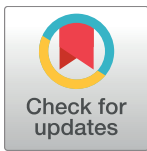


RESEARCH ARTICLE

Associations of angiogenesis-related proteins with specific prognostic factors, breast cancer subtypes and survival outcome in early-stage breast cancer patients. A Hellenic Cooperative Oncology Group (HeCOG) trial

Anna Goussia^{1*}, Nafsika Simou¹, Flora Zagouri², Kyriaki Manousou³, Georgios Lazaridis⁴, Helen Gogas⁵, Angelos Koutras⁶, Maria Sotiropoulou⁷, George Pentheroudakis⁸, Dimitrios Bafaloukos⁹, Christos Markopoulos¹⁰, Helen Patsea¹¹, Christos Christodoulou¹², Pavlos Papakostas¹³, Thomas Zaramboukas¹⁴, Epaminontas Samantas¹⁵, Paris Kosmidis¹⁶, Vasileios Venizelos¹⁷, Charisios Karanikiotis¹⁸, George Papatsibas¹⁹, Grigorios Xepapadakis²⁰, Konstantine T. Kalogeras^{21,22}, Christina Bamia²³, Meletios-Athanassios Dimopoulos², Vassiliki Malamou-Mitsi^{1†}, George Fountzilias^{22,24}, Anna Batistatou¹



OPEN ACCESS

Citation: Goussia A, Simou N, Zagouri F, Manousou K, Lazaridis G, Gogas H, et al. (2018) Associations of angiogenesis-related proteins with specific prognostic factors, breast cancer subtypes and survival outcome in early-stage breast cancer patients. A Hellenic Cooperative Oncology Group (HeCOG) trial. *PLoS ONE* 13(7): e0200302. <https://doi.org/10.1371/journal.pone.0200302>

Editor: Soumitro Pal, Children's Hospital Boston, UNITED STATES

Received: February 1, 2018

Accepted: June 22, 2018

Published: July 31, 2018

Copyright: © 2018 Goussia et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: This study was supported by an internal Hellenic Cooperative Oncology Group (HeCOG) translational research grant (HE TRANS_BR). The funders played no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

1 Department of Pathology, Ioannina University Hospital, Faculty of Medicine, School of Health Sciences, University of Ioannina, Ioannina, Greece, **2** Department of Clinical Therapeutics, Alexandra Hospital, National and Kapodistrian University of Athens School of Medicine, Athens, Greece, **3** Section of Biostatistics, Hellenic Cooperative Oncology Group, Athens, Greece, **4** Department of Medical Oncology, Papageorgiou Hospital, Aristotle University of Thessaloniki, School of Health Sciences, Faculty of Medicine, Thessaloniki, Greece, **5** First Department of Medicine, Laiko General Hospital, National and Kapodistrian University of Athens School of Medicine, Athens, Greece, **6** Division of Oncology, Department of Medicine, University Hospital, University of Patras Medical School, Patras, Greece, **7** Department of Pathology, Alexandra Hospital, Athens, Greece, **8** Department of Medical Oncology, Ioannina University Hospital, Ioannina, Greece, **9** First Department of Medical Oncology, Metropolitan Hospital, Piraeus, Greece, **10** Second Department of Prop. Surgery, Laiko General Hospital, National and Kapodistrian University of Athens School of Medicine, Athens, Greece, **11** Department of Pathology, IASSO General Hospital, Athens, Greece, **12** Second Department of Medical Oncology, Metropolitan Hospital, Piraeus, Greece, **13** Oncology Unit, Hippokraton Hospital, Athens, Greece, **14** Department of Pathology, Aristotle University of Thessaloniki, School of Health Sciences, Faculty of Medicine, Thessaloniki, Greece, **15** Third Department of Medical Oncology, Agii Anargiri Cancer Hospital, Athens, Greece, **16** Second Department of Medical Oncology, Hygeia Hospital, Athens, Greece, **17** Breast Unit, Metropolitan Hospital, Piraeus, Greece, **18** Department of Medical Oncology, 424 Army General Hospital, Thessaloniki, Greece, **19** Oncology Department, University General Hospital of Larissa, Larissa, Greece, **20** Breast Clinic, REA Hospital, Piraeus, Greece, **21** Translational Research Section, Hellenic Cooperative Oncology Group, Athens, Greece, **22** Laboratory of Molecular Oncology, Hellenic Foundation for Cancer Research/Aristotle University of Thessaloniki, Thessaloniki, Greece, **23** Department of Hygiene, Epidemiology and Medical Statistics, National and Kapodistrian University of Athens School of Medicine, Athens, Greece, **24** Aristotle University of Thessaloniki, Thessaloniki, Greece

† Deceased.
* agoussia@uoi.gr

Abstract

Several studies support an important role of angiogenesis in breast cancer growth and metastasis. The main objectives of the study were to investigate the immunohistochemical expression of vascular endothelial growth factor (VEGF) family ligands (VEGF-A and VEGF-C) and receptors (VEGFR1, VEGFR2 and VEGFR3) in breast cancer and their associations with clinicopathological parameters, cancer subtypes/subgroups and patient

Competing interests: As per Journal requirements, and on behalf of all authors, the authors confirm that they have the following interests: HG: Honoraria from BMS, MSD, Roche, Novartis, AMGEN. Research grants from BMS, Roche, Novartis. ES: Advisory Board of Merck, MSD, AstraZeneca, Roche, Amgen and Genesis. GF: Advisory Board of Pfizer, Sanofi and Roche. Honoraria from AstraZeneca. This does not alter the authors' adherence to all the PLOS ONE policies on sharing data and materials. The rest of the authors have declared that no competing interests exist.

outcome. Formalin-fixed paraffin-embedded tumor tissue samples were collected from early-stage breast cancer patients treated with anthracycline-based chemotherapy within a randomized trial. Immunohistochemistry was performed on serial 2.5 μm thick tissue sections from tissue microarray blocks. High VEGF-A, VEGF-C, VEGFR1, VEGFR2 and VEGFR3 protein expression was observed in 11.8% (N = 87), 80.8% (N = 585), 28.1% (N = 202), 64.6% (N = 359) and 71.8% (N = 517) of the cases, respectively. Significant associations were observed among all proteins (all p-values <0.05), with the exception of the one between VEGF-C and VEGFR1 (chi-square test, $p = 0.15$). Tumors with high VEGF-A protein expression, as compared to tumors with low expression were more frequently ER/PgR-negative (33.3% vs. 20.8%, chi-square test, $p = 0.009$) and HER2-positive (44.8% vs. 20.6%, $p < 0.001$). In addition, tumors with high VEGFR1 expression, were more frequently HER2-positive (32.8% vs. 19.6%, $p < 0.001$), while tumors with high VEGFR3 expression were more frequently ER/PgR-negative (24.9% vs. 17.0%, $p = 0.024$) and HER2-positive (26.9% vs. 14.8%, $p = 0.001$). High VEGF-A and VEGF-C protein expression was associated with increased DFS in the entire cohort (HR = 0.57, 95% CI 0.36–0.92, Wald's $p = 0.020$ and HR = 0.71, 95% CI 0.52–0.96, $p = 0.025$, respectively), as well as in specific subtypes/subgroups, such as HER2-positive (VEGF-A, HR = 0.32, 95% CI 0.14–0.74, $p = 0.008$) and triple-negative (VEGF-C, HR = 0.44, 95% CI 0.21–0.91, $p = 0.027$) patients. High vs. low VEGFR1 expression was an unfavorable factor for DFS in triple-negative patients (HR = 2.74, 95% CI 1.26–5.98, $p = 0.011$), whereas the opposite was observed among the ER/PgR-positive patients (HR = 0.69, 95% CI 0.48–0.98, $p = 0.041$). Regarding OS, high VEGF-C protein expression was associated with increased OS in the entire cohort (HR = 0.64, 95% CI 0.46–0.89, Wald's $p = 0.008$), as well as in in specific subtypes/subgroups, such as ER/PgR-negative (HR = 0.37, 95% CI 0.20–0.71, $p = 0.003$) and triple-negative (HR = 0.42, 95% CI 0.19–0.90, $p = 0.026$) patients. In conclusion, high expression of angiogenesis-related proteins is associated with adverse clinicopathological parameters in early-stage breast cancer patients and may be surrogate markers of biologically distinct subgroups of ER/PgR-negative or triple-negative tumors with superior outcome. Further validation of our findings in independent cohorts is needed.

Introduction

Experimental and clinical studies suggest that breast cancer is an angiogenic dependent disease and that angiogenesis plays an important role in tumor development and metastasis[1, 2]. A number of angiogenic factors are expressed by several human tumors, including breast cancer. Among them, the most important are the members of the vascular endothelial growth factor (VEGF) family and their receptors (VEGFRs). The VEGF family consists of five structurally homologous proteins: VEGF-A, -B, -C, -D and -E, with the first three being better characterized in terms of mechanism of action. VEGF-A and -B are considered mainly angiogenic, while VEGF-C mediates lymphangiogenesis. The biological effects of VEGF-A are mediated by two tyrosine kinase receptors: VEGFR1 and VEGFR2. Both receptors are predominantly expressed in vascular endothelial cells[3]. VEGFR2 mediates major growth and permeability actions of VEGF-A[4], whereas VEGFR1 has a weaker signal transducing ability either by acting as a decoy receptor or by suppressing signaling through VEGFR2[5]. VEGF-B forms

heterodimers with VEGF-A and has two binding receptors: VEGFR1 and neuropilin-1(3). VEGF-C mediates lymphangiogenesis through binding to VEGFR3 and angiogenesis through binding to VEGFR2[6].

The main prognostic biomarkers in early-stage breast cancer are tumor size, grade, lymph node status, number of positive lymph nodes, estrogen and progesterone receptor (ER, PgR) status and epidermal growth factor receptor 2 (HER2) status[7]. These variables are used to identify patients who are more likely to benefit from hormonal therapy and adjuvant chemotherapy. To improve the therapeutic ratio of breast cancer patients, research efforts focus on the identification of other prognostic and predictive biomarkers. Current evidence indicates that breast carcinoma cells express angiogenic factors at the mRNA or protein level[1, 8–21] and their clinicopathological significance has been studied in both, node-negative and node-positive breast cancers with inconsistent results[8, 10, 13–15, 18, 20–23]. The conflicting findings are in part due to the molecular heterogeneity of the disease, to different antibodies used and to variability in the selected cut-offs. Although the role of VEGF-A is more well studied and in several reports its expression has been correlated with adverse clinicopathological parameters[8, 14], the significance of the other ligands and receptors is yet unclear.

In the present study, we evaluated the immunohistochemical expression of VEGF-A, VEGF-C, VEGFR1, VEGFR2 and VEGFR3 in tumor tissue sections of early-stage breast cancer patients who had participated in a randomized adjuvant chemo-hormonotherapy trial of the Hellenic Cooperative Oncology Group (HeCOG)[24] and correlated it with clinicopathological parameters, cancer subtypes and survival outcome.

Materials and methods

Study population

This is translational research study among 1,086 early-stage breast cancer patients, enrolled in a prospective randomized phase III trial (HE10/00) of the Hellenic Cooperative Oncology Group (HeCOG), that was included in the Australian New Zealand Clinical Trials Registry (ANZCTR) and allocated Registration Number ACTRN12609001036202. The clinical protocol was approved by the HeCOG Protocol Review Committee, the Institutional Review Board of the AHEPA University and by the Bioethics Committee of the Aristotle University of Thessaloniki, School of Medicine. All patients signed a written informed consent for the use of their biological material for future research purposes.

The patients were randomized to receive concurrent or dose-dense sequential administration of epirubicin (E) and paclitaxel (T), followed by dose-dense cyclophosphamide/methotrexate/fluorouracil (CMF) (ET-CMF versus E-T-CMF)(24). By study design, all patients were female, with the cumulative doses and the chemotherapy duration being identical in the two arms but dose intensity of epirubicin and paclitaxel being double in the E-T-CMF arm. Prophylactic administration of granulocyte-colony stimulating factor (G-CSF) was administered in all cycles with CMF. Premenopausal women received additional treatment with lutenizing hormone-releasing hormone (LH-RH) analogs for two years. Postmenopausal patients received tamoxifen 20 mg daily for two-three years followed two-three years of daily exemestane 25 mg. Hormonal therapy or radiation therapy was administered after the completion of chemotherapy.

Baseline characteristics and clinical outcomes of this trial have recently been described(24). Tumor size, histological grade and lymph node status were obtained from the pathology report.

Tumor tissue samples

Formalin-fixed paraffin embedded (FFPE) tumor tissue samples were retrieved from the HeCOG Tumor Tissue Repository and a hematoxylin-eosin evaluation round was employed

for obtaining cores for tissue microarrays (TMAs) construction. In 749 out of 1,086 patients the tumor tissue was adequate for the construction of TMAs containing 2 cores for each tumor, as well as positive and negative controls for the tested antibodies. Cases not represented or inadequate on the TMAs sections were re-cut from the original blocks and whole tissue sections were used for immunohistochemical analysis. The REMARK diagram is shown in Fig 1.

Immunohistochemical procedure and interpretation

Immunohistochemistry was performed on serial 2.5 µm thick tissue sections from the TMAs or the original blocks. The histological sections were prepared at the Laboratory of Molecular

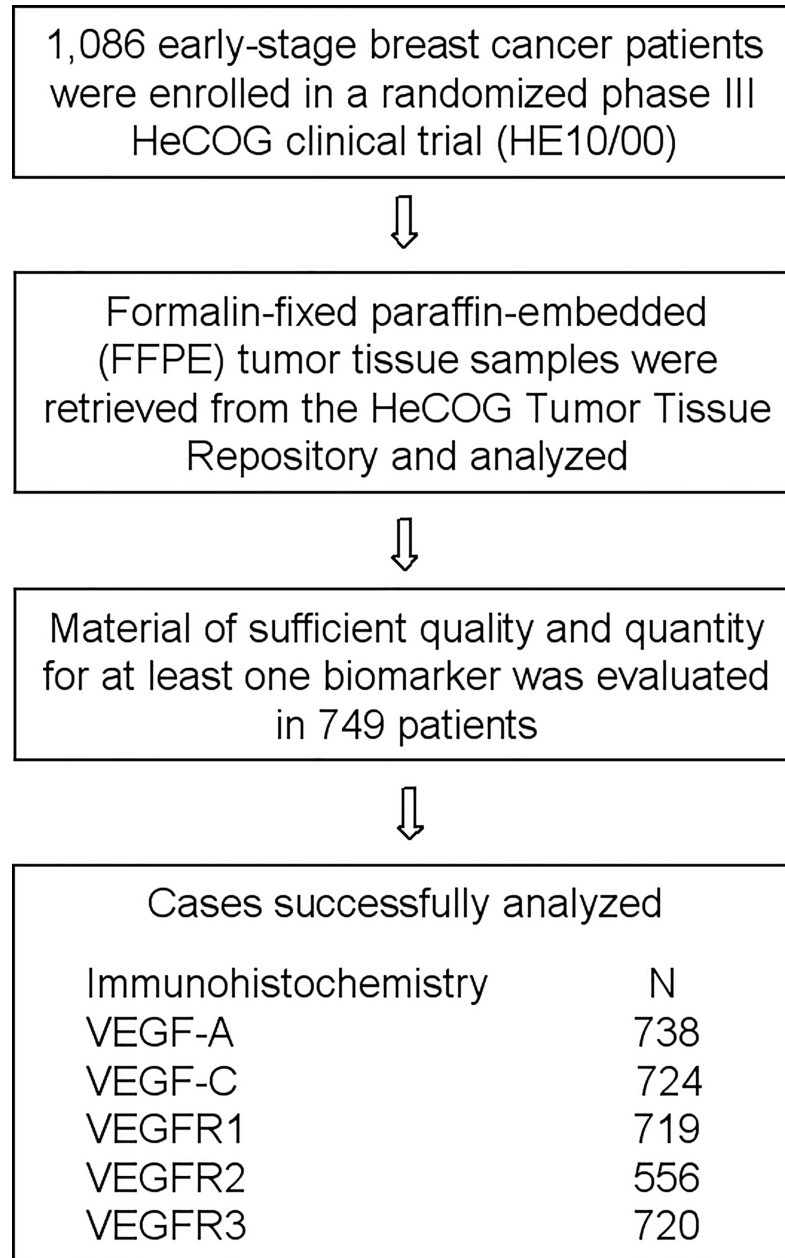


Fig 1. REMARK diagram detailing FFPE tumor tissue sample availability for the determination of immunohistochemical protein expression.

<https://doi.org/10.1371/journal.pone.0200302.g001>

Oncology of the Hellenic Foundation of Cancer Research/Aristotle University of Thessaloniki. The immunohistochemical procedure was performed using a Bond Max™ autostainer machine (Leica Microsystems, Wetzlar, Germany). The primary antibodies used, their source, dilutions and staining conditions are presented in Table 1, as previously described[21].

All tissue sections were evaluated independently by two pathologists blinded to the patients’ clinicopathological data. The immunostaining was estimated only in areas with well-preserved tumor morphology and the assessment examined for the expression of the angiogenesis-related proteins in neoplastic cells. Positive tumoral stromal cells, inflammatory cells or endothelial cells were used as internal positive controls. Different scoring methods of tumor staining for VEGF-A, VEGF-C, VEGFR1, VEGFR2 and VEGFR3 were assessed: a) the percentage of stained neoplastic cells, irrespective of staining intensity, b) the H-score (range 0–300) and the Allred score (sum 0–8), taking account the percentage of stained neoplastic cells and the staining intensity. For each of the above biomarkers, cut-offs determining high and low protein expression groups were calculated through: a) ROC curves enquiring for the “optimal” cut-off value of each biomarker to predict the probability of patients having 5-year disease-free survival (DFS), b) median for % intensity, 50 for H-score (0–50 negative, 51–300 positive), 2 for Allred score (0–2 negative, 3–8 positive), c) distribution quartiles. We evaluated the prognostic significance of the scoring methods described above and selected the percentage of tumor stained cells with cut-offs determined using the 5-year DFS ROC curves.

ER, PgR, HER2 and Ki67 protein expression was centrally evaluated for the characterization of immunophenotypical breast cancer subtypes. The staining procedures for estrogen receptor (ER, clone 6F11, dilution 1:70, Novocastra™, Leica Biosystems, Newcastle, U.K), progesterone receptor (PgR, clone 1A6, dilution 1:70, Novocastra™, Leica Biosystems), HER2 (A0485 polyclonal antibody, dilution 1:200, Dako, Glostrup, Denmark) and Ki67 (clone MIB-1, dilution 1:70, Dako) were performed using a Bond Max™ autostainer (Leica Microsystems), as previously described in detail(25). Fluorescence in situ hybridization (FISH) analysis, using the ZytoLight® SPEC HER2/TOP2A/CEP17 triple-color probe (Z-2073, ZytoVision, Bremerhaven, Germany) was performed in all HER2 immunohistochemistry 2+ cases [25].

The evaluation of all sections was done by two experienced breast cancer pathologists, blinded as to the patients’ clinical characteristics and survival data. Briefly, HER2 protein expression was scored in a scale from 0 to 3+, the latter corresponding to uniform, intense membrane staining in >30% invasive tumor cells[26]; ER and PgR were considered positive if staining was present in ≥1% of tumor cell nuclei[27]; and, for Ki67, the expression was defined

Table 1. Primary antibodies, clone, source, dilution and staining conditions used in the present study.

Antibody	Clone/Source	Dilution	Antigen Retrieval	Incubation Time
VEGF-A (m)	VG1 (1)	1:75	20/EDTA	60
VEGF-C (r, PL)	Z-CVC7 (2)	1:250	20/CA	Overnight
VEGFR1 (r)	RB-1527 (3)	1:450	15/CA	Overnight
VEGFR2 (r)	55B11 (4)	1:450	20/EDTA	Overnight
VEGFR3 (m)	KLT9 (5)	1:50	15/CA	Overnight

m, mouse; r, rabbit; PL, polyclonal; EDTA, ethylene diamine tetraacetate, PH 8.8; CA, citric acid, PH 6.0

(1), Dako, Glostrup, Denmark

(2), Zymed™, Invitrogen, Carlsbad, CA

(3), Thermo Fisher Scientific, Fremont, CA

(4), Cell Signalling Technology, Beverly, MA

(5), Novocastra™, Leica Biosystems, Newcastle Upon Tyne, UK.

<https://doi.org/10.1371/journal.pone.0200302.t001>

as low (<20%) or high (\geq 20%) based on the percentage of stained/unstained nuclei from the tumor areas[28].

Statistical analysis

For the description of continuous variables the mean (standard deviation), median and range (min-max) were used, while categorical variables were presented as frequencies (%). The chi-square test was used for group comparisons of categorical data.

Statistical analyses focused on the examination of the aforementioned immunohistochemical markers with overall survival (OS) and DFS after adjusting for certain clinicopathological characteristics. Overall survival (OS) was defined as the time from the date of diagnosis with breast cancer to the date of patient's death or last contact, while DFS was defined as the time from the date of diagnosis to documented first relapse, death without prior documented relapse or last contact, whichever occurred first. Surviving patients were censored at the date of last contact. Women who died without prior relapse were treated as events, that is as having had relapsed at the date of their death. Survival curves were estimated using the Kaplan-Meier method and compared across groups with the log-rank test. The associations between the examined factors and relapse/mortality rates were evaluated with hazard ratios (HRs) estimated with the Cox proportional hazards model. The proportional hazards assumption was tested by evaluating the statistical significance of the time-dependent association between each variable and relapse/mortality rates. The following parameters were studied in relation to DFS/OS: 1) clinicopathological, such as age ($>$ median, \leq median), number of positive lymph nodes (0–3, \geq 4), tumor size (\leq 2, $>$ 2cm), type of operation (breast-conserving surgery, modified radical mastectomy), breast cancer subtypes (luminal A, luminal B, luminal-HER2, HER2-enriched, triple-negative) 2) angiogenesis-related proteins (high vs. low expression), the cut-off values resulting from the ROC curve analyses (see [methods](#) above) hereafter referred to as 5-years DFS ROC curve cut-off. In multivariate analysis, we estimated the effect (HR) of each immunohistochemical marker adjusted for the effect of the clinicopathological parameters that were statistically significant ($p < 0.05$) in the univariate analysis.

The above-indicated analyses were performed in the entire cohort of patients with available data for each marker examined and in the patient subgroups defined by: breast cancer subtypes (luminal A, luminal B, luminal-HER2, HER2-enriched, triple-negative), ER/PgR status (ER/PgR-positive, ER/PgR-negative) and HER2 status (HER2-positive, HER2-negative).

The statistical analyses were performed using the SAS software (SAS for Windows, version 9.3, SAS Institute Inc., Cary, NC). Statistical significance was set at 2-sided $p = 0.05$.

Results

Patient and tumor characteristics

Selected patient and tumor characteristics are shown in [Table 2](#) for the entire cohort and by treatment arm (these characteristics are also tabulated according to expression of one or more of the VEGF markers in [S3 Table](#)). The median age was 53.7 years (range 22–79). Most patients were postmenopausal (55.8%) and had invasive ductal carcinomas not otherwise specified (NOS, 81.8%), tumor size $>$ 2 cm (69.6%), positive lymph nodes \geq 4 (52.0%), ER/PgR-positive (77.7%) and HER2-negative (76.9%) status. According to ER, PgR, HER2 and Ki67 status, tumors were classified as luminal A (ER- and/or PgR-positive, HER2-negative and Ki67 $<$ 20%; 40.0%), luminal B (ER- and/or PgR-positive, HER2-negative and Ki67 \geq 20%; 22.8%), luminal-HER2 (ER- and/or PgR-positive and HER2-positive; 14.5%), HER2-enriched (ER-negative, PgR-negative, HER2-positive; 9.1%) and triple-negative breast cancer (TNBC, ER/PgR/HER2-negative; 13.6%).

Table 2. Basic patient and tumor characteristics in the entire cohort and by treatment arm (E-T-CMF, ET-CMF).

Parameter	Entire cohort (N = 749)	E-T-CMF (N = 373)	ET-CMF (N = 376)
	N (%)		
Age			
Mean (standard deviation)	53.5 (11.1)	53.2 (11.1)	53.9 (11.1)
Range	22–79	29–79	22–76
Menopausal status			
Postmenopausal	418 (55.8)	201 (53.9)	217 (57.7)
Premenopausal	325 (43.4)	171 (45.8)	154 (41.0)
Not reported	6 (0.8)	1 (0.3)	5 (1.3)
Type of surgery			
Modified radical mastectomy	482 (64.4)	246 (66)	236 (62.8)
Partial/Simple mastectomy	259 (34.6)	124 (33.2)	135 (36.0)
Not reported	8 (1.0)	3 (0.8)	5 (1.4)
Tumor size			
≤2	222 (29.6)	107 (28.7)	115 (30.6)
>2	521 (69.6)	265 (71.0)	256 (68.1)
Not reported	6 (0.8)	1 (0.3)	5 (1.3)
Number of positive nodes			
0–3	354 (47.2)	170 (45.6)	184 (49.0)
≥4	389 (52.0)	202 (54.1)	187 (49.7)
Not reported	6 (0.8)	1 (0.3)	5 (1.3)
Histological classification			
Invasive ductal	612 (81.8)	309 (82.8)	303 (80.6)
Invasive lobular	72 (9.6)	29 (7.8)	43 (11.5)
Mixed	47 (6.2)	27 (7.2)	20 (5.3)
Other	12 (1.6)	7 (1.9)	5 (1.3)
Not reported	6 (0.8)	1 (0.3)	5 (1.3)
ER/PgR status			
<i>Informative*</i>	736 (98.3)	364 (97.6)	372 (98.9)
Positive (ER and/or PgR positive)	572 (77.7)	280 (76.9)	292 (78.5)
Negative (ER and PgR negative)	164 (22.3)	84 (23.1)	80 (21.5)
HER2 status			
<i>Informative**</i>	745 (99.5)	372 (9.7)	373 (99.2)
Positive	172 (23.1)	89 (23.9)	83 (22.3)
Negative	573 (76.9)	283 (76.1)	290 (77.7)
Subtypes			
<i>Informative***</i>	718 (95.9)	356 (95.4)	362 (96.3)
Luminal A	287 (40.0)	139 (39.0)	148 (40.9)
Luminal B	164 (22.8)	82 (23.0)	82 (22.7)
Luminal-HER2	104 (14.5)	51 (14.3)	53 (14.6)
HER2-enriched	65 (9.1)	36 (10.1)	29 (8.0)
Triple-negative	98 (13.6)	48 (13.5)	50 (13.8)
Histological grade			
I-II	371 (49.6)	182 (48.8)	189 (50.2)
III-IV	368 (49.2)	189 (50.6)	179 (47.6)
Not reported	10 (1.4)	2 (0.6)	8 (2.2)
Hormonal therapy			

(Continued)

Table 2. (Continued)

Parameter	Entire cohort (N = 749)	E-T-CMF (N = 373)	ET-CMF (N = 376)
	N (%)		
Yes	543 (72.4)	263 (70.5)	280 (74.5)
No	182 (24.2)	100 (26.8)	82 (21.8)
Unknown	24 (3.2)	10 (2.7)	14 (3.7)
Radiotherapy			
Yes	546 (72.8)	261 (70.0)	285 (75.8)
No	171 (22.8)	98 (26.3)	73 (19.4)
Unknown	32 (4.2)	14 (3.7)	18 (4.8)

*Data available from central evaluation of ER and PgR protein expression

**Data available from central evaluation of HER2 protein expression and HER2 amplification in 2+ cases

***Data available from central evaluation of ER/PgR and HER2 status and Ki67 protein expression.

<https://doi.org/10.1371/journal.pone.0200302.t002>

Associations among the angiogenic factors

The basic descriptive statistics for each of the angiogenic factors protein expression are shown in **S1 Table**. The cut-off values, identified through ROC analyses and used to dichotomize these markers according to their high/low expression, are also shown in this Table. The median percentage of stained tumor cells were 2.5%, 90.0%, 8.5%, 70.0% and 47.5% for VEGF-A, VEGF-C, VEGFR1, VEGFR2 and VEGFR3, respectively. High protein expression was observed in 11.8% (N = 87; cut-off = 55.0% VEGF-A positive cells), 80.8% (N = 585; cut-off = 72.5% VEGF-C positive cells), 28.1% (N = 202; cut-off = 43.5% VEGFR1 positive cells), 64.6% (N = 359; cut-off = 55.0% VEGFR2 positive cells) and 71.8% (N = 517; cut-off = 13.5% VEGFR3 positive cells) of the cases, respectively. The associations among these proteins are presented in **S2 Table**. Significant associations were observed among almost all proteins (all p-values ≤ 0.05), with the exception of the one between VEGF-C and VEGFR1 (chi-square test, $p = 0.15$).

Associations between the angiogenic factors and clinicopathological parameters

The associations between the angiogenic factors and clinicopathological characteristics are presented in **S3 Table**. As is evident in this Table, some comparisons were carried out in the presence of very small cell counts, and therefore should be interpreted with care. Tumors with high VEGF-A protein expression, as compared to tumors with low expression were more frequently ER/PgR-negative (33.3% vs. 20.8%, chi-square test, $p = 0.009$) and HER2-positive (44.8% vs. 20.6%, $p < 0.001$). In addition, tumors with high VEGFR1 expression, as compared to tumors with low expression, were more frequently HER2-positive (32.8% vs. 19.6%, $p < 0.001$). Finally, tumors with high VEGFR3 expression were more frequently ER/PgR-negative (24.9% vs. 17.0%, $p = 0.024$) and HER2-positive (26.9% vs. 14.8%, $p = 0.001$).

Regarding the associations of the angiogenic factors with breast cancer subtypes, it was demonstrated that tumors with high VEGF-A expression, as compared to tumors with low expression, were more frequently of the HER2-positive subtypes (overall chi-square $p < 0.001$). Moreover, tumors with high VEGFR1 expression were more frequently of the HER2-enriched subtype ($p < 0.001$).

Effect of angiogenic factors on outcome

Latest available survival status of the patients was retrieved from the HeCOG's electronic database on April 2018. After a median follow-up period of 123.8 months (range 0.5–188.3), 257 DFS events and 201 deaths were recorded. Median OS was 172.5 (95% CI 172.5-not reached), while median DFS was not reached up to the date of the analyses.

The age-adjusted and multivariable-adjusted Cox regression analyses, for the entire cohort and for subgroups defined by breast cancer subtypes, ER/PgR status and HER2 status are presented in **Tables 3** and **4** for DFS and OS, respectively. In multivariable analyses, HRs were adjusted for breast surgery, tumor size, number of positive lymph nodes and breast cancer subtypes/subgroups (where appropriate). The number of patients/events in some of these subgroups is small and therefore results from these analyses should be interpreted with care.

Disease-free survival

The direction and statistical significance of the estimated HRs were the same in both age-adjusted and multivariate analyses. Among all patients, high VEGF-A and VEGF-C protein expression was associated with increased DFS (**Table 3**). When patients were analyzed according to breast cancer subtypes, high as compared to low expression of VEGF-A was a favorable factor for DFS among luminal-HER2 and HER2-positive patients. High VEGF-C expression was associated with increased DFS in patients with luminal B, TNBC, ER/PgR-negative and HER2-negative tumors. In the ER/PgR-positive subgroup, high VEGFR3 expression was associated with increased relapse (HR = 1.43, 95% CI 1.01–2.01, Wald's $p = 0.042$). High vs. low expression of VEGFR1 protein was deemed unfavorable for the risk of relapse in the TNBC subgroup (HR = 2.74, 95% CI 1.26–5.98, $p = 0.011$), whereas the opposite was observed among the ER/PgR positive breast cancer patients (HR = 0.69, 95% CI 0.48–0.98, $p = 0.041$). Of note the association of high VEGFR1 expression with DFS was not statistically significant in the entire population of women (HR = 0.80, 95% CI 0.59–1.09, $p = 0.151$). None of the examined factors was statistically significant among women with HER-enriched or luminal A tumors.

Overall survival

In the entire population of women only high as compared to low VEGF-C protein expression was associated with increased survival (HR = 0.64, 95% CI 0.46–0.89, Wald's $p = 0.008$) (**Table 4**). High expression of VEGF-C was also favorably associated with survival in patients with luminal B, HER-enriched and TNBC breast cancer subtypes and ER/PgR-negative and HER2-negative tumors. In addition, high vs. low expression of VEGFR2 was associated with prolonged survival among luminal B patients but only in the age-adjusted analysis. Among ER/PgR-positive patients VEGFR3 (high vs. low) was marginally statistically significantly associated with increased mortality (HR = 1.49, 95% CI 1.00–2.21, $p = 0.048$). None of the examined factors was statistically significantly associated with survival among women with HER2-positive, luminal-HER2 and luminal A breast cancer subtypes. Kaplan-Meier curves of DFS probability and OS probability according to VEGF-A and VEGF-C expression are presented in **Fig 2**.

Results from the univariate Cox regression analyses in the entire cohort for each of the clinicopathological parameters are presented in **S4 Table**. Breast conserving surgery, lower tumor size, number of positive lymph nodes and positive ER/PgR status were associated with improved outcome in terms of both DFS and OS.

Table 3. Hazard ratios and 95% CIs estimated from age-adjusted and multivariable-adjusted* Cox regression analyses with respect to DFS for the high vs. low expression of each of the angiogenesis-related proteins in the entire cohort and in selected subgroups.

	N of patients		N of events		Age-adjusted			Multivariable-adjusted*		
	High vs. low	High vs. low	HR	95% CI	p-value	HR	95% CI	p-value		
Entire Cohort										
VEGF-A	87 vs. 651	20 vs. 234	0.60	0.38–0.95	0.030	0.57	0.36–0.92	0.020		
VEGF-C	585 vs. 139	193 vs. 59	0.72	0.54–0.97	0.028	0.71	0.52–0.96	0.025		
VEGFR1	202 vs. 517	61 vs. 187	0.82	0.61–1.09	0.174	0.80	0.59–1.09	0.151		
VEGFR2	359 vs. 197	119 vs. 75	0.82	0.61–1.09	0.170	0.83	0.61–1.12	0.217		
VEGFR3	517 vs. 203	183 vs. 63	1.20	0.90–1.60	0.209	1.29	0.95–1.74	0.102		
Luminal A										
VEGF-A	22 vs. 258	6 vs. 82	0.91	0.40–2.08	0.815	1.17	0.50–2.71	0.716		
VEGF-C	216 vs. 60	64 vs. 21	0.86	0.53–1.42	0.567	0.85	0.52–1.39	0.519		
VEGFR1	74 vs. 202	18 vs. 70	0.64	0.38–1.08	0.094	0.69	0.41–1.15	0.156		
VEGFR2	143 vs. 75	39 vs. 26	0.75	0.46–1.24	0.267	0.71	0.43–1.18	0.182		
VEGFR3	185 vs. 89	59 vs. 27	1.20	0.76–1.90	0.426	1.28	0.81–2.03	0.297		
Luminal B										
VEGF-A	14 vs. 149	5 vs. 51	0.99	0.40–2.49	0.990	1.53	0.58–3.98	0.388		
VEGF-C	132 vs. 31	41 vs. 16	0.52	0.29–0.93	0.028	0.53	0.30–0.95	0.034		
VEGFR1	44 vs. 114	11 vs. 42	0.63	0.32–1.22	0.171	0.57	0.29–1.12	0.103		
VEGFR2	80 vs. 38	24 vs. 17	0.59	0.32–1.10	0.098	0.66	0.35–1.24	0.192		
VEGFR3	111 vs. 48	41 vs. 12	1.34	0.69–2.60	0.382	1.65	0.86–3.17	0.130		
Luminal-HER2										
VEGF-A	21 vs. 83	3 vs. 36	0.27	0.08–0.89	0.031	0.27	0.08–0.90	0.034		
VEGF-C	86 vs. 17	35 vs. 4	1.87	0.66–5.30	0.240	1.67	0.59–4.76	0.335		
VEGFR1	30 vs. 72	10 vs. 28	0.85	0.41–1.75	0.656	0.74	0.35–1.54	0.420		
VEGFR2	48 vs. 25	19 vs. 11	0.86	0.41–1.83	0.703	0.97	0.46–2.07	0.941		
VEGFR3	80 vs. 22	32 vs. 6	1.67	0.70–4.02	0.251	1.77	0.73–4.29	0.206		
HER-enriched										
VEGF-A	18 vs. 47	3 vs. 21	0.34	0.10–1.15	0.083	0.44	0.13–1.53	0.198		
VEGF-C	57 vs. 7	20 vs. 4	0.53	0.18–1.57	0.254	0.57	0.19–1.73	0.319		
VEGFR1	36 vs. 27	11 vs. 13	0.60	0.27–1.35	0.215	0.70	0.31–1.58	0.387		
VEGFR2	30 vs. 17	11 vs. 8	0.74	0.29–1.88	0.526	0.87	0.33–2.26	0.771		
VEGFR3	56 vs. 8	19 vs. 4	0.57	0.19–1.68	0.305	0.69	0.23–2.04	0.504		
TNBC										
VEGF-A	11 vs. 85	3 vs. 34	0.66	0.20–2.21	0.503	0.52	0.16–1.74	0.291		
VEGF-C	74 vs. 19	25 vs. 11	0.43	0.21–0.88	0.020	0.44	0.21–0.91	0.027		
VEGFR1	13 vs. 82	9 vs. 27	2.92	1.36–6.27	0.006	2.74	1.26–5.98	0.011		
VEGFR2	49 vs. 33	22 vs. 10	1.44	0.68–3.08	0.344	1.35	0.63–2.88	0.443		
VEGFR3	70 vs. 26	28 vs. 9	1.25	0.59–2.67	0.566	1.05	0.49–2.28	0.899		
ER/PgR-positive										
VEGF-A	58 vs. 505	14 vs. 173	0.67	0.39–1.15	0.143	0.71	0.40–1.25	0.236		
VEGF-C	444 vs. 112	143 vs. 43	0.81	0.58–1.15	0.239	0.80	0.57–1.14	0.223		
VEGFR1	151 vs. 398	40 vs. 142	0.70	0.49–0.99	0.046	0.69	0.48–0.98	0.041		
VEGFR2	276 vs. 141	84 vs. 54	0.74	0.52–1.04	0.081	0.76	0.53–1.07	0.115		
VEGFR3	383 vs. 166	133 vs. 47	1.32	0.95–1.85	0.099	1.43	1.01–2.01	0.042		
ER/PgR-negative										
VEGF-A	29 vs. 133	6 vs. 55	0.47	0.20–1.09	0.078	0.47	0.20–1.11	0.086		
VEGF-C	131 vs. 26	45 vs. 15	0.46	0.26–0.84	0.011	0.46	0.25–0.85	0.013		

(Continued)

Table 3. (Continued)

	N of patients	N of events	Age-adjusted			Multivariable-adjusted*		
	High vs. low	High vs. low	HR	95% CI	p-value	HR	95% CI	p-value
VEGFR1	49 vs. 110	20 vs. 40	1.23	0.72–2.12	0.444	1.32	0.70–2.47	0.391
VEGFR2	79 vs. 50	33 vs. 18	1.09	0.61–1.95	0.762	1.06	0.59–1.89	0.842
VEGFR3	127 vs. 34	47 vs. 13	1.00	0.54–1.86	0.993	0.93	0.50–1.75	0.827
HER2-positive								
VEGF-A	39 vs. 133	6 vs. 58	0.30	0.13–0.71	0.006	0.32	0.14–0.74	0.008
VEGF-C	145 vs. 24	56 vs. 8	1.20	0.57–2.52	0.639	1.12	0.53–2.37	0.773
VEGFR1	66 vs. 101	21 vs. 42	0.74	0.44–1.26	0.270	0.71	0.41–1.23	0.224
VEGFR2	80 vs. 42	31 vs. 19	0.81	0.46–1.45	0.486	0.91	0.51–1.64	0.765
VEGFR3	138 vs. 30	52 vs. 10	1.20	0.60–2.36	0.608	1.17	0.59–2.33	0.648
HER2-negative								
VEGF-A	48 vs. 514	14 vs. 174	0.84	0.49–1.45	0.528	0.89	0.51–1.55	0.680
VEGF-C	437 vs. 115	135 vs. 51	0.62	0.45–0.86	0.004	0.64	0.46–0.89	0.008
VEGFR1	135 vs. 413	40 vs. 143	0.82	0.58–1.17	0.269	0.85	0.59–1.23	0.396
VEGFR2	278 vs. 153	88 vs. 54	0.83	0.59–1.16	0.269	0.80	0.56–1.13	0.197
VEGFR3	375 vs. 173	129 vs. 53	1.18	0.86–1.63	0.305	1.32	0.94–1.84	0.108

*Adjusted for breast surgery, tumor size, number of positive lymph nodes and breast cancer subtypes (where appropriate).

N, number; HR, hazard ratio; CI, confidence interval; TNBC, triple-negative breast cancer.

Significant p-values are shown in bold.

<https://doi.org/10.1371/journal.pone.0200302.t003>

Discussion

Herein we analyzed the immunohistochemical expression of VEGF family members in a large cohort of early breast cancer patients treated in a randomized trial with long-term follow-up and evaluated their association with clinicopathological parameters, clinical subtypes and survival outcome.

Up-regulation of VEGF family members has been found in invasive breast carcinomas by immunohistochemical, PCR or Western blot approaches[1, 8–21]. However, in most series the analysis of the angiogenic markers has been investigated by immunohistochemistry and several studies have examined their prognostic and/or predictive value with some supporting[8, 14, 18, 22] and others refuting an adverse effect[10, 13, 15, 23, 29]. The conflicting results may be due to the molecular heterogeneity of the disease, to different antibodies used and to variability in the selected cut-offs.

In our study, conducted in the pre-trastuzumab era, we found that high VEGF-A, VEGF-C, VEGFR2 and VEGFR3 protein expression was associated with certain adverse prognostic factors, such as ER/PgR-negative and/or HER2-positive status, suggesting that high protein expression of VEGF family members might have a negative prognostic impact on DFS or OS. Our findings are consistent with the results of recent studies[19, 29, 30] and suggest that subgroups of patients with enhanced tumor angiogenesis seem to have an unfavorable profile, while signaling through VEGF receptors in cancer cells can promote events associated with tumor progression. Existing evidence suggests that HER2 activation is one of the several mechanisms that promote angiogenesis and that HER2-amplified breast cancers have increased angiogenesis[31]. Data from preclinical models report that in NIH 3T3 fibroblasts, which have been transfected to overexpress HER2, VEGF-A production was increased and this overproduction was blocked using a monoclonal antibody directed to HER2[32]. Therefore, the evaluation of VEGF family members could prove to be more useful when analyzed in combination

Table 4. Hazard ratios and 95% CIs estimated from age-adjusted and multivariable-adjusted* Cox regression analyses with respect to OS for the high vs. low expression of each of the angiogenesis-related proteins in the entire cohort and in selected subgroups.

	N of patients		N of events		Age-adjusted			Multivariable-adjusted*		
	High vs. low	High vs. low	HR	95% CI	p-value	HR	95% CI	p-value		
Entire Cohort										
VEGF-A	87 vs. 651	19 vs. 180	0.79	0.49–1.26	0.316	0.77	0.48–1.26	0.301		
VEGF-C	585 vs. 139	148 vs. 49	0.68	0.49–0.94	0.020	0.64	0.46–0.89	0.008		
VEGFR1	202 vs. 517	46 vs. 147	0.81	0.58–1.14	0.227	0.80	0.57–1.14	0.221		
VEGFR2	359 vs. 197	90 vs. 58	0.82	0.59–1.15	0.255	0.81	0.58–1.14	0.236		
VEGFR3	517 vs. 203	142 vs. 49	1.22	0.88–1.70	0.225	1.29	0.92–1.81	0.142		
Luminal A										
VEGF-A	22 vs. 258	5 vs. 53	1.23	0.49–3.09	0.656	1.72	0.68–4.38	0.253		
VEGF-C	216 vs. 60	41 vs. 15	0.82	0.45–1.49	0.518	0.79	0.44–1.44	0.442		
VEGFR1	74 vs. 202	12 vs. 46	0.69	0.36–1.30	0.247	0.76	0.40–1.43	0.392		
VEGFR2	143 vs. 75	23 vs. 18	0.67	0.36–1.24	0.202	0.61	0.32–1.15	0.128		
VEGFR3	185 vs. 89	38 vs. 18	1.21	0.69–2.13	0.510	1.25	0.71–2.22	0.436		
Luminal B										
VEGF-A	14 vs. 149	5 vs. 46	1.19	0.47–3.00	0.719	1.85	0.70–4.88	0.217		
VEGF-C	132 vs. 31	36 vs. 15	0.54	0.29–0.98	0.044	0.53	0.29–0.98	0.043		
VEGFR1	44 vs. 114	10 vs. 38	0.66	0.33–1.32	0.235	0.64	0.31–1.29	0.212		
VEGFR2	80 vs. 38	20 vs. 17	0.48	0.25–0.91	0.026	0.56	0.29–1.09	0.090		
VEGFR3	111 vs. 48	37 vs. 11	1.41	0.70–2.83	0.334	1.68	0.85–3.32	0.135		
Luminal-HER2										
VEGF-A	21 vs. 83	3 vs. 29	0.38	0.12–1.26	0.115	0.36	0.11–1.21	0.098		
VEGF-C	86 vs. 17	29 vs. 3	2.32	0.70–7.66	0.167	2.18	0.65–7.25	0.205		
VEGFR1	30 vs. 72	8 vs. 23	0.91	0.41–2.05	0.825	0.82	0.36–1.86	0.639		
VEGFR2	48 vs. 25	17 vs. 6	1.57	0.62–4.00	0.343	1.72	0.66–4.44	0.265		
VEGFR3	80 vs. 22	26 vs. 5	1.69	0.65–4.44	0.283	1.94	0.73–5.16	0.184		
HER-enriched										
VEGF-A	18 vs. 47	3 vs. 15	0.59	0.17–2.06	0.404	1.04	0.29–3.82	0.948		
VEGF-C	57 vs. 7	14 vs. 4	0.28	0.09–0.89	0.031	0.27	0.08–0.89	0.032		
VEGFR1	36 vs. 27	7 vs. 11	0.52	0.20–1.38	0.192	0.61	0.23–1.61	0.316		
VEGFR2	30 vs. 17	8 vs. 6	0.94	0.30–2.89	0.912	0.99	0.31–3.19	0.991		
VEGFR3	56 vs. 8	13 vs. 4	0.58	0.16–2.06	0.400	0.67	0.19–2.37	0.538		
TNBC										
VEGF-A	11 vs. 85	3 vs. 30	0.65	0.19–2.19	0.490	0.61	0.18–2.06	0.428		
VEGF-C	74 vs. 19	22 vs. 10	0.42	0.20–0.90	0.025	0.42	0.19–0.90	0.026		
VEGFR1	13 vs. 82	7 vs. 25	1.95	0.84–4.55	0.123	1.80	0.76–4.28	0.184		
VEGFR2	49 vs. 33	19 vs. 10	1.14	0.53–2.47	0.740	1.10	0.50–2.39	0.815		
VEGFR3	70 vs. 26	25 vs. 8	1.28	0.57–2.87	0.550	1.06	0.47–2.40	0.895		
ER/PgR-positive										
VEGF-A	58 vs. 505	13 vs. 131	0.86	0.49–1.53	0.617	0.98	0.54–1.77	0.934		
VEGF-C	444 vs. 112	109 vs. 34	0.83	0.56–1.22	0.349	0.77	0.52–1.13	0.182		
VEGFR1	151 vs. 398	31 vs. 108	0.75	0.50–1.12	0.159	0.74	0.49–1.12	0.155		
VEGFR2	276 vs. 141	61 vs. 41	0.73	0.49–1.08	0.118	0.77	0.51–1.15	0.202		
VEGFR3	383 vs. 166	102 vs. 35	1.40	0.95–2.07	0.086	1.49	1.00–2.21	0.048		
ER/PgR-negative										
VEGF-A	29 vs. 133	6 vs. 45	0.60	0.25–1.42	0.243	0.67	0.28–1.62	0.376		
VEGF-C	131 vs. 26	36 vs. 14	0.37	0.20–0.69	0.002	0.37	0.20–0.71	0.003		

(Continued)

Table 4. (Continued)

	N of patients	N of events	Age-adjusted			Multivariable-adjusted*		
			High vs. low	High vs. low	HR	95% CI	p-value	HR
VEGFR1	49 vs. 110	14 vs. 36	0.94	0.51–1.76	0.852	1.07	0.53–2.15	0.854
VEGFR2	79 vs. 50	27 vs. 16	1.07	0.57–2.02	0.836	1.01	0.53–1.91	0.977
VEGFR3	127 vs. 34	38 vs. 12	0.97	0.49–1.90	0.921	0.92	0.46–1.84	0.820
HER2-positive								
VEGF-A	39 vs. 133	6 vs. 45	0.46	0.20–1.09	0.079	0.48	0.20–1.13	0.094
VEGF-C	145 vs. 24	44 vs. 7	1.06	0.47–2.35	0.895	0.97	0.43–2.18	0.935
VEGFR1	66 vs. 101	15 vs. 35	0.71	0.39–1.31	0.275	0.71	0.38–1.33	0.286
VEGFR2	80 vs. 42	26 vs. 12	1.21	0.60–2.41	0.593	1.29	0.63–2.62	0.481
VEGFR3	138 vs. 30	40 vs. 9	1.11	0.53–2.29	0.787	1.18	0.56–2.49	0.658
HER2-negative								
VEGF-A	48 vs. 514	13 vs. 134	1.02	0.58–1.81	0.935	1.07	0.60–1.92	0.811
VEGF-C	437 vs. 115	103 vs. 42	0.61	0.43–0.88	0.008	0.59	0.41–0.86	0.005
VEGFR1	135 vs. 413	31 vs. 111	0.84	0.56–1.25	0.378	0.85	0.56–1.29	0.449
VEGFR2	278 vs. 153	64 vs. 45	0.73	0.50–1.07	0.108	0.70	0.47–1.03	0.069
VEGFR3	375 vs. 173	101 vs. 40	1.27	0.87–1.83	0.214	1.31	0.90–1.92	0.162

*Adjusted for breast surgery, tumor size, number of positive lymph nodes and breast cancer subtypes (where appropriate).

N, number; HR, hazard ratio; CI, confidence interval; TNBC, triple-negative breast cancer.

Significant p-values are shown in bold.

<https://doi.org/10.1371/journal.pone.0200302.t004>

with other markers, with potential to provide information regarding the profile of breast cancer aggressiveness.

In the analysis of angiogenic factors coexpression, an association between the ligands and receptors was observed. This observation suggests an “intracrine/autocrine” regulatory mechanism, supporting cancer cell autonomy, as has previously been suggested [18, 33] and supports the view of complementary functions of the angiogenesis pathways in the neoplastic cells.

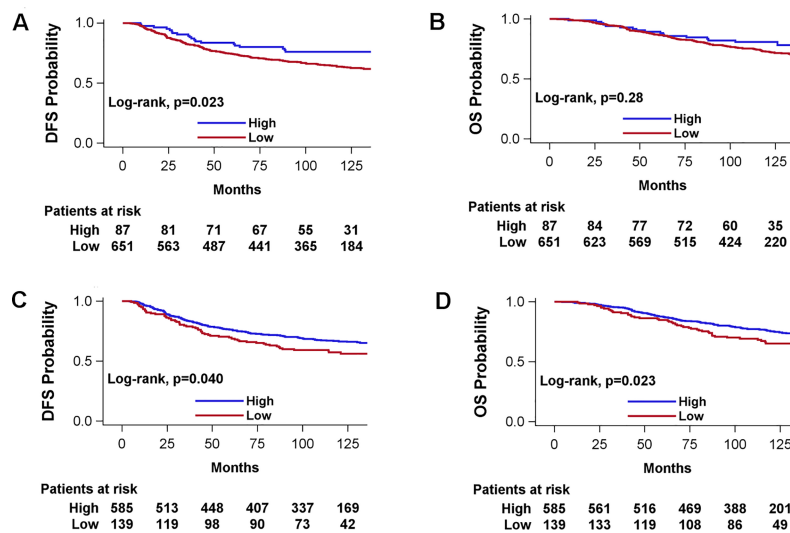


Fig 2. Kaplan-Meier curves according to VEGF-A (A, B) and VEGF-C (C, D) protein expression (5-years DFS ROC curve cut-off). A, C: DFS; B, D: OS.

<https://doi.org/10.1371/journal.pone.0200302.g002>

The issue of whether angiogenesis is enhanced in distinct breast cancer subtypes has been discussed in the literature[29, 30, 34–36]. Gene expression profiling has been used to classify breast carcinomas into molecular subtypes with significant differences in incidence, risk factors, prognosis and treatment sensitivity[37]. By using a panel of immunohistochemical markers, breast carcinomas have been classified into phenotypic subtypes, quite similar to those identified by gene expression profiling[25, 29, 34, 35, 38–40], although the correlation between gene expression and immunohistochemical subtyping has not been shown to be perfect[41]. Several studies have reported an enhanced angiogenesis, as assessed by VEGF-A, VEGF-A/VEGFR2, VEGF-C or VEGFR3 immunohistochemical or mRNA expression in luminal B, luminal-HER2, HER2-enriched and TNBC/basal-like tumors compared to the luminal A subtype[29, 30, 34, 35, 42]. In our study, tumors with high expression of VEGF-A and VEGFR1, compared with tumors with low expression, were more frequently of the HER2-positive subtypes (luminal-HER2, HER2-enriched), while tumors with high VEGFR3 expression were more frequently of the HER2-positive and TNBC subtypes. Breast cancer subtypes, defined by our classification scheme, have shown different prognosis, with luminal A tumors having better prognosis[25]. It seems that the expression of VEGF family members in breast cancer, when elevated, is associated with more aggressive cancer phenotypes and therefore more aggressive tumor biology.

According to our findings, the association of some angiogenic proteins with an aggressive tumor profile, namely the fact that higher expression was to be expected when the tumor was more aggressive, was not reflected by a negative prognostic effect on DFS or OS. Existing data regarding the prognostic significance of angiogenic markers, when studied by immunohistochemistry, are controversial[8, 10, 13–15, 18, 22, 23, 29]. Contrary to expectations, high VEGF-A expression, when analyzed in the entire cohort, was associated with favorable DFS, in both age-adjusted and multivariable-adjusted analyses. In the subgroup analyses, VEGF-A retained its favorable prognostic value for DFS in luminal-HER2 and HER2-positive tumors, however, this should be interpreted with caution, due to the small number of events in the high VEGF-A category. Furthermore, high VEGF-C expression, when analyzed in the entire cohort, was associated with favorable DFS and OS, in both age-adjusted and multivariable-adjusted analyses. Additionally, high VEGF-C expression predicted for better DFS and OS in HER2-negative patients and in patients of the luminal B and TNBC subtype, while high VEGFR1 expression was associated with favorable DFS in ER/PgR-positive patients and unfavorable DFS in patients of the TNBC subtype. Using a large number of breast cancer cases identified from the Nurses' Health Study, Liu et al. found that the VEGF-A associated adverse effects on breast cancer specific mortality and distant recurrence was only observed in luminal A tumors and not in luminal B, HER2-positive and basal-like/triple-negative cancers(29). Interestingly, the authors reported a VEGF-A associated decreased risk of overall mortality in patients with basal-like tumors. In the study by Kourea et al, where no direct anti-VEGF treatment was administered, high immunohistochemical expression of VEGFR1 and co-expression of VEGFR1/VEGFR2 were associated with better survival, irrespectively of breast cancer subtyping[21]. Based on our observations, one could hypothesize that among ER/PgR-negative or TNBC tumors, subsets of less aggressive cancers with better prognosis may be identified, according to their angiogenic profile and suggest that more than one of the components of the VEGF signaling pathway rather than an individual member may affect prognosis of breast cancer patients. This issue should be addressed in larger cohorts, where the expression of individual VEGF ligands would be evaluated in conjunction with their respective receptors, which might eventually resolve the many discrepant results appearing in the literature and shed more light in the apparent interactions between the multiple players in the VEGF signaling pathway. For instance, when looking more carefully at the 9 TNBC patients with high VEGFR1 protein

expression that was associated with significantly (HR = 2.74, 95% CI 1.26–5.98, $p = 0.011$) decreased DFS, 7 of the 9 TNBC patients were found to have low VEGF-A protein expression. It is unclear whether the low VEGF-A protein expression seen in most of these patients was responsible for the up-regulation of the VEGFR1 and the observed decreased DFS in these patients.

The contradiction among the original studies, as well as among some interesting recent meta-analyses[43–44], concerning the prognostic significance of most of the angiogenesis-related proteins has hindered their clinical utility in breast cancer patients. One has to remember however, that in most of the existing studies, including ours, the findings are not purely prognostic, since most if not all studies included some type of treatment (hormonal treatment or chemotherapy). In our trial, concurrent or dose-dense sequential epirubicin and paclitaxel were administered, followed by dose-dense CMF. It appears therefore that high expression of some VEGF proteins in early-stage breast cancer patients with aggressive tumors, could plausibly lead to better DFS and OS, when such patients had received in the adjuvant setting a very effective regimen, like the one administered in our study.

The present study has some limitations. High VEGF-A expression was observed in a rather small proportion of tumors (11.8%), therefore the results concerning its prognostic utility may not be conclusive. Limitations regarding immunohistochemistry, as a method for assessing angiogenesis-related proteins in FFPE samples, have been described[45–46]. In addition, caution is warranted when comparing the present data related with immunohistochemical subtyping with those from studies employing gene expression profiling for breast cancer subtyping; the two approaches do not perfectly fit. Moreover, our study was conducted in the pre-trastuzumab era, thus, it is unclear if the results related to associations of angiogenic markers with HER2-positive tumors are applicable to patients treated in the trastuzumab era.

In conclusion, high expression of angiogenesis-related proteins is associated with adverse clinicopathological parameters in early-stage breast cancer patients and may be surrogate markers of biologically distinct subgroups of ER/PgR-negative or TNBC tumors with superior outcome. The multiple associations identified among ligands and receptors highlight potential intracellular pathways in the tumor cells. Further studies are undoubtedly needed in order to validate our results in independent cohorts.

Supporting information

S1 Table. Descriptive statistics for the five angiogenesis-related proteins (calculated as the percentage of tumor stained cells).

(PDF)

S2 Table. Associations among the angiogenesis-related proteins (using 5-years ROC curve cut-offs). Data presented as N (%). p-values of the chi-square test are shown.

(PDF)

S3 Table. Associations between the angiogenesis-related proteins (using 5-years ROC curve cut-offs) and selected clinicopathological parameters. Data presented as N (%). p-values of the chi-square test are shown.

(PDF)

S4 Table. Hazard ratios (95% CIs) estimated from univariate Cox regression for selected clinicopathological parameters.

(PDF)

S1 Dataset.
(XLSX)

Acknowledgments

The authors are indebted to all patients and their families for their trust and participation in the Hellenic Cooperative Oncology Group trials and for the provision of biological material for research purposes. The authors would also like to thank Eneida Jaupaj for tissue collection and Maria Moschoni for coordinating the data management.

Author Contributions

Conceptualization: Anna Goussia, Nafsika Simou, George Pentheroudakis, Vassiliki Malamou-Mitsi, George Fountzilias, Anna Batistatou.

Formal analysis: Kyriaki Manousou.

Resources: Anna Goussia, Nafsika Simou, Flora Zagouri, Georgios Lazaridis, Helen Gogas, Angelos Koutras, Maria Sotiropoulou, George Pentheroudakis, Dimitrios Bafaloukos, Christos Markopoulos, Helen Patsea, Christos Christodoulou, Pavlos Papakostas, Thomas Zaramboukas, Epaminontas Samantas, Paris Kosmidis, Vasileios Venizelos, Charisios Karanikiotis, George Papatsibas, Grigorios Xepapadakis, Meletios-Athanassios Dimopoulos, Vassiliki Malamou-Mitsi, George Fountzilias, Anna Batistatou.

Visualization: Anna Goussia, Nafsika Simou.

Writing – original draft: Anna Goussia.

Writing – review & editing: Anna Goussia, Nafsika Simou, George Pentheroudakis, Konstantine T. Kalogeras, Christina Bamia, Vassiliki Malamou-Mitsi, George Fountzilias, Anna Batistatou.

References

1. Toi M, Inada K, Suzuki H, Tominaga T. Tumor angiogenesis in breast cancer: its importance as a prognostic indicator and the association with vascular endothelial growth factor expression. *Breast Cancer Res Treat.* 1995; 36(2):193–204. PMID: [8534867](https://pubmed.ncbi.nlm.nih.gov/8534867/)
2. Fox SB, Generali DG, Harris AL. Breast tumour angiogenesis. *Breast Cancer Res.* 2007; 9(6):216. <https://doi.org/10.1186/bcr1796> PMID: [18190723](https://pubmed.ncbi.nlm.nih.gov/18190723/)
3. Kowanzet M, Ferrara N. Vascular endothelial growth factor signaling pathways: therapeutic perspective. *Clin Cancer Res.* 2006; 12(17):5018–22. <https://doi.org/10.1158/1078-0432.CCR-06-1520> PMID: [16951216](https://pubmed.ncbi.nlm.nih.gov/16951216/)
4. Shibuya M. Differential roles of vascular endothelial growth factor receptor-1 and receptor-2 in angiogenesis. *J Biochem Mol Biol.* 2006; 39(5):469–78. PMID: [17002866](https://pubmed.ncbi.nlm.nih.gov/17002866/)
5. Yancopoulos GD, Davis S, Gale NW, Rudge JS, Wiegand SJ, Holash J. Vascular-specific growth factors and blood vessel formation. *Nature.* 2000; 407(6801):242–8. <https://doi.org/10.1038/35025215> PMID: [11001067](https://pubmed.ncbi.nlm.nih.gov/11001067/)
6. Jussila L, Alitalo K. Vascular growth factors and lymphangiogenesis. *Physiol Rev.* 2002; 82(3):673–700. <https://doi.org/10.1152/physrev.00005.2002> PMID: [12087132](https://pubmed.ncbi.nlm.nih.gov/12087132/)
7. Cianfrocca M, Goldstein LJ. Prognostic and predictive factors in early-stage breast cancer. *Oncologist.* 2004; 9(6):606–16. <https://doi.org/10.1634/theoncologist.9-6-606> PMID: [15561805](https://pubmed.ncbi.nlm.nih.gov/15561805/)
8. Toi M, Hoshina S, Takayanagi T, Tominaga T. Association of vascular endothelial growth factor expression with tumor angiogenesis and with early relapse in primary breast cancer. *Jpn J Cancer Res.* 1994; 85(10):1045–9. <https://doi.org/10.1111/j.1349-7006.1994.tb02904.x> PMID: [7525523](https://pubmed.ncbi.nlm.nih.gov/7525523/)
9. Valtola R, Salven P, Heikkila P, Taipale J, Joensuu H, Rehn M, et al. VEGFR-3 and its ligand VEGF-C are associated with angiogenesis in breast cancer. *Am J Pathol.* 1999; 154(5):1381–90. [https://doi.org/10.1016/S0002-9440\(10\)65392-8](https://doi.org/10.1016/S0002-9440(10)65392-8) PMID: [10329591](https://pubmed.ncbi.nlm.nih.gov/10329591/)

10. De Paola F, Granato AM, Scarpi E, Monti F, Medri L, Bianchi S, et al. Vascular endothelial growth factor and prognosis in patients with node-negative breast cancer. *Int J Cancer*. 2002; 98(2):228–33. PMID: [11857413](#)
11. Dales JP, Garcia S, Bonnier P, Duffaud F, Carpentier S, Djemli A, et al. [Prognostic significance of VEGF receptors, VEGFR-1 (Flt-1) and VEGFR-2 (KDR/Flk-1) in breast carcinoma]. *Ann Pathol*. 2003; 23(4):297–305. PMID: [14597894](#)
12. Hoar FJ, Chaudhri S, Wadley MS, Stonelake PS. Co-expression of vascular endothelial growth factor C (VEGF-C) and c-erbB2 in human breast carcinoma. *Eur J Cancer*. 2003; 39(12):1698–703. PMID: [12888364](#)
13. Kostopoulos I, Arapantoni-Dadioti P, Gogas H, Papadopoulos S, Malamou-Mitsi V, Scopa CD, et al. Evaluation of the prognostic value of HER-2 and VEGF in breast cancer patients participating in a randomized study with dose-dense sequential adjuvant chemotherapy. *Breast Cancer Res Treat*. 2006; 96(3):251–61. <https://doi.org/10.1007/s10549-005-9062-2> PMID: [16538542](#)
14. Mohammed RA, Green A, El-Shikh S, Paish EC, Ellis IO, Martin SG. Prognostic significance of vascular endothelial cell growth factors -A, -C and -D in breast cancer and their relationship with angio- and lymphangiogenesis. *Br J Cancer*. 2007; 96(7):1092–100. <https://doi.org/10.1038/sj.bjc.6603678> PMID: [17353919](#)
15. Mylona E, Alexandrou P, Giannopoulou I, Liapis G, Sofia M, Keramopoulos A, et al. The prognostic value of vascular endothelial growth factors (VEGFs)-A and -B and their receptor, VEGFR-1, in invasive breast carcinoma. *Gynecol Oncol*. 2007; 104(3):557–63. <https://doi.org/10.1016/j.ygyno.2006.09.031> PMID: [17150246](#)
16. Linderholm BK, Hellborg H, Johansson U, Elmberger G, Skoog L, Lehtio J, et al. Significantly higher levels of vascular endothelial growth factor (VEGF) and shorter survival times for patients with primary operable triple-negative breast cancer. *Ann Oncol*. 2009; 20(10):1639–46. <https://doi.org/10.1093/annonc/mdp062> PMID: [19549711](#)
17. Guo S, Colbert LS, Fuller M, Zhang Y, Gonzalez-Perez RR. Vascular endothelial growth factor receptor-2 in breast cancer. *Biochim Biophys Acta*. 2010; 1806(1):108–21. <https://doi.org/10.1016/j.bbcan.2010.04.004> PMID: [20462514](#)
18. Fountzilas G, Kourea HP, Bobos M, Televantou D, Kotoula V, Papadimitriou C, et al. Paclitaxel and bevacizumab as first line combined treatment in patients with metastatic breast cancer: the Hellenic Cooperative Oncology Group experience with biological marker evaluation. *Anticancer Res*. 2011; 31(9):3007–18. PMID: [21868552](#)
19. Linardou H, Kalogeras KT, Kronenwett R, Kouvatseas G, Wirtz RM, Zagouri F, et al. The prognostic and predictive value of mRNA expression of vascular endothelial growth factor family members in breast cancer: a study in primary tumors of high-risk early breast cancer patients participating in a randomized Hellenic Cooperative Oncology Group trial. *Breast Cancer Res*. 2012; 14(6):R145. <https://doi.org/10.1186/bcr3354> PMID: [23146280](#)
20. Linardou H, Kalogeras KT, Kronenwett R, Alexopoulou Z, Wirtz RM, Zagouri F, et al. Prognostic Significance of VEGFC and VEGFR1 mRNA Expression According to HER2 Status in Breast Cancer: A Study of Primary Tumors from Patients with High-risk Early Breast Cancer Participating in a Randomized Hellenic Cooperative Oncology Group Trial. *Anticancer Res*. 2015; 35(7):4023–36. PMID: [26124351](#)
21. Kourea HP, Kotoula V, Koutras A, Alexopoulou Z, Papaspiroiu I, Skarlos DV, et al. Impact of tumor angiogenic profile on the outcome of patients with metastatic breast carcinoma treated with weekly docetaxel. A Hellenic Cooperative Oncology Group (HeCOG) study. *Histol Histopathol*. 2015; 30(9):1129–41. <https://doi.org/10.14670/HH-11-612> PMID: [25807307](#)
22. Ryden L, Jirstrom K, Haglund M, Stal O, Ferno M. 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. *Breast Cancer Res Treat*. 2010; 120(2):491–8. <https://doi.org/10.1007/s10549-010-0758-6> PMID: [20135347](#)
23. Zhang XH, Huang DP, Guo GL, Chen GR, Zhang HX, Wan L, et al. Coexpression of VEGF-C and COX-2 and its association with lymphangiogenesis in human breast cancer. *BMC Cancer*. 2008; 8:4. <https://doi.org/10.1186/1471-2407-8-4> PMID: [18190720](#)
24. Gogas H, Dafni U, Karina M, Papadimitriou C, Batistatou A, Bobos M, et al. Postoperative dose-dense sequential versus concomitant administration of epirubicin and paclitaxel in patients with node-positive breast cancer: 5-year results of the Hellenic Cooperative Oncology Group HE 10/00 phase III Trial. *Breast Cancer Res Treat*. 2012; 132(2):609–19. <https://doi.org/10.1007/s10549-011-1913-4> PMID: [22187126](#)
25. Fountzilas G, Dafni U, Bobos M, Batistatou A, Kotoula V, Trihia H, et al. Differential response of immunohistochemically defined breast cancer subtypes to anthracycline-based adjuvant chemotherapy with

- or without paclitaxel. *PLoS One*. 2012; 7(6):e37946. <https://doi.org/10.1371/journal.pone.0037946> PMID: 22679488
26. Wolff AC, Hammond ME, Hicks DG, Dowsett M, McShane LM, Allison KH, et al. Recommendations for human epidermal growth factor receptor 2 testing in breast cancer: American Society of Clinical Oncology/College of American Pathologists clinical practice guideline update. *Arch Pathol Lab Med*. 2014; 138(2):241–56. <https://doi.org/10.5858/arpa.2013-0953-SA> PMID: 24099077
 27. Hammond ME, Hayes DF, Dowsett M, Allred DC, Hagerty KL, Badve S, et al. American Society of Clinical Oncology/College of American Pathologists guideline recommendations for immunohistochemical testing of estrogen and progesterone receptors in breast cancer. *Arch Pathol Lab Med*. 2010; 134(6):907–22. <https://doi.org/10.1043/1543-2165-134.6.907> PMID: 20524868
 28. Romero Q, Bendahl PO, Ferno M, Grabau D, Borgquist S (2014) A novel model for Ki67 assessment in breast cancer. *Diagn Pathol*. 2014; 9:118. <https://doi.org/10.1186/1746-1596-9-118> PMID: 24934660
 29. Liu Y, Tamimi RM, Collins LC, Schnitt SJ, Gilmore HL, Connolly JL, et al. The association between vascular endothelial growth factor expression in invasive breast cancer and survival varies with intrinsic subtypes and use of adjuvant systemic therapy: results from the Nurses' Health Study. *Breast Cancer Res Treat*. 2011; 129(1):175–84. <https://doi.org/10.1007/s10549-011-1432-3> PMID: 21390493
 30. Sa-Nguanraksa D, Chuangsuwanich T, Pongpruttipan T, P OC. High vascular endothelial growth factor gene expression predicts poor outcome in patients with non-luminal A breast cancer. *Mol Clin Oncol*. 2015; 3(5):1103–8. <https://doi.org/10.3892/mco.2015.574> PMID: 26623059
 31. Blackwell KL, Dewhirst MW, Liotcheva V, Snyder S, Broadwater G, Bentley R, et al. HER-2 gene amplification correlates with higher levels of angiogenesis and lower levels of hypoxia in primary breast tumors. *Clin Cancer Res*. 2004; 10(12 Pt 1):4083–8.
 32. Petit AM, Rak J, Hung MC, Rockwell P, Goldstein N, Fendly B, et al. Neutralizing antibodies against epidermal growth factor and ErbB-2/neu receptor tyrosine kinases down-regulate vascular endothelial growth factor production by tumor cells in vitro and in vivo: angiogenic implications for signal transduction therapy of solid tumors. *Am J Pathol*. 1997; 151(6):1523–30. PMID: 9403702
 33. Kerbel RS. Tumor angiogenesis. *N Engl J Med*. 2008; 358(19):2039–49. <https://doi.org/10.1056/NEJMra0706596> PMID: 18463380
 34. Ribeiro-Silva A, Ribeiro do Vale F, Zucoloto S. Vascular endothelial growth factor expression in the basal subtype of breast carcinoma. *Am J Clin Pathol*. 2006; 125(4):512–8. <https://doi.org/10.1309/D744-C4NM-15J3-B00D> PMID: 16627261
 35. Yehia L, Boulos F, Jabbour M, Mahfoud Z, Fakhruddin N, El-Sabban M. Expression of HIF-1alpha and Markers of Angiogenesis Are Not Significantly Different in Triple Negative Breast Cancer Compared to Other Breast Cancer Molecular Subtypes: Implications for Future Therapy. *PLoS One*. 2015; 10(6):e0129356. <https://doi.org/10.1371/journal.pone.0129356> PMID: 26046764
 36. Ribatti D, Nico B, Ruggieri S, Tamma R, Simone G, Mangia A. Angiogenesis and Antiangiogenesis in Triple-Negative Breast cancer. *Transl Oncol*. 2016; 9(5):453–7. <https://doi.org/10.1016/j.tranon.2016.07.002> PMID: 27751350
 37. Sorlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, et al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc Natl Acad Sci U S A*. 2001; 98(19):10869–74. <https://doi.org/10.1073/pnas.191367098> PMID: 11553815
 38. Cheang MC, Voduc D, Bajdik C, Leung S, McKinney S, Chia SK, et al. Basal-like breast cancer defined by five biomarkers has superior prognostic value than triple-negative phenotype. *Clin Cancer Res*. 2008; 14(5):1368–76. <https://doi.org/10.1158/1078-0432.CCR-07-1658> PMID: 18316557
 39. Cheang MC, Martin M, Nielsen TO, Prat A, Voduc D, Rodriguez-Lescure A, et al. Defining breast cancer intrinsic subtypes by quantitative receptor expression. *Oncologist*. 2015; 20(5):474–82. <https://doi.org/10.1634/theoncologist.2014-0372> PMID: 25908555
 40. Dawood S, Hu R, Homes MD, Collins LC, Schnitt SJ, Connolly J, et al. Defining breast cancer prognosis based on molecular phenotypes: results from a large cohort study. *Breast Cancer Res Treat*. 2011; 126(1):185–92. <https://doi.org/10.1007/s10549-010-1113-7> PMID: 20711652
 41. Prat A, Pineda E, Adamo B, Galvan P, Fernandez A, Gaba L, et al. Clinical implications of the intrinsic molecular subtypes of breast cancer. *Breast*. 2015; 24 Suppl 2:S26–35.
 42. Liu HT, Ma R, Yang QF, Du G, Zhang CJ. Lymphangiogenic characteristics of triple negativity in node-negative breast cancer. *Int J Surg Pathol*. 2009; 17(6):426–31. <https://doi.org/10.1177/1066896909337505> PMID: 19493918
 43. Zhang Z, Luo G, Tang H, Cheng C, Wang P. Prognostic significance of high VEGF-C expression for patients with breast cancer: An update Meta Analysis. *PLoS One*. 2016; 11:e0165725. <https://doi.org/10.1371/journal.pone.0165725> PMID: 27812168

44. Wang F, Li S, Zhao Y, Yang K, Chen M, Niu H, et al. Predictive role of the overexpression for CXCR4, C-Met, and VEGF-C among breast cancer patients: A meta-analysis. *Breast*. 2016; 28: 45–53. <https://doi.org/10.1016/j.breast.2016.04.016> PMID: 27214240
45. de Matos LL, Trufelli DC, de Matos MG, da Silva Pinhal MA. Immunohistochemistry as an important tool in biomarkers detection and clinical practice. *Biomark Insights*. 2010; 5:9–20. PMID: 20212918
46. Jacobsen J, Grankvist K, Rasmuson T, Bergh A, Landberg G, Ljungberg B. Expression of vascular endothelial growth factor protein in human renal cell carcinoma. *BJU Int*. 2004; 93:297–302. PMID: 14764126