGF expression [7]; this contradiction has not been cleared so far. This dual and bi-directional angiogenesis regulatory mechanism becomes more complex by the interference of hypoxia. Under hypoxic conditions, there is an activation of the c-Src pathway leading to HIF-1α up-regulation and finally to increased VEGF production [8, 9], which induces NO production via VEGFR-2 and its downstream effector c-Src [10–14]. Additionally, hypoxia seems to induce NO production [15]. On the contrary, high NO concentrations inhibit c-Src leading to HIF-1α down-regulation, thus decreasing VEGF production [2].

The current knowledge regarding NO/NOS and their relationship with angiogenesis in breast cancer is based primarily on in vitro experiments, since there are only a few studies reporting on the relationship of NO/NOS expression in the prognosis of human tumors [16–19]. To our own best of knowledge there is no study in the literature investigating the relationship between nitric oxide synthases and other angiogenic factors or receptors on clinical specimens.

The aim of the present study was to investigate whether molecules of the above interrelating pathways are expressed in human breast cancers.

Patients, Materials and Methods

Patients and Molecular Subtyping Two hundred and 42 cases of operated early breast cancer (invasive ductal breast carcinomas) were randomly retrieved from the archives of the Pathology Department of the University General Hospital of Heraklion, Crete, Greece. All patients had undergone primary surgical treatment (breast surgery ± axillary sentinel lymph node dissection ± complete axillary lymph node dissection). No patient had received preoperative chemotherapy. Archival data regarding estrogen receptors (ER), progesterone receptors (PR) and Her-2 status on each patient was retrieved as well. All cases were subgrouped according to their molecular profile as previously described [20]: a) Luminal A [ER (+) or PR (+), Her-2 (−)], b) Luminal B [ER (+) or PR (+), Her-2 (+)], c) Triple negative [ER (−), PR (−), Her-2 (−)] and d) Her-2 positive [ER (−), PR (−), Her-2 (+)].

Immunohistochemistry for Detection of VEGF, VEGFR-2, eNOS, iNOS, HIF-1α Expression Immunodetection protocol details are presented in Table 1. Briefly, formalin-fixed and paraffin-embedded tissue sections (3 μm thick) were deparaffinized in xylene and rehydrated through graded concentrations of ethanol. Antigen retrieval via microwave treatment (3 cycles of 5 min. each) followed. After incubation with the primary antibodies, positivity was revealed by using a commercially available immunodetection system (UltraVision LP, TL-125-AL, Lab Vision, CA, USA with fast red as chromogen) followed by Mayer's hematoxylin counterstaining for 3 min. Slides were then

Table 1 Basic features of the immunohistochemistry detection performed in the study

Antibody/Origin/Source	Antigen retrieval	Dilution in TBS	Incubation	Positive Control	Detection System
Anti-VEGF, mouse, MS-1467-P, Neomarkers, CA, USA	MW[1]	1/50	1 h	Angiosarcoma	UltraVision LP/AP with Fast-Red (Lab Vision, CA, USA)
Anti-HIF1a, mouse, MS 1164, *Thermo scientific, CA, USA*	MW[1]	1/20	overnight	Oral squamous cell carcinoma	
Anti-VEGFR-2, mouse, SC-6251, SantaCruz, CA, USA	MW[1]	1/40	2 h	Human colon carcinoma	
Anti- eNOS, rabbit, RB-1711-P, Neomarkers, CA, USA	MW[2]	1/100	overnight	Capillary endothelium	
Anti-iNOS, rabbit, RB-9242, Thermo scientific, CA, USA	MW[1]	1/300	1 h	Lung macrophages	

[1] MW (350 W, three times for 5 min. each) in 0.01 M Citrate Buffer, pH 6

[2] MW (350 W, three times for 5 min. each) in 0.001 M EDTA, pH 8

rinsed with ammonia water, washed in tap water and covered using Glycergel (Dako, CA, USA). Positive controls (according to the manufacturer's protocol) were included in the study. Sections stained with the same protocol by omitting the primary antibodies were used as negative controls.

Scoring of Immunoreactivity

The evaluation of all immunostainings was performed by two independent observers (MK and ENS) working blindly to the clinical and histopathological data, and the mean of the two recorded observations was considered as the final value. In cases with greater than 10% discordance in reading results, the final conclusion was reached in consensus [21]. Immunostaining results were evaluated in a Nikon, Eclipse, E-400 photomicroscope equipped, among others, with a 40X/0.65-∞/0.17 WD 0.65 objective lens.

Nuclear reactivity for HIF-1a was scored as previously reported [22], based on reaction intensity and percentage of positive cells as follows: a) staining not detected: 0, b) positive cells less than 1%: 1+, c) 1–10% cells with slight to moderate intensity or 11–50% slightly positive cells: 2+, d) 11–50% positive cells with moderate to marked staining: 3+ and e) more than 50% positive cells with moderate to marked staining: 4+.

The VEGFR2 expression was evaluated as previously described [23]: a) carcinomas with less than 10% positive cells/10 hpf (x400): 0, b) 11%–30% positive cells/10 hpf: 1, c) 31%–50% positive cells/10 hpf: 2, and d) more than 50% positive cells/10 hpf: 3 [23].

The eNOS immunostaining was evaluated as previously reported [16]. Intensity of cytoplasmic immunostaining was scored from 0 to 4+ (no staining, weak staining, moderate staining, strong staining, very strong staining, respectively). There was no need for positive tumor cells counting, since in any single case all cells were equally either positive or negative.

Regarding iNOS, there was no need for positive tumor cells counting and grading, since in any individual case all cancer cells were mostly equally either positive, with a granular cytoplasmic positivity pattern without differences in intensity from cell to cell, or negative.

VEGF staining was evaluated as previously described [24]. In brief, intensity of cytoplasmic immunostaining was scored from 0 to 3+ (no staining, weak staining, moderate staining, strong staining, respectively).

Each section was also characterised as negative (IHC score=0) or positive (IHC score≥1+) according to previously mentioned reports [16, 25–27].

Statistical Analysis Potential correlations between expressions of molecules involved in angiogenesis were assessed either by evaluating the Gamma correlation co-efficient or by applying the chi-square test (when appropriate). Every association presented with $p<0.05$ was considered statistically significant.

Results

Patient Clinicopathological Features All cases enrolled in this study were invasive ductal carcinoma. Among these, 146 (60.33%) were ER positive, 135 (55.79%) were PR positive and 36 (14.88%) were Her-2 positive. According to ER/PR/Her-2 expression status, cases were grouped as Luminal A ($n=156$, 64.46%), Luminal B ($n=16$, 6.61%), Her-2 expressing ($n=19$, 7.85%) and triple negative ($n=51$, 21.07%). Axillary lymph node dissection was performed on 227 cases; 138 (60.79%) were found to have one or more positive lymph nodes (Table 2).

The selected tissue sections of 200 and 42 cases of early primary invasive ductal breast carcinomas examined immunohistochemically for eNOS, VEGF, VEGFR2, HIF1α and iNOS expression were representative of the individual tumors architectural and cytological pattern. Representative figures of immunostained slides are presented in Fig. 1.

Distribution of IHC Positivity Endothelial NOS (eNOS) positivity was cytoplasmic, mainly on tumor cells; 36 (14.88%), 80 (33.06%), 29 (11.98%) and 14 (5.79%) were scored 1+, 2+, 3+ and 4+ respectively (Table 2). VEGF was expressed in the cytoplasm of tumor cells, and 81 (33.5%), 44 (18.2%) and 6 (2.4%) were scored as weak, moderate and strong positive, respectively (Table 2). HIF1α was found to be expressed in the nucleus of tumor cells; 82 cases (33.88%) presented as 2+, 18 (7.44%) as 3+ and 39 (16.12%) as 4+ (Table 2). VEGFR2 was expressed on tumor cells as well as on endothelial cell membranes and was found in 11 (4.55%) cases as weakly positive, in 6 cases (2.48%) as moderately positive and in 168 (69.42) cases as strongly positive (Table 2). Finally, iNOS was detected in 22 cases (9.09%) in a cytoplasmic pattern (Table 2).

Sixty five cases (26.85%) were positive for all the four molecules included in the VEGF-eNOS pathway (eNOS, VEGF, VEGFR2, HIF1α) (Fig. 1b). Thirty nine cases were of luminal A, 7 were of luminal B, 5 were of Her-2 expressing type and 14 were triple negative cases. This distribution however was not significant ($p>0.05$).

Correlation Analysis eNOS, VEGF, HIF-1a, VEGFR2 and iNOS Endothelial NOS expression was detected in 104 out of 131 (79.39%) of the VEGF positive cases and in 55 out

Table 2 Major clinicopathological features and IHC scores of the molecules of the current study

			n		(%)
Histology					
Invasive Ductal Carcinoma			242		100
Grade					
G1			38		15.70
G2			113		46.69
G3			91		37.60
Nodal Status (*n*=227)					
N_0			89		39.21
N^+			138		60.79
Estrogen Receptors (ER)					
Positive			146		60.33
Negative			96		39.67
Progesterone Receptors (PR)					
Positive			135		55.79
Negative			107		44.21
Her-2					
Positive			36		14.88
Negative			206		85.12
Molecular subtypes					
Luminal A			156		64.46
Luminal B			16		6.61
Her-2			19		7.85
Triple Negative			51		21.07
Immunohistochemistry scores					
eNOS	n	(%)	HIF-1a	n	(%)
0	83	34.30	0	103	42.56
1	36	14.88	1	0	0
2	80	33.06	2	82	33.88
3	29	11.98	3	18	7.44
4	14	5.79	4	39	16.12
VEGF	n	(%)	VEGFR2	n	(%)
0	111	45.87	0	57	23.55
1	81	33.47	1	11	4.55
2	44	18.18	2	6	2.48
3	6	2.48	3	168	69.42
iNOS		n		(%)	
Positive		22		9.09	
Negative		220		90.91	

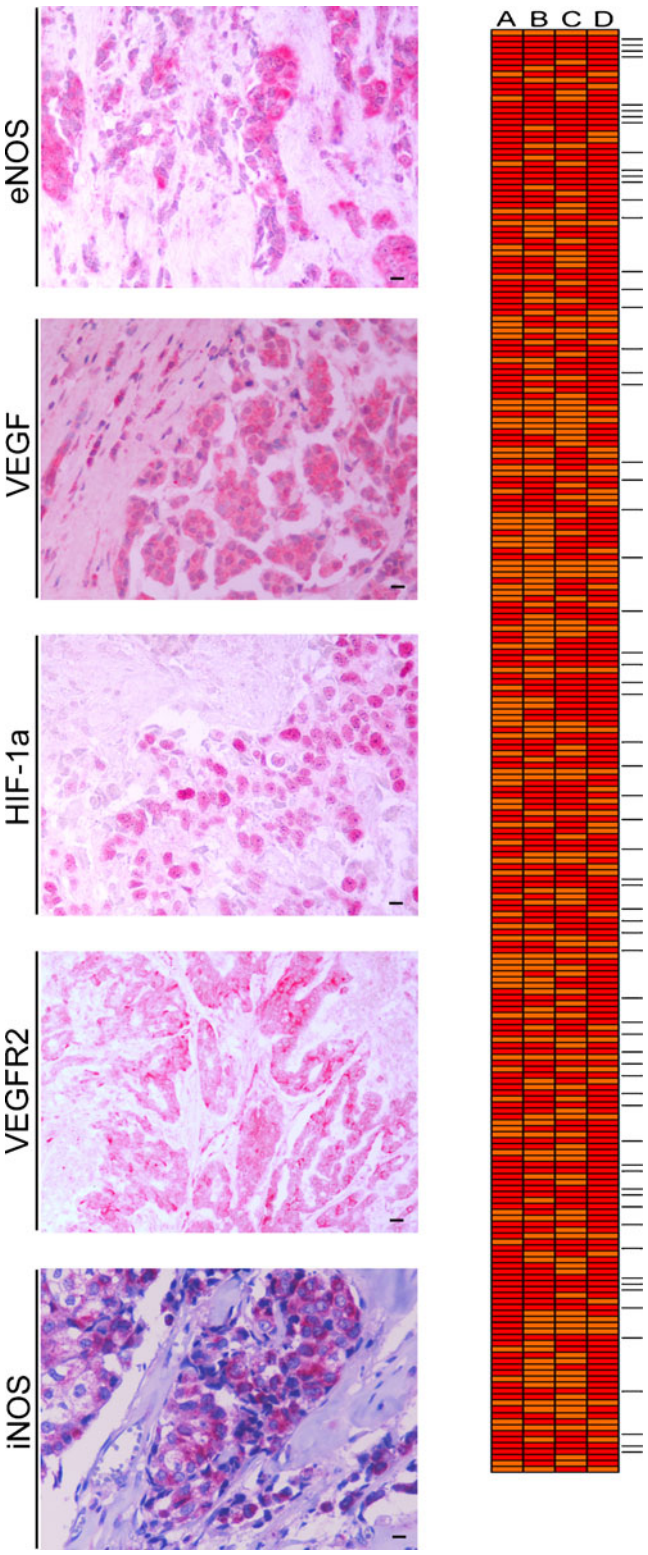

Fig. 1 Representative sections of breast cancer specimens, immunostained for the molecules studied (the scale bar represents 10 μm) and schematic presentation of the sample regarding eNOS (**a**), VEGF (**b**), HIF-1a (**c**) and VEGFR2 (**d**). In sixty five cases all the molecules co-exist (*marked with a dash*). *Orange*: Negative IHC staining, *Red*: Positive IHC staining

of 111 (49.55%) of the VEGF negative cases. Moreover, 104 out of 159 (65.40%) of the eNOS positive cases were found to express VEGF compared to 27 out of 83 (32.53%) of the eNOS negative cases (Table 3). The correlation between eNOS and VEGF was significant (gamma co-efficient: 0.377, $p<10^{-6}$) (Table 4). Concurrently eNOS was detected in 133 out of 185 (71.89%) of

Table 3 Cross-tabulation of data according to the expression of the molecules involved in the current study

	VEGF		HIF 1a		VEGFR2	
	Negative (n=111)	Positive (n=131)	Negative (n=103)	Positive (n=139)	Negative (n=57)	Positive (n=185)
eNOS negative (n=83)	56	27	44	39	31	52
eNOS positive (n=159)	55	104	59	100	26	133
iNOS negative (n=220)	104	116	95	125	55	165
iNOS positive (n=22)	7	15	8	14	2	20
	VEGF negative (n=111)		60	51	40	71
	VEGF positive (n=131)		43	88	17	114
			HIF 1a negative (n=103)		29	74
			HIF 1a positive (n=139)		28	111

the VEGFR2 positive cases and in 26 out of 57 (45.61%) of the VEGFR2 negative cases, while 133 out of 159 (83.64%) of the eNOS positive cases compared to 52 out of 83 (62.65%) of the eNOS negative cases were found to express VEGFR2 (Table 3). The correlation between eNOS and VEGFR2 was significant (gamma co-efficient: 0.244, p=0.000848), as well (Table 4). Finally, eNOS was detected in 100 out of 139 (71.94%) of the HIF-1α positive cases and in 59/103 (57.28%) of the HIF-1α negative cases, whereas 100/159 (62.89%) of the eNOS positive cases were found to express HIF-1α compared to 39/83 (46.99%) of the eNOS negative cases (Table 3). The correlation between eNOS and HIF1α was also significant (gamma co-efficient: 0.130, p=0.031229) (Table 4).

Regarding VEGFR-2, its expression was found to be significantly correlated to the expression of VEGF (gamma co-efficient: 0.385, $p=10^{-6}$) (Table 4). VEGFR2 was detected in 114 out of 131 (87.02%) of the VEGF positive cases and in 71 out of 111 (63.96%) of the VEGF negative cases. One hundred and 14 (61.6%) out of 185 VEGFR-2 positive cases were found expressing VEGF, compared to 17 out of 57 (29.82%) of the VEGFR-2 negative cases (Table 3). Finally a significant correlation was found between VEGF and HIF-1α expression (Table 4).

The correlations between VEGFR2-HIF-1α, iNOS-VEGF, iNOS-VEGFR2, iNOS-HIF1α were not statistically significant (Table 4).

Table 4 Pair-wise correlations of the molecules under study

Pairwise correlations		
	Gamma correlation	
	Gamma	p
eNOS & VEGF	0.377	$<10^{-6*}$
eNOS & HIF 1	0.130	0.031229*
eNOS & VEGFR	0.244	0.000848*
VEGF & HIF 1	0.307	0.000001*
VEGF & VEGFR	0.385	0.000001*
HIF 1 & VEGFR	0.131	0.094057
	Chi square test (χ^2), df=3	p
iNOS & VEGF	6.135	0.10519
iNOS & HIF 1	3.086	0.37847
iNOS & VEGFR	2.925	0.40327

Significant observations are marked with an asterisk

Correlation Analysis Between Clinicopathological Features and the Molecules of the Study All the clinicopathological features were examined for possible correlations with the molecules of the study. Estrogen receptor status (Table 5) and progesterone receptors status (Table 6a) was significantly correlated to eNOS expression. Indeed 72.6% and 71.1% of the ER/PR positive cases respectively, express eNOS, while only 55.2% and 58.8% of the ER/PR negative cases respectively are eNOS reactive (Table 6a).

Additionally a significant correlation was revealed between PR and HIF-1a expression (Table 5), since 49.6% of the PR positive and 67.28% of the PR negative cases express HIF-1a (Table 6b). Analysis between HIF-1a groups and different IHC scores (Table 6b) also revealed that intense HIF-1a IHC score (i.e. 4+) is significantly more common (p=0.028) in ER negative (23.9%) than in ER positive (10.9%) cases and in HER-2 positive (25%) than in HER-2 negative (14.5%) cases (p=0.004).

Taking into account the molecular subtyping, it is shown that HIF-1a expressing cases are significantly more common (p=0.036) in Luminal B (75%), HER-2 (68.42%) and triple negative (68.62%) cases, than in Luminal A (50.64%) cases (Table 7). HIF-1a, VEGF and VEGFR2 expressions were positively correlated to the histological grade (Table 5). The rest of the investigated correlations were not significant (Table 5).

Table 5 Correlations between salient clinicopathologic features and the molecules of the study. Significant results are underlined. Analysis on ER, PR, Her-2 and lymph node status has been performed by applying chi square. Grade and tumor diameter analysis has been performed by applying gamma correlation statistic

	ER (p)	PR (p)	HER-2 (p)	Grade (p)	Tumor Diameter (p)	Lymph node status (p)
eNOS $_{(df=4)}$	*0.042*	0.078	0.483	0.837	0.986	0.729
VEGF$_{(df=3)}$	0.362	0.842	0.559	*0.001*	0.942	0.763
HIF-1a$_{(df=3)}$	*0.028*	*0.027*	*0.004*	$\leq 10^{-3}$	0.305	0.588
VEGFR2$_{(df=3)}$	0.520	0.626	0.978	*0.028*	0.986	0.756
iNOS$_{(df=1)}$	0.212	0.219	0.153	0.396	0.766	0.863

Discussion

Angiogenesis is an essential process for tumor growth and dissemination. The key-role in angiogenesis is held by VEGF [28], being regulated among others by hypoxia and NO. Hypoxia and NO production are expected to be strongly associated, since it has already been shown that HIF1α is enrolled in the NO-induced VEGF production [5, 29].

Although efforts have been made to identify and study VEGF, HIF1α, eNOS, iNOS and VEGFR-2 and the in between them relation using in vitro models, little progress has been made in studying these molecules on human breast cancer specimens. Most of the studies so far (frequently reporting contradictory results) have focused either on NO production or on hypoxia, in the aim of identifying significant correlations regarding clinical features such as overall or disease-free survival [18, 30–32].

To the best of our knowledge, there are no studies in humans investigating the major components of the two pathways involving VEGF-induced NO production, and vice versa, as an intergraded system of molecules, interacting or opposing or even being complementary to each other. Our work, using a statistically adequate sample (n=242 breast cancer cases), is the first one clearly demonstrating that the above molecules can co-exist in human breast cancers, since 26.85% of the cases in our sample are positive for all the relevant molecules.

We have shown statistically significant co-expression between eNOS and VEGF on the one hand, and eNOS and HIF1α on the other; this observation indicates an important role in angiogenesis for all the above three molecules,

Table 6 A & B. Selective detailed presentation of the significant correlations. Further analysis (by a 2×2 contingency table) of eNOS expression has been performed by grouping all positive IHC scores together, as previously performed. (df: degrees of freedom)

A.							
	eNOS						
	0	**1+**	**2+**	**3+**	**+4**	**p (df=4)**	**p (df=1)**
ER							
Positive	40	21	54	22	9	*0.042*	*0.005*
Negative	43	15	26	7	5		
PR							
Positive	39	17	50	21	8	0.078	*0.046*
Negative	44	19	30	8	6		
B.							
		HIF-1a					
		0	**2+**	**3+**	**4+**	**p (df=3)**	
Her-2							
Positive		10	10	7	9	*0.004*	
Negative		93	72	11	30		
ER							
Positive		66	55	9	16	*0.028*	
Negative		37	27	9	23		
PR							
Positive		68	43	8	16	*0.027*	
Negative		35	39	10	23		

Table 7 Distribution of the molecules of the study among the molecular subtypes

	Luminal A	Luminal B	Her-2	Triple Negative	p
eNOS					
Positive	108	13	12	26	0.056
Negative	48	3	7	25	
VEGF					
Positive	84	13	9	25	0.131
Negative	72	3	10	26	
HIF-1a					
Positive	79	12	13	35	*0.036*
Negative	77	4	6	16	
VRGFR2					
Positive	115	11	15	44	0.264
Negative	41	5	4	7	
iNOS					
Positive	17	1	0	4	0.428
Negative	139	15	19	47	

which have already been demonstrated to participate in NO-induced VEGF production [2]. Co-expression of VEGF and HIF1α has been previously reported [8] and it is in consistence with our results (Table 4). Additionally, VEGF-mediated NO production has been reported via eNOS up-regulation by VEGFR-2 signaling [13]. The above finding is in agreement with the significant co-expression of VEGFR-2 and eNOS that was found in our series. Finally, the lack of statistical significance found in our results (Table 4), when a tentative HIF1α and VEGFR-2 co-expression was evaluated, seems to indicate that both in human breast cancers and in vitro [2], VEGF-induced NO-production may not be -at least directly- HIF-1a-dependent.

Another interesting finding of our study is that eNOS, but not iNOS, is strongly correlated with VEGF, VEGFR-2, and HIF-1α. iNOS is considered essential for tumor angiogenesis, since it is responsible for NO production in the stroma [33], and has been reported as a predictor of survival in axilla negative breast cancer cases [34]. Nevertheless, as our results indicate, it seems that in breast cancer, its contribution to the mechanism suggested and discussed herein is not significant.

The association of eNOS and estrogen receptors shown in our series (Tables 5 and 6a) is in concordance with previous knowledge. In the past, few reports demonstrated the current ER-eNOS association in breast cancer patients [16, 35]. Since then, it has been reported that ER induces eNOS expression [36], explaining why estrogens induce eNOS but not iNOS production in the MCF-7 breast cancer cell line [37]. Recently an association between ERα and eNOS expression has been found in healthy women as well [38]. Our findings verify previous results and thus contribute to the establishment of the thesis that in breast cancer eNOS and ER are significantly associated.

We have, also, shown a marginally significant association of progesterone receptors and eNOS expression, being in accordance with a previous report. The effect however of progesterone upon eNOS expression is not clear yet. Progesterone has been described to oppose to estrogen by down-regulating eNOS expression [39, 40]. Such an observation might seem as discordance to our results. However the evidence regarding the progesterone effect in breast cancer is weak so far. It could be hypothesized though that as long as eNOS is expressed, the significant co-existence of progesterone receptors could be a part of a negative feedback mechanism.

In cancer, the major role of hypoxia has been established by an expression profiling study [41], in which, genes affected by hypoxia were clustered to form the "hypoxia signature". This hypoxia signature was proved to be of clinical importance, since it could significantly predict survival rates [41, 42]. The key role in this hypoxia signature is held by the HIF family molecules via which other genes and proteins are regulated [42]. In our study, we analysed the effect of HIF-1a as part of the hypoxia signature and we demonstrated that in addition to the above discussed correlations, HIF-1a is inversely related to ER and PR expression and positively related to Her-2 expression. The inverse association of HIF-1a with the hormone receptors can be justified since it has been reported that hypoxia downregulates ER and PR expression in breast cancer cell lines [43]. However to our knowledge our report is one of the few verifying that HIF-1a (demonstrated by IHC and not by expression profiling) is clinically associated with reduced ER and PR expression in breast cancer cases [44]. Few reports correlate HER-2 with HIF-1a expression as well [45, 46]. Our results are in agreement with these reports.

Finally, HIF-1a was found to be more abundant in the more aggressive breast cancer subtypes (luminal B, Her-2 expressing and triple negative). Such an observation is in concordance with a recent report associating HIF-1a expression with aggressive breast cancer and worse disease free and overall survival [44].

All the above taken together produce evidence that in human breast cancer there is, at least, a proportion of cases that can express the key-molecules needed for the regulation of VEGF and NO production. Patients within this group are likely to benefit from targeted treatments, including VEGF inhibition, such as the commercially available anti-VEGF antibody Bevacizumab [47, 48]. Additionally, HIF1α and thus VEGF inhibition is nowadays an appealing solution. Several HIF1α inhibitors (microtubule modifiers, HSP90 inhibitors, topoisomerase inhibitors and other molecules) have been identified; however most of

them are not as specific as required in order to be clinically exploited [49, 50].

However, interactions between NO, hypoxia factors and VEGF might be more complex than hypothesized in the present work, since a) HIF-1α is regulated by oncogenic signaling pathways which are cell-type specific [49] and b) hypoxia elements for HIF-2 have already been identified on the eNOS promoter region [51]. Consequently, combined functional, molecular and immunohistochemical studies are necessary to further clarify the relationship between hypoxia, NO pathways and angiogenesis.

Conclusions

In conclusion, we have shown that in breast cancer cases, the major molecules regulating NO and VEGF production can co-exist, implying-but not proving-functional activity of the relevant pathways. However appropriate experiments, such as functional assays, remain to be conducted to strengthen such a hypothesis. This finding could be of clinical importance for designing future anti-angiogenic interventions. More extended studies are certainly required in order for the above concept to be applied to breast cancer therapy.

Conflict of Interest The authors declare that they have no conflict of interest.

References

1. Fox SB, Generali DG, Harris AL (2007) Breast tumour angiogenesis. Breast Canc Res 9:216
2. Kimura H, Esumi H (2003) Reciprocal regulation between nitric oxide and vascular endothelial growth factor in angiogenesis. Acta Biochim Pol 50:49–59
3. Amano K, Matsubara H, Iba O et al (2003) Enhancement of ischemia-induced angiogenesis by eNOS overexpression. Hypertension 41:156–62
4. Fukumura D, Gohongi T, Kadambi A et al (2001) Predominant role of endothelial nitric oxide synthase in vascular endothelial growth factor-induced angiogenesis and vascular permeability. Proc Natl Acad Sci USA 98:2604–9
5. Sandau KB, Fandrey J, Brune B (2001) Accumulation of HIF-1alpha under the influence of nitric oxide. Blood 97:1009–15
6. Sandau KB, Faus HG, Brune B (2000) Induction of hypoxia-inducible-factor 1 by nitric oxide is mediated via the PI 3 K pathway. Biochem Biophys Res Commun 278:263–7
7. Ghiso N, Rohan RM, Amano S, Garland R, Adamis AP (1999) Suppression of hypoxia-associated vascular endothelial growth factor gene expression by nitric oxide via cGMP. Invest Ophthalmol Vis Sci 40:1033–9
8. Choi KS, Bae MK, Jeong JW, Moon HE, Kim KW (2003) Hypoxia-induced angiogenesis during carcinogenesis. J Biochem Mol Biol 36:120–7
9. Tsuzuki Y, Fukumura D, Oosthuyse B, Koike C, Carmeliet P, Jain RK (2000) Vascular endothelial growth factor (VEGF) modulation by targeting hypoxia-inducible factor-1alpha–>hypoxia response element–>VEGF cascade differentially regulates vascular response and growth rate in tumors. Cancer Res 60:6248–52
10. Chou MT, Wang J, Fujita DJ (2002) Src kinase becomes preferentially associated with the VEGFR, KDR/Flk-1, following VEGF stimulation of vascular endothelial cells. BMC Biochem 3:32
11. Duval M, Le Boeuf F, Huot J, Gratton JP (2007) Src-mediated phosphorylation of Hsp90 in response to vascular endothelial growth factor (VEGF) is required for VEGF receptor-2 signaling to endothelial NO synthase. Mol Biol Cell 18:4659–68
12. He H, Venema VJ, Gu X, Venema RC, Marrero MB, Caldwell RB (1999) Vascular endothelial growth factor signals endothelial cell production of nitric oxide and prostacyclin through flk-1/KDR activation of c-Src. J Biol Chem 274:25130–5
13. Hood JD, Meininger CJ, Ziche M, Granger HJ (1998) VEGF upregulates ecNOS message, protein, and NO production in human endothelial cells. Am J Physiol 274:H1054–8
14. Shen BQ, Lee DY, Zioncheck TF (1999) Vascular endothelial growth factor governs endothelial nitric-oxide synthase expression via a KDR/Flk-1 receptor and a protein kinase C signaling pathway. J Biol Chem 274:33057–63
15. Beleslin-Cokic BB, Cokic VP, Yu X, Weksler BB, Schechter AN, Noguchi CT (2004) Erythropoietin and hypoxia stimulate erythropoietin receptor and nitric oxide production by endothelial cells. Blood 104:2073–80
16. Loibl S, Strank C, von Minckwitz G et al (2005) Immunohistochemical evaluation of endothelial nitric oxide synthase expression in primary breast cancer. Breast 14:230–5
17. Mortensen K, Christensen IJ, Nielsen HJ, Hansen U, Larsson LI (2004) High expression of endothelial cell nitric oxide synthase in peritumoral microvessels predicts increased disease-free survival in colorectal cancer. Cancer Lett 216:109–14
18. Mortensen K, Holck S, Christensen IJ et al (1999) Endothelial cell nitric oxide synthase in peritumoral microvessels is a favorable prognostic indicator in premenopausal breast cancer patients. Clin Cancer Res 5:1093–7
19. Nakamura Y, Yasuoka H, Tsujimoto M et al (2006) Nitric oxide in breast cancer: induction of vascular endothelial growth factor-C and correlation with metastasis and poor prognosis. Clin Cancer Res 12:1201–7
20. Kwan ML, Kushi LH, Weltzien E et al (2009) Epidemiology of breast cancer subtypes in two prospective cohort studies of breast cancer survivors. Breast Cancer Res 11:R31
21. Kalmanti L, Dampaki K, Dimitriou H, Stiakaki E, Chaniotis V, Stathopoulos E (2005) A morphometric approach for the evaluation of angiogenesis in acute lymphoblastic leukemia of childhood. Leuk Res 29:673–7
22. Aebersold DM, Burri P, Beer KT et al (2001) Expression of hypoxia-inducible factor-1alpha: a novel predictive and prognostic parameter in the radiotherapy of oropharyngeal cancer. Cancer Res 61:2911–16
23. Nakopoulou L, Stefanaki K, Panayotopoulou E et al (2002) Expression of the vascular endothelial growth factor receptor-2/Flk-1 in breast carcinomas: correlation with proliferation. Hum Pathol 33:863–70
24. Takahashi Y, Tucker SL, Kitadai Y et al (1997) Vessel counts and expression of vascular endothelial growth factor as prognostic factors in node-negative colon cancer. Arch Surg 132:541–6
25. Meunier-Carpentier S, Dales JP, Djemli A et al (2005) Comparison of the prognosis indication of VEGFR-1 and VEGFR-2 and Tie2 receptor expression in breast carcinoma. Int J Oncol 26:977–84
26. Mylona E, Alexandrou P, Giannopoulou I et al (2007) The prognostic value of vascular endothelial growth factors

(VEGFs)-A and -B and their receptor, VEGFR-1, in invasive breast carcinoma. Gynecol Oncol 104:557–63
27. Trastour C, Benizri E, Ettore F et al (2007) HIF-1alpha and CA IX staining in invasive breast carcinomas: prognosis and treatment outcome. Int J Cancer 120:1451–8
28. Hayes DF, Miller K, Sledge G (2007) Angiogenesis as targeted breast cancer therapy. Breast 16(Suppl 2):S17–19
29. Namba T, Koike H, Murakami K et al (2003) Angiogenesis induced by endothelial nitric oxide synthase gene through vascular endothelial growth factor expression in a rat hindlimb ischemia model. Circulation 108:2250–7
30. Duenas-Gonzalez A, Isales CM, del Mar Abad-Hernandez M, Gonzalez-Sarmiento R, Sangueza O, Rodriguez-Commes J (1997) Expression of inducible nitric oxide synthase in breast cancer correlates with metastatic disease. Mod Pathol 10:645–9
31. Vleugel MM, Greijer AE, Shvarts A et al (2005) Differential prognostic impact of hypoxia induced and diffuse HIF-1alpha expression in invasive breast cancer. J Clin Pathol 58:172–7
32. Lundgren K, Holm C, Landberg G (2007) Hypoxia and breast cancer: prognostic and therapeutic implications. Cell Mol Life Sci 64:3233–47
33. Lechner M, Lirk P, Rieder J (2005) Inducible nitric oxide synthase (iNOS) in tumor biology: the two sides of the same coin. Semin Cancer Biol 15:277–89
34. Loibl S, Buck A, Strank C et al (2005) The role of early expression of inducible nitric oxide synthase in human breast cancer. Eur J Cancer 41:265–71
35. Vakkala M, Paakko P, Soini Y (2000) eNOS expression is associated with the estrogen and progesterone receptor status in invasive breast carcinoma. Int J Oncol 17:667–71
36. Sumi D, Ignarro LJ (2003) Estrogen-related receptor alpha 1 up-regulates endothelial nitric oxide synthase expression. Proc Natl Acad Sci USA 100:14451–6
37. Loibl S, Bratengeier J, Farines V et al (2006) Investigations on the inducible and endothelial nitric oxide synthases in human breast cancer cell line MCF-7 - estrogen has an influence on e-NOS, but not on i-NOS. Pathol Res Pract 202:1–7
38. Gavin KM, Seals DR, Silver AE, Moreau KL (2009) Vascular endothelial estrogen receptor alpha is modulated by estrogen status and related to endothelial function and endothelial nitric oxide synthase in healthy women. J Clin Endocrinol Metab 94:3513–20
39. Oishi A, Ohmichi M, Takahashi K et al (2004) Medroxyprogesterone acetate attenuates estrogen-induced nitric oxide production in human umbilical vein endothelial cells. Biochem Biophys Res Commun 324:193–8
40. Zerr-Fouineau M, Chataigneau M, Blot C, Schini-Kerth VB (2007) Progestins overcome inhibition of platelet aggregation by endothelial cells by down-regulating endothelial NO synthase via glucocorticoid receptors. FASEB J 21:265–73
41. Chi JT, Wang Z, Nuyten DS et al (2006) Gene expression programs in response to hypoxia: cell type specificity and prognostic significance in human cancers. PLoS Med 3:e47
42. Milani M, Harris AL (2008) Targeting tumour hypoxia in breast cancer. Eur J Cancer 44:2766–73
43. Kurebayashi J, Otsuki T, Moriya T, Sonoo H (2001) Hypoxia reduces hormone responsiveness of human breast cancer cells. Jpn J Cancer Res 92:1093–101
44. Yamamoto Y, Ibusuki M, Okumura Y et al (2008) Hypoxia-inducible factor 1alpha is closely linked to an aggressive phenotype in breast cancer. Breast Cancer Res Treat 110:465–75
45. Laughner E, Taghavi P, Chiles K, Mahon PC, Semenza GL (2001) HER2 (neu) signaling increases the rate of hypoxia-inducible factor 1alpha (HIF-1alpha) synthesis: novel mechanism for HIF-1-mediated vascular endothelial growth factor expression. Mol Cell Biol 21:3995–4004
46. Giatromanolaki A, Koukourakis MI, Simopoulos C et al (2004) c-erbB-2 related aggressiveness in breast cancer is hypoxia inducible factor-1alpha dependent. Clin Cancer Res 10:7972–7
47. Traina TA, Rugo HS, Dickler M (2007) Bevacizumab for advanced breast cancer. Hematol Oncol Clin North Am 21:303–19
48. Marty M, Pivot X (2008) The potential of anti-vascular endothelial growth factor therapy in metastatic breast cancer: Clinical experience with anti-angiogenic agents, focusing on bevacizumab. Eur J Cancer 44:912–20
49. Patiar S, Harris AL (2006) Role of hypoxia-inducible factor-1alpha as a cancer therapy target. Endocr Relat Cancer 13(Suppl 1):S61–75
50. Melillo G (2007) Targeting hypoxia cell signaling for cancer therapy. Cancer Metastasis Rev 26:341–52
51. Coulet F, Nadaud S, Agrapart M, Soubrier F (2003) Identification of hypoxia-response element in the human endothelial nitric-oxide synthase gene promoter. J Biol Chem 278:46230–40