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Prolonged tamoxifen treatment increases relapse-free survival for patients with primary breast cancer expressing high levels of VEGF

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ABSTRACT

Previous retrospective studies have shown that high intratumoural levels of vascular endothelial growth factor (VEGF) correlate with an inferior outcome for patients treated with adjuvant tamoxifen. Our objectives were to validate the impact of VEGF on survival after adjuvant tamoxifen and to investigate the interaction between VEGF and treatment duration. For this purpose tumour homogenates from 402 patients with operable oestrogen receptor positive breast cancer (BC), treated with tamoxifen for 2 ($n = 149$) or 5 years ($n = 253$) as the only systemic adjuvant therapy were included. The median follow-up time for surviving patients was 9.8 years (range 0.5–14.8 years). Expression of VEGF was assessed by an enzyme-linked immunosorbent assay and investigated in relation to the standard BC parameters and survival. In the total population, higher VEGF was significantly correlated with shorter recurrence-free survival (RFS) (HR = 1.63, 95%CI = 1.11–2.39, $p = 0.010$), breast cancer corrected survival (BCCS) (HR = 1.82, 95%CI = 1.13–2.93, $p = 0.014$) and overall survival (OS) (HR = 1.51, 95%CI = 1.11–2.05, $p = 0.009$). High VEGF was significantly associated with reduced RFS (HR = 2.61, 95%CI = 1.45–4.70, $p = 0.001$) after two years of tamoxifen, whilst no difference was seen in patients treated for five years (HR = 1.09, 95%CI = 0.64–1.84, $p = 0.760$). A statistically significant interaction was observed between high VEGF expression and improved RFS after 5-year tamoxifen ($p = 0.034$). In concordance with previous studies, high VEGF was significantly correlated with shorter survival. We present data not reported previously revealing that patients expressing high levels of VEGF display a better outcome provided that tamoxifen is given for five years. Further studies on the impact of VEGF on a 5-year regimen are motivated.

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1. Introduction

Throughout the last forty years both detection and treatment of breast cancer (BC) have markedly improved, nevertheless BC still remains a leading cause of cancer deaths in women.[1] There is strong evidence that oestrogen plays a key-role in development and progression of the disease which is why endocrine treatment aims to reduce the adverse effect of oestrogen. This can be achieved by using the selective oestrogen receptor modulator, tamoxifen, or by ovarian suppression in premenopausal women, alternatively by inhibition of oestrogen synthesis with aromatase inhibitors (AIs) in postmenopausal women.[2] Endocrine therapy is usually given for five years and ongoing studies are being conducted to confirm whether or not further prolongation will enhance survival. Tamoxifen has been a corner stone in BC treatment for decades; however some patients will fail at this therapy.[3] At present, the only well-established predictive factors for response to endocrine therapy are the oestrogen receptor (ER) and the progesterone receptor (PR). Receptor positivity in tumours suggests for a beneficial response to endocrine therapy in approximately 80% of ER positive BC in the adjuvant setting.[4] However, many tamoxifen-resistant tumours remain oestrogen-dependent and respond to second-line endocrine therapy. In fact, loss of ER is not a major mechanism of resistance.[5] ER and growth factor signalling are potential key factors in development of the resistance. The responses of tumour cells to growth factors include enhancement of proliferation, cell survival, motility, invasiveness and angiogenesis. Thus, an increase in growth factor signalling, either through enhanced supply of growth factor ligands or via increased activation of the corresponding receptor and their downstream signalling pathways, has been associated with a more aggressive tumour phenotype.[6] So far, most attention has been given to the epidermal growth factor receptor (EGFR) family. An increased interest has emerged in vascular endothelial growth factor (VEGF) and its receptor VEGFR2. VEGF being the main driver of angiogenesis is regulated by a variety of genetic and environmental factors such as hypoxia.[7] Earlier studies have shown that high VEGF levels in primary breast tumours correlate to shorter survival times for patients treated with adjuvant tamoxifen.[8–11] Similar results were obtained when tamoxifen was given after first relapse.[12–14] These findings have been supported by various preclinical studies demonstrating the importance of VEGF for the survival of tamoxifen-resistant BC cells.[15,16] Our primary objectives with this study were to validate the effect of VEGF expression on survival after adjuvant tamoxifen and to investigate a possible interaction between VEGF levels and tamoxifen treatment duration.

2. Patients and methods

2.1. Patient characteristics

Information regarding patient characteristics was obtained from the Breast Cancer database at the Regional Oncologic Centre, Linköping University Hospital, Linköping, Sweden. This database contains information regarding all patients from the Southeast Sweden Health Care Region with primary BC. The database takes into account several parameters at time for diagnosis such as age, detection method (mammogram screening or not), type of surgery, tumour size, S-phase fraction, number of removed axillary lymph nodes and metastases, as well as ER and PR levels. Although the histopathological type was reported, data on grade were not available. Information on adjuvant therapy, participation in clinical trials including which treatment patients were randomised to, date and type of first relapse, as well as date and cause of death, were registered. A total of 711 frozen cytosols with a known hormone receptor status from patients diagnosed with primary operable BC from 1991 to 1996 were identified. In this study 449 patients with ER positive BC of stages I–III subjected to tamoxifen as the only adjuvant therapy were included. Patients with locally advanced BC, displaying distant metastases at diagnosis, or having received neoadjuvant therapy, were excluded. Additional analyses included in work presented elsewhere reduced the available material to 402 patients. The median age of all patients included was 63 years with a median tumour size of 20 mm. Data on node involvement were available for 384 (96%) of the patients. Of these, 200 had a node-negative and 184 had a node-positive breast cancer. A description of the patients' characteristics is listed in Table 1.

2.2. Treatment and follow-up

All patients underwent either breast conserving surgery as standardised sector resection or modified radical mastectomy. Dissection of levels 1–2 of the axilla was performed in 384 (96%) patients with a medium of nine identified lymph nodes. The other 18 (4%) patients were not subjected to axillary dissection due to concomitant diseases or high age. Patients treated with breast conserving surgery, received radiotherapy in 25 daily fractions of 2 Gy to a total dose of 50 Gy during five weeks. All node-positive patients received radiotherapy against loco-regional lymph node stations. As part of phase III clinical trial, patients were randomised into two arms; two or five years of adjuvant tamoxifen until 1995 as the 5-year regimen became standard therapy. After primary treatment the patients were observed for 10 years by annual clinical examinations and mammograms at the departments of Oncology or Surgery within the South East Health Care Region of Sweden. Recurrences were defined as the first documented evidence of new disease manifestations in the loco-regional area, distant sites or a combination of these. The median follow-up time in relapse-free patients was 9.8 years (range 0.5–14.8 years). The study design was approved by the research ethics board of Linköping University, Sweden.

2.3. Tumour tissue preparation

Immediately after surgery, representative tumour tissue was dissected and frozen in liquid nitrogen. Frozen tumour tissue was homogenised in a microdismembrator (Braun, Melsungen, Germany) and suspended in cold potassium phosphate buffer (5 mM, pH 7.4, 10% glycerol v/v, 1 mM dithiothreitol). Supernatants containing the cytosolic fractions

Table 1 – Clinicopathological characteristics of steroid receptor positive patients receiving adjuvant endocrine therapy.

Feature	Tamoxifen 2 years *n* (%)	Tamoxifen 5 years *n* (%)
Patients enrolled	149 (37)	253 (63)
Age, years		
Median	62	63
Range	30–88	31–96
<50	42 (28)	45 (18)
⩾50	107 (72)	208 (82)
Tumour size, mm		
Median	20	20
Range	8–120	3–150
Stage		
T1	90 (60)	132 (52)
T2–T3	59 (40)	121 (48)
S-phase[*]		
<10%	87 (67)	180 (78)
⩾10%	43 (33)	51(22)
Lymph-node status[a]		
Node-negative	71 (50)	129 (54)
Node-positive	72 (50)	112 (46)
PR, fmol/μg DNA		
Median	3.5	2.3
Range	0.0–36.0	0.0–34.2
neg (<0.09)	19 (13)	26 (10)
pos (⩾0.09)	130 (87)	227 (90)
VEGF, ρg/μg DNA		
Median	2.74	2.35
Range	0.0–49.4	0.0–85.3
Low (<2.4)	69 (46)	132 (52)
High (⩾2.4)	80 (54)	121 (48)

Abbreviations: PR, progesterone receptor; VEGF, vascular endothelial growth factor.

[*] S-phase was determined in 361 patients.

[a] Lymph node data were available for 384 patients.

were collected after refrigerated centrifugation at 20,000 g, used for steroid receptor content analysis and stored at −70 °C. The pellet fractions were analysed by the method of Burton, in order to evaluate DNA concentration.

2.4. Steroid receptor analysis

ER and PR contents were measured with enzyme immunoassays (Abbott Laboratories, Diagnostic Division, Chicago, Illinois, USA). Receptor concentrations were expressed as femtomole of receptor per microgram of DNA where samples with receptor content ⩾0.30 fmol receptor/μg DNA were classified as ER or PR positive, and samples with values below as ER or PR negative. Determination of steroid receptor levels for patients within the South East Region of Sweden was centralised to Linköping University Hospital. Linköping University Hospital participated in the external quality assessment programme arranged by the EORTC-Receptor and Biomarker Group at the Quality Assessment Laboratory, University Medical Centre Nijmegen, Netherlands.[17]

2.5. S-phase fraction (SPF)

DNA analysis on frozen tumour tissue was performed with a FACScan flow cytometer (Beckton Dickinson, USA). Tumours with a SPF ⩾10% were considered to have a high SPF whilst those with values below 10% had a low one. SPF and steroid receptor analyses from all patients were performed prospectively in accordance with the regional treatment protocol of the South East Breast Cancer Group.

2.6. Measurement of VEGF

The protein levels of VEGF were measured on breast tumour cytosols by using commercial quantitative enzyme-linked immunoassay kits for human VEGF-A (Quantikine, R & D Systems, Minneapolis, MN, USA) according to protocol of the manufacturer as in previous studies.[9,10,18] The protein levels of VEGF were expressed as pg/μg DNA.

2.7. Statistical methods

In order to validate VEGF as a possible candidate predictive marker for tamoxifen sensitivity we used a pre-defined cut-off point (2.40 pg/μg DNA). This level was set according to previous publications including patients from the Northern Health Care Region of Sweden, in which the median of VEGF expression was 2.40 pg/μg DNA (range 0.11–144.79) in node-negative and 2.33 pg/μg DNA (range 0.04–134.29) in node-positive patients.[10,18] The median values were used as cut-off points and significantly correlated to survival in both groups. In a recent publication, the median of VEGF was 2.40 pg/μg DNA (range 0.0–5.86) in a series of patients the majority being steroid receptor positive and receiving tamoxifen as only adjuvant systemic treatment.[9] Determination of VEGF expression in these studies was performed in the same way as the present.[9,10,18] Pearson χ^2-test was used to analyse associations between VEGF levels and standard BC parameters. Factors investigated (VEGF, tumour size, lymph-node status, SPF and age) were dichotomised. Possible differences in VEGF levels throughout years of diagnosis were investigated by ANOVA. Survival was estimated using the Kaplan–Meier method, and the comparison between study groups was performed with the log-rank test. Recurrence-Free Survival (RFS), Distant Disease-Free Survival (DDFS), overall survival (OS) and Breast Cancer Corrected Survival (BCCS) were calculated as time from diagnosis to first documented recurrence, distant metastases, death by any reason or death specific to BC, respectively. BCCS was calculated since a large proportion of the deaths were unrelated to BC. The Cox's proportional hazard model was used for the multivariate analysis of pathological parameters, treatment and survival. All tests were two sided and values <0.05 were considered significant.

3. Results

3.1. Patient outcome

Within the follow-up time with a median of 9.8 years, a total of 128 recurrences (29 local and 99 distant, of which 18 were both local and distant) and 139 deaths (72 due to BC and 67

unrelated to BC, i.e. in patients without any documented relapses from the disease) were recorded in the total patient material.

3.2. Expression of VEGF

All samples except three expressed detectable VEGF levels. The median level of VEGF in all patients was 2.41 pg/μg DNA (range 0.0–85.3). The distribution of VEGF levels was overall constant without exhibiting any statistically significant differences between the years of diagnosis ($p = 0.21$). However, the samples from the year 1993 ($n = 22$) exhibited a wider range between the 10th and 90th percentile (1.38–27.3) compared to that of all patients (0.54–12.5) pg/μg DNA.

3.3. Associations between VEGF and other prognostic or biological factors

In this ER positive patient population, no statistically significant correlations were found between VEGF and tumour size ($p = 0.23$), presence of lymph-node metastases ($p = 0.99$), SPF ($p = 0.70$), age ($p = 0.18$) and high PR expression ($p = 0.22$).

3.4. Survival – univariate analysis

VEGF levels in the samples were correlated with clinical data to investigate the possible prognostic significance. Univariate analyses of the total patient population showed higher VEGF content to be significantly correlated with shorter RFS resulting in a hazard ratio (HR) of 1.63 ($p = 0.010$) (Fig. 1a); BCCS (HR = 1.82, $p = 0.014$); DDFS (HR = 1.58, $p = 0.025$); OS (HR = 1.51, $p = 0.009$). When investigating patients subjected to two years of tamoxifen ($n = 149$), high VEGF content was significantly associated with reduced RFS (HR = 2.61, $p = 0.001$) (Fig. 1b). By contrast, no difference in RFS was found between high and low VEGF in the group of patients on 5 years of tamoxifen ($n = 253$); RFS (HR = 1.09, $p = 0.760$) (Fig. 1c).

In analyses on patients with low VEGF content, no increased benefit was seen for either of the two regimens (HR = 1.11, $p = 0.740$) (Fig. 2a). In contrast, patients expressing high levels of VEGF exhibited a statistically significant improvement in RFS when receiving the 5-year regimen (HR = 0.48, $p = 0.004$). In the latter group of patients the approximate 10-year RFS for the 2-year and 5-year regimen was calculated to be 52% and 74%, respectively (Fig. 2b). A test for interaction between VEGF and benefit from prolonged treatment was statistically significant ($p = 0.034$). Concordant results were found for BCCS as seen in Table 2. The tested interaction was not found to be dependent on age. In terms of RFS the test of interaction was statistically significant in patients <50 years ($p = 0.024$) ($n = 87$) although the low number of patients included in this analysis may result in less robust data.

3.5. Survival – multivariate analysis

A Cox proportional hazard regression model was used to estimate HR, where a value above 1.0 indicates a greater risk of recurrence or death than for the comparative group set as a reference. Factors included were VEGF, 5-year regimen of

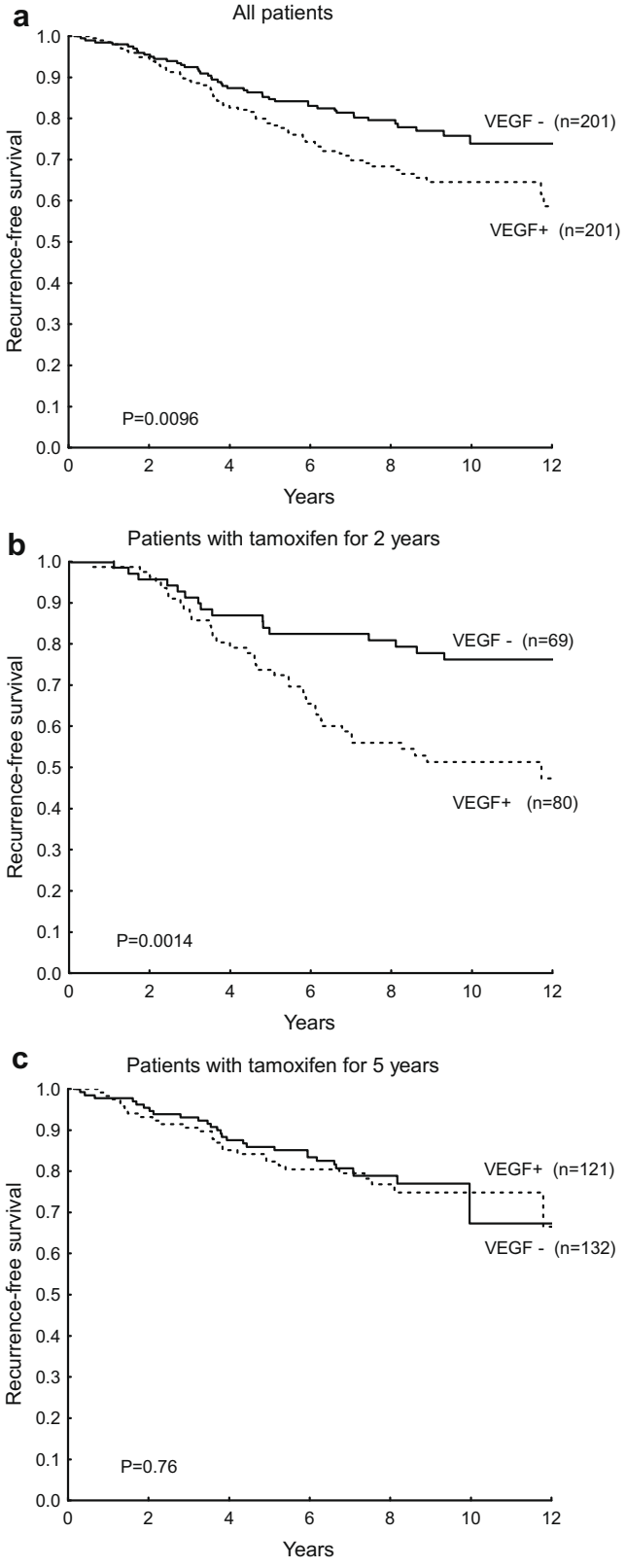

Fig. 1 – (a) Recurrence-free survival for all steroid receptor positive patients treated with adjuvant tamoxifen considering their expression of vascular endothelial growth factor (VEGF) ($n = 402$). The predefined cut-off value for VEGF was 2.40 pg/μg DNA. Recurrence-free survival according to VEGF in patients treated for two years (b) and five years (c) of tamoxifen.

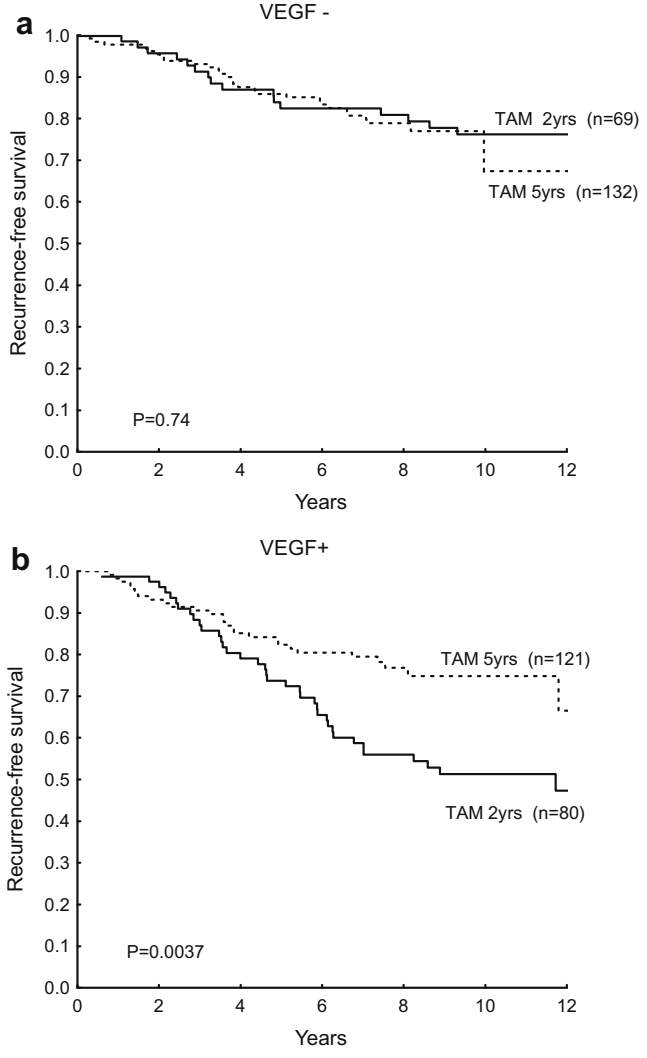

Fig. 2 – Recurrence-free survival according to tamoxifen treatment duration for patients expressing low levels (a) and high levels (b) of vascular endothelial growth factor.

tamoxifen and the interaction between VEGF and treatment duration as well as SPF, age, nodal status, tumour size. VEGF remained as a statistically significant factor for reduced survival times regarding RFS, (HR = 2.57, $p = 0.005$); BCCS (HR = 2.36, $p = 0.043$); OS (HR = 2.17, $p = 0.007$) and DDFS (HR = 2.34, $p = 0.016$). By contrast, 5 years of tamoxifen was not statistically significant in relation to survival. The interaction between VEGF and treatment duration exhibited a border-line value for RFS (HR = 0.43, $p = 0.054$). Similar results were found for SPF; RFS (HR = 1.53, $p = 0.047$), BCCS (HR = 1.68, $p = 0.045$), DDFS (HR = 1.50, $p = 0.070$) and OS (HR = 1.18, $p = 0.380$). Exclusion of SPF increased the otherwise limited number of patients without having a major influence on the results (not shown). Age was only found to be significantly correlated to shorter OS (HR = 1.04, $p < 0.001$) and not to RFS or BCCS as shown in Table 3. Lymph-node status and tumour size remained as independent prognostic factors in all analyses.

4. Discussion

Results from the present study confirm significantly reduced survival times for patients with high intratumoural VEGF when taking the total patient cohort into account. This conclusion coincides well with results from several retrospective studies, as well as one prospective randomised phase III trial suggesting high VEGF levels as a potential marker of impaired survival following adjuvant tamoxifen.[8–11] However, three of the retrospective studies mentioned above include patients subjected to different treatment durations of adjuvant tamoxifen although the majority were treated for two years.[8–10] It is noteworthy that in the randomised trial by Rydén and colleagues, patients were allocated to two years of tamoxifen versus no adjuvant endocrine therapy.[11] The effect of a prolonged tamoxifen regimen in relation to VEGF expression remains an issue that so far has not been specifically addressed. In the present study, more than 60% of the patients received tamoxifen for five years. When studying all patients according to therapy duration we found VEGF to be a strong marker for shorter survival times only for those receiving two years of tamoxifen. Moreover, when patients were separated according to VEGF expression we found that in the subset of high VEGF a significantly improved RFS was seen for those on a 5-year regimen. However, the positive effect of the prolonged tamoxifen regimen on RFS was not seen in patients exhibiting low VEGF levels. To the best of our knowledge the fact that patients with high VEGF expression benefit from a prolonged tamoxifen treatment in terms of RFS has not been previously reported.

Hypoxia has been suggested as the major key-player in regulation of VEGF transcription in cancer, although a growing

Table 2 – Logrank test analysis determining the univariate significance of these variables in relation to survival.

Variables	HR	RFS 95% CI		P-value	HR	BCCS 95% CI		P-value
		Lower	Upper			Lower	Upper	
VEGF (high versus low) in all patients ($n = 402$)	1.63	1.11	2.39	0.010	1.82	1.13	2.93	0.014
VEGF (high versus low) in patients on 2 years of tam ($n = 149$)	2.61	1.45	4.70	0.001	2.67	1.24	5.74	0.012
VEGF (high versus low) in patients on 5 years of tam ($n = 253$)	1.09	0.64	1.84	0.760	1.37	0.73	2.57	0.330
Tam (5 versus 2) years in patients with low VEGF ($n = 201$)	1.11	0.59	2.09	0.740	1.13	0.51	2.53	0.760
Tam (5 versus 2) years in patients with high VEGF ($n = 201$)	0.48	0.29	0.79	0.004	0.59	0.33	1.06	0.079

Abbreviations: RFS, relapse-free survival; BCCS, breast cancer corrected survival; HR, hazard ratio; CI, confidence interval; VEGF, vascular endothelial growth factor; tam, tamoxifen.

Table 3 – Cox-regression analysis of vascular endothelial growth factor expression and treatment duration as well as pathological parameters in relation to survival.

Variables	HR	RFS 95 % CI		P-value	HR	BCCS 95 % CI		P-value
		Lower	Upper			Lower	Upper	
VEGF	2.57	1.34	4.94	0.005	2.36	1.03	5.43	0.043
Tam 5 years	1.36	0.69	2.69	0.380	1.62	0.69	3.80	0.270
VEGF and treatment	0.43	0.18	1.02	0.054	0.53	0.66	5.37	0.240
SPF	1.53	1.01	2.33	0.047	1.68	1.01	2.80	0.045
Age	0.99	0.98	1.01	0.260	1.00	0.98	1.02	0.910
Lymph-node status	2.49	1.58	3.93	<0.001	2.89	1.61	5.19	<0.001
Tumour size	2.08	1.36	3.19	<0.001	2.61	1.53	4.46	<0.001

Abbreviations: RFS, relapse-free survival; BCCS, breast cancer corrected survival; HR, hazard ratio; CI, confidence interval; VEGF, vascular endothelial growth factor; tam, tamoxifen; SPF, S-phase fraction.

body of evidence underlining the importance of the endocrine regulation of VEGF has emerged during recent years. Oestrogens as well as progestins have been shown to stimulate VEGF transcription in BC cells[19] through an oestrogen response element (ERE) binding region within the promoter region of the VEGF gene.[20] However, results observed from preclinical studies have shown contradictory results regarding the ability of tamoxifen to affect VEGF expression. *In vivo* studies in MCF-7 tumours have found tamoxifen to be able to oppose the effect of oestradiol-induced transcription and secretion of VEGF resulting in reduced vascularisation.[21] By contrast, the increased VEGF levels induced by hypoxia in MCF-7 cells were not counteracted by tamoxifen supporting the hypothesis involving VEGF as a mediator of endocrine resistance.[8] Moreover, tamoxifen was found to increase the production of VEGF likely by an ER-independent pathway.[19]

Peritumoural vascular invasion (PVI) is considered as a negative prognostic marker in BC and shown to correlate to VEGF levels in colorectal cancer.[22] Interestingly, a recent publication investigated PVI in relation to survival in a large randomised trial by the International Breast Cancer Study Group. The adverse prognostic impact of PVI was not seen in postmenopausal patients with steroid receptor-positive BC subjected to adjuvant tamoxifen for five years.[23] The authors conclude that high PVI fails as an adverse prognostic marker provided that tamoxifen was used for a proper time period, i.e. five years. HER2 poses as another factor shown to correlate to higher VEGF levels and less benefit from endocrine therapy.[24] Moreover, experiments in nude mice have shown a possibility to affect angiogenesis by targeting EGFR. Blockade of EFGR1 by a tyrosine kinase inhibitor resulted in reduced VEGF and vessel density as well as reduced tumour growth and proliferation.[25] Although HER2 targeted therapy is likely of benefit for BC patients expressing high levels of VEGF it still has not been fully elucidated.

At present the majority of postmenopausal patients receive adjuvant endocrine therapy with an AI. Interestingly, there are studies suggesting that treatment with AIs may be of increased benefit to patients expressing high VEGF levels compared to tamoxifen. Levels of circulating VEGF were measured after 5 and 12 weeks of adjuvant endocrine therapy, respectively, and found to have increased for patients on tamoxifen whilst no change in VEGF levels was seen after treatment with AIs.[26,27] Although the data suggest that the agonistic character of tamoxifen may be responsible for the induction of VEGF the AI's failure in reducing VEGF implies that VEGF induction is also ER-independent. Furthermore, a benefit in terms of treatment response was seen in patients on tamoxifen exhibiting a high baseline serum VEGF in contrast to low VEGF. No such correlation was found for patients treated with the AI.[27]

An important point that is also being discussed is the carry-over effect of tamoxifen believed to extend the benefit well beyond the end of therapy. Although patients included in our study have a relatively long follow-up time we cannot disregard from the fact that the prolonged tamoxifen regimen only delays relapses which a more extended follow-up would possibly detect. Another disadvantage with our study is that not only randomised patients were included. With these drawbacks in mind, we consider our data to be more of a hypothesis-generating character.

Together with the introduction of anti-VEGF compounds, a demand for reliable predictive markers for the identification of patients likely to benefit from this type of therapy has emerged during recent years pushing to optimise VEGF determination. So far VEGF determined at mRNA or protein level with different techniques have failed to give relevant predictive information for selecting patients with advanced disease to angiogenesis-inhibiting therapy. Although tumour cells are the predominantly source of VEGF more recent observations have detected VEGF in infiltrating tumour-associated stroma.[28] Moreover, based on the results from an *in vivo* model it has been hypothesised that VEGF is involved in recruitment of stromal cells that in turn benefit both tumour and additional stromal cell growth.[16] Thus measuring VEGF content in tumour tissue homogenates containing various cell types may implicate difficulties determining the specific source of the protein and more importantly whether or not these values truly reflect VEGF activity.[29] In patient material, discrepancies between VEGF determined by ELISA and IHC and/or microvessel density are reported.[30] Several studies using ELISAs have shown conforming results relating high VEGF levels to impaired survival whilst studies using IHC have reported varying results suggesting for both a positive and negative association between VEGF and survival following adjuvant tamoxifen.[11,31]

We have previously shown that in a comparative study between VEGF determined by ELISA on cytosols and IHC on a tissue micro array, a significantly relevant association between VEGF and survival was only obtained with the ELISA-based results.[32] So far there is no standard test assuring completely reliable results for the determination of VEGF.[29] We find our results reliable considering that measurements from different institutions and time periods are in accordance with each other in terms of median and range of the VEGF values. Moreover, VEGF levels did not exhibit major differences in our samples despite different years of patient inclusion. In summary, as previously reported high VEGF was significantly correlated to an impaired survival when studying the whole patient population. However, an improved survival was observed for patients with high VEGF-expressing tumours provided they were given tamoxifen for a prolonged time period. Further investigation of VEGF in relation to effect of five years of tamoxifen is justified.

Conflict of interest statement

None declared.

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