Quantifying tumour vascularity in non-luminal breast cancers

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Abstract

Aims

Microvessel density (MVD), proliferating MVD (pMVD) and vascular proliferation index (VPI) are methods used to quantify tumour vascularity in histopathological sections. In this study we assessed MVD, pMVD and VPI in non-luminal subtypes of breast cancer. Differences between subtypes were studied, and the prognostic value of each method was assessed.

Methods

All non-luminal subtypes (61 basal phenotype (BP), 60 HER2 type and 30 five negative phenotype (5NP)) were selected from a series comprising 909 cases of breast cancer. Sections were stained for Ki67 and von Willebrand factor. Associations between MVD, pMVD and VPI, molecular subtypes and prognosis were studied.

Results

MVD was highest in 5NP (Δ54.3 microvessels/mm^2 compared to BP, 95% confidence interval (CI) 30.3-78.3), whereas no clear difference was found between HER2 type and BP (Δ8.8 microvessels/mm^2, 95% CI -9.6-27.1). pMVD and VPI did not differ between subtypes. For MVD, hazard ratio (HR) was 1.07 (95% CI 1.03-1.11) per 10 vessel increase and 1.93 (95% CI 1.21-3.07) if MVD was > median value. High MVD was associated with poor prognosis in the HER2 type (HR 1.07 (95% CI 1.02-1.12)) and 5NP (HR 1.13 (95% CI 1.03-1.23)), but not in BP (HR 1.04 (95% CI 0.94-1.14) per 10 vessel increase). pMVD and VPI were not associated with prognosis.

Conclusion

MVD appears to be an independent prognostic factor in HER2 and 5NP subtypes of breast cancer, where high MVD is associated with poor survival. MVD was higher in the 5NP compared to both BP and HER2 type.

INTRODUCTION

Inducing angiogenesis is one of the six original hallmarks of cancer (1). Tumour angiogenesis occurs when cancer cells stimulate adjacent blood vessels to proliferate and infiltrate the tumour tissue (2-4). The result is a vascularised tumour with potential for exponential tumour growth and metastasis (4, 5). Knowledge of tumour vascularity might provide information about the biology of a tumour, patient prognosis, and may ultimately aid in choice of therapy.

As a measure of angiogenesis, Weidner *et al* introduced microvessel density (MVD), which is the average number of vessels per mm^2 in the most vascularised area of the tumour (6). However, although some studies found MVD to have prognostic value in cancer (6-9), it is merely a descriptive measure of vascular density in a tumour, and does not describe ongoing angiogenesis. Therefore, it has been suggested that vascular proliferation may be a better estimate of ongoing tumour angiogenesis. Proliferating microvessel density (pMVD), defined as the average number of proliferating vessels per mm^2, and vascular proliferation index (VPI) which is the ratio of proliferating vessels to the total amount of vessels, can be used to assess vascular proliferation (10).

Breast cancer is a heterogeneous group of diseases. Gene expression analyses enable sub-classification of breast cancers into molecular subtypes with differing biology and prognosis (11-13). Immunohistochemistry (IHC) and *in situ* hybridisation (ISH) can be used as surrogates for gene expression analysis, and thus, reclassify breast cancers into molecular subtypes as shown in Figure 1 (14). Each subtype displays a distinct survival pattern (14-16). The Luminal A subtype has a relatively constant rate of death over time, while mortality rates in the other subtypes are initially high, peak within five years after diagnosis and then decrease, with few deaths occurring from five years and onwards (15). Before the advent of trastuzumab the HER2 type had the poorest prognosis (14, 17, 18), followed by the Five Negative Phenotype (5NP) and the Basal Phenotype (BP) (18), often classified together as triple negative (TN) tumours (14). Patients with non-luminal subtypes do not qualify for endocrine therapy, leaving chemotherapy as the only systemic adjuvant treatment available for TN tumours (19). There is an urgent need for a better understanding of the biological characteristics of non-luminal subtypes, and for new prognostic and predictive markers.

The aims of this study were to assess MVD, pMVD and VPI in non-luminal subtypes of breast cancer, and to evaluate if they differ between subtypes. In addition, we wished to study the prognostic impact of MVD, pMVD and VPI for the non-luminal subtypes combined and in each subtype separately.

MATERIALS AND METHODS

Study population and specimen characteristics

The study material comprised 909 primary breast carcinomas from women born between 1886 and 1928 in Nord-Trøndelag County, Norway. These women were invited to participate in a survey for

early breast cancer detection organized by the Norwegian Cancer Society, between 1956 and 1959. They were followed for breast cancer occurrence from 1961 to 2008, through information linkage with the Cancer Registry of Norway. After diagnosis, the women were followed until death from breast cancer, death from other causes or December 31st 2010, whichever came first. Date and cause of death was obtained from the Cancer Registry of Norway after linkage with the Causes of Death Registry (20, 21).

Pathology reports and formalin-fixed, paraffin embedded (FFPE) tissue from tumours were available from the Department of Pathology and Medical Genetics, St Olav's Hospital, Trondheim University Hospital, Norway. As described by Engstrøm *et al* (14), tumours were classified into histopathological type according to the WHO Classification of Tumours of the Breast (22), and grade according to the Nottingham Grading System (23). Next, they were reclassified into molecular subtypes using IHC and ISH as surrogates for gene expression analysis. Briefly, tissue microarrays (TMA) were constructed using Tissue Arrayer MiniCore® with TMA Designer2 software (Alphelys). Three 1mm in diameter tissue cores were extracted from peripheral regions of each tumour and inserted into the TMA recipient blocks. Sections were stained with HES and IHC for oestrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2), the proliferation marker Ki67, cytokeratin 5 (CK5) and epidermal growth factor receptor (EGFR). Chromogenic ISH was used for the assessment of HER2 gene copy number. Based on biomarker expression, tumours were classified into molecular subtypes as outlined in Figure 1 (14).

Dual-colour immunohistochemical staining

For the present study, full-face sections from all non-luminal tumours were included. Sections, 4µm thick, were retrieved from storage at -20^{0}C and placed into a heating cabinet at 42^{0}C overnight. Heat Induced Epitope Retrieval was performed in a Pre-Treatment link. Sections were immersed in Dako Target Retrieval Solution buffer pH 6, S1699 (DAKO). Temperature was raised from 80^{0}C to 97^{0}C, and maintained for 20 minutes before cooling. Sections were then immersed for 3 minutes x2 in Dako Wash Buffer, S3006 10x diluted with deionized water (dH_2O).

Immunostaining was done at room temperature, using Dako Autostainer Plus (DAKO Denmark A/S, Produksjonsvej 42 DK-2600 Glostrup, Denmark). Enzyme blocking was performed for 8 minutes, using Dual Endogenous Enzyme Block, S2003 (DAKO). Sections were incubated with a cocktail of primary antibodies for 60 minutes. The cocktail contained rabbit von Willebrand factor, 3.8µg/L (Polyclonal rabbit, A0082, DAKO) and mouse Ki67 antibody, 160µg/L (Clone MIB1, M7240, DAKO). A mixture of detection systems containing Southern Biotech alkaline phosphatase/goat anti-mouse for Ki67 diluted 1:100 and EnVision Detection System-Peroxidase/rabbit for von Willebrand factor, was incubated for 30 minutes. Each step in the immunostaining process was followed by 5

minutes of rinsing with Dako Wash Buffer (DAKO). To visualise Ki67, Ferangi blue (BIOCARE medical) FB81335S diluted 1:50 with Ferangi Blue Buffer was incubated for 15 minutes followed by three dH_2O rinses. For von Willebrand Factor, sections were incubated for 15 minutes with amino-ethyl-carbazole substrate chromogen, K3469 (DAKO) and then rinsed twice with dH_2O. Sections were put into lukewarm water after immunostaining and coverslipped with Dako Faramount aqueous medium, S025 (DAKO). Von Willebrand factor positive cells displayed reddish-brown cytoplasm and Ki67 positive cells displayed blue nuclei (Figure 2).

Scoring and reporting

All cases were assessed by one observer (MRK) who had undergone training on a test series of 24 colon cancer sections prior to the study material (9). The most vascularised area of each tumour was marked by two authors (AMB and MRK) at low magnification (100x). Within the marked area, microvessels and proliferating microvessels were counted in 10 high-power fields (400x) with tumour tissue in at least 50% of each field of vision. Areas with sclerosis, necrosis, fibrotic scars and normal breast tissue were avoided. A vessel was defined as a von Willebrand factor positively stained endothelial cell or cell cluster. In cases with glomeruloid microvascular proliferations or long, twisted branches of endothelial cells, each lumen was counted as a separate vascular unit. A proliferating vessel was defined as a vascular unit containing at least one nucleus with distinct staining for Ki67. MVD and pMVD were expressed as the number of vessels per square millimetre (mm^2), and estimated as the average number of vessels per field of vision divided by the visual field area of the microscope. VPI is the ratio between pMVD and MVD, given in percent. The study is reported in accordance with the REMARK recommendations for tumour marker studies (24).

Statistical analyses

Multiple linear regression analyses were performed to study the association between non-luminal breast cancer subtypes and MVD, pMVD and VPI. Adjustments were made for age at diagnosis (<65, 65-79, ≥80 years) alone, and for age, grade (1, 2 and 3) and stage (I, II, III and IV) together. For survival analyses, the median was set as cut-off to distinguish high and low values of MVD, pMVD and VPI. Cumulative incidence of death from breast cancer was calculated, where death from other causes was treated as a competing event. The cumulative risk of death from breast cancer can be interpreted as the risk of dying from breast cancer before dying from other causes (25). To compare the equality of the cumulative incidence curves, Gray's test was used (26). Multiple Cox proportional hazards regression analyses were performed to estimate relative risk of death from breast cancer, given as hazard ratios (HR) with 95% confidence intervals (CI). HR was assessed per unit increase in MVD, pMVD and VPI, as well as for values above and below the median. Adjustments were made for age, grade, stage and subtype together (if applicable). The proportional hazard assumption was met for all models, as assessed from log-minus-log plots and tests based on Schoenfeld residuals. Survival analyses were performed for all cases combined and separately for each molecular subtype. To

evaluate the robustness of the findings, analyses were also performed adjusting for age as a continuous variable, adjusting for decade of diagnosis and by using the 75th percentile as cut-off. All analyses were performed using STATA 13.1.

Ethics

The study was granted approval including dispensation from the general requirement of patient consent by the Regional Committee for Medical and Health Sciences Research Ethics (REK, Midt-Norge, ref. nr: 836/2009).

RESULTS

Characteristics of the study participants are presented in Table 1. Median age at diagnosis was 72 years for BP patients, 67 for HER2 patients and 73.5 for 5NP patients. Fifty-three percent died from breast cancer during follow-up. Among BP patients, 44.3% died from breast cancer, compared to 61.7% of the HER2 patients and 53.3% of the 5NP patients.

For all tumours combined, median MVD was 83.6 microvessels/mm^2 (interquartile range [IQR] 55.5-120.0), median pMVD was 2.3 proliferating microvessels/mm^2 (IQR 1.2-5.2) and median VPI was 3.6% (IQR 1.2-6.7). Figure 3 shows the distribution of MVD and pMVD for all tumours combined, and for each molecular subtype separately.

There was no difference in mean MVD between HER2 and BP tumours (Δ8.8, 95% CI -9.6-27.1, Table 2). In both unadjusted and adjusted analyses, 5NP had higher MVD than BP (Δ54.3 microvessels/mm^2, 95% CI 30.3- 78.3, p<0.001) (Table 2). Since the 95% CIs between HER2 type and 5NP did not overlap, the 5NP also had higher MVD than the HER2 type. For pMVD and VPI, no difference in mean value was found between subtypes.

Figure 4 shows the cumulative risk of breast cancer death according to MVD in all patients. Cases with high MVD had a poorer prognosis compared to those with a lower MVD, regardless of cut-off level. With the median as cut-off, cumulative risk of death for patients with low MVD was 34.2% (95% CI 24.7-46.0) five years after diagnosis, and 41.6% (95% CI 31.3-53.7) 15 years after diagnosis. The corresponding cumulative risks for patients with high MVD were 56.0% (95% CI 45.2-67.4) and 64.1% (95% CI 53.3-74.8), respectively (Table 3). After adjustments for age, grade, stage and molecular subtype, HR was 1.07 (95% CI 1.03-1.11) per 10 vessels increase, and 1.93 (95% CI 1.21-3.07) for those with high MVD compared to those with low MVD (Table 4).

Table 1 Descriptive characteristics for the 151 breast cancer cases.

	BP	HER2 type	5NP	Total
Number of cases (%)	61 (40.4)	60 (39.7)	30 (19.5)	151 (100)
Median age at diagnosis, years (IQR)	72 (65-80)	67 (58.5-74.5)	73.5 (66-83)	71 (64-78)
Median follow-up time, years (IQR)	4.9 (2.4-11.2)	3.2 (1.3-9.4)	3.9 (2.5-10.3)	3.8 (1.6-10.5)
Median time to breast cancer death, years (IQR)	2.5 (1.3-3.7)	1.7 (1.0-3.1)	3.0 (2.0-4.5)	2.4 (1.2-3.7)
Death from breast cancer, *n* (%)				
Yes	34 (55.7)	23 (38.3)	14 (46.7)	71 (47.0)
No	27 (44.3)	37 (61.7)	16 (53.3)	80 (53.0)
Grade, *n* (%)				
1	2 (3.3)	0 (0.0)	0 (0.0)	2 (1.3)
2	7 (11.5)	10 (16.7)	18 (60.0)	35 (23.2)
3	52 (85.3)	50 (83.3)	12 (40.0)	114 (75.5)
Tumour diameter (mm), *n* (%)				
≤20	25 (41.0)	19 (31.7)	9 (30.0)	53 (35.1)
>20, ≤50	12 (19.7)	3 (5.0)	4 (13.3)	19 (12.6)
>50	2 (3.3)	1 (1.7)	2 (6.7)	5 (3.3)
Uncertain, but ≥20	10 (16.4)	22 (36. 7)	10 (33. 3)	42 (27.8)
Unknown	12 (19.7)	15 (25.0)	5 (16.7)	32 (21.2)
Lymph node status, *n* (%)				
Negative	19 (31.2)	15 (25.0)	5 (16.7)	39 (25.8)
Negative, less than 5 measures	5 (8.2)	4 (6.7)	3 (10.0)	12 (8.0)
Positive	26 (42.6)	33 (55.0)	14 (46.7)	73 (48.3)
Unknown (not examined)	11 (18.0)	8 (13.3)	8 (26.7)	27 (17.9)
Stage, *n* (%)				
I	25 (41.0)	23 (38.3)	13 (43.3)	61 (40.4)
II	30 (49.2)	27 (45.0)	13 (43.3)	70 (46.4)
III+IV	6 (9.8)	10 (16.7)	4 (13.3)	20 (13.3)
Ki67 status, *n (%)*				
<15%	10 (16.4)	12 (20.0)	17 (56.7)	39 (25.8)
≥15%	50 (82.0)	48 (80.0)	13 (43.3)	111 (73.5)
Median MVD, microvessels/mm2 (IQR)	72.7 (49.6-99.8)	83.6 (60.6-125.7)	122.0 (70.4-182.8)	83.6 (55.5-120.0)
Median pMVD, microvessels/mm2 (IQR)	2.3 (1.2-4.6)	2.9 (1.2-6.3)	2.5 (1.2-6.9)	2.3 (1.2-5.2)

Median VPI, percentage points (IQR)	3.6 (1.4-7.1)	4.1 (1.6-6.3)	2.3 (0.8-6.5)	3.6 (1.2-6.7)

Table 2 Mean differences in MVD, pMVD and VPI according to tumour subtype based on linear regression. Basal phenotype (BP), HER2 type and five negative phenotype (5NP).

	Δ Unadjusted	95% CI	*Δ* Adjusted[1]	95% CI	*Δ* Adjusted[2]	95% CI
MVD, microvessels/mm²						
BP		Ref		Ref		Ref
HER2 type*	14.7	-3.2-32.6	12.3	-5.9-30.6	8.8	-9.6-27.1
5NP	55.7	33.8-77.5	55.9	34.1-77.7	54.3	30.3-78.3
pMVD, microvessels/mm²						
BP		Ref		Ref		Ref
HER2 type*	0.6	-1.3-2.6	0.6	-1.4-2.5	0.5	-1.5-2.5
5NP	1.6	-0.8-3.9	1.5	-0.8-3.9	1.9	-0.7-4.5
VPI, percentage points						
BP		Ref		Ref		Ref
HER2 type*	0.0	-1.7-1.6	0.1	-1.6-1.8	0.2	-1.5-1.9
5NP	-0.2	-2.2-1.8	-0.3	-2.3-1.7	0.1	-2.1-2.3

[1]Adjusted for age. [2]Adjusted for age, grade, stage.
*One outlier, which was HER2 type, was excluded from linear regression analyses.

Table 3 Risk of death from breast cancer (cumulative incidence) according to MVD. Cut-off set at median value.

		5 years after diagnosis		15 years after diagnosis	
MVD median	Total (*n*)	Breast cancer deaths (*n*)	Risk (%), (95% CI)	Breast cancer deaths (*n*)	Risk (%), (95% CI)
All cases					
≤83.6	76	26	34.2 (24.7-46.0)	31	41.6 (31.3-53.7)
>83.6	75	42	56.0 (45.2-67.4)	48	64.1 (53.3-74.8)
BP					
≤72.7	31	11	35.5 (21.5-54.9)	13	42.7 (27.4-62.1)
>72.7	30	11	36.7 (22.3-56.4)	14	46.7 (30.9-65.7)
HER2 type					
≤83.6	30	12	40.0 (25.1-59.6)	13	43.5 (28.0-62.9)
>83.6	30	21	70.0 (53.5-85.0)	23	76.7 (60.6-89.7)
5NP					
≤122.0	15	4	26.7 (11.0-56.4)	7	46.7 (25.6-73.7)
>122.0	15	9	60.0 (37.2-83.5)	9	60.0 (37.2-83.5)

Table 4 Relative risk of death from breast cancer according to MVD, pMVD and VPI in all non-luminal breast cancers. Cut-off set at median value.

	Unadjusted HR	95% CI	Adjusted[1] HR	95% CI	Adjusted[2] HR	95% CI
MVD, microvessels/mm²						
≤83.6*	1	Ref	1	Ref	1	Ref
>83.6*	2.0	1.3-3.1	1.9	1.2-3.0	1.9	1.2-3.1
Per 10 vessels increase	1.07	1.03-1.10	1.07	1.03-1.10	1.07	1.03- 1.11
pMVD, microvessels/mm²						
≤2.3*	1	Ref	1	Ref	1	Ref
>2.3*	0.9	0.6-1.4	0.9	0.6-1.4	0.8	0.5-1.3
Per 1 vessel increase	1.03	1.00-1.07	1.04	1.00-1.07	1.04	1.01- 1.07
VPI, percentage points						
≤3.6*	1	Ref	1	Ref	1	Ref
>3.6*	1.0	0.6- 1.5	1.0	0.6- 1.5	1.0	0.6- 1.5
Per percentage point increase	1.01	0.95-1.06	1.00	0.95-1.06	1.02	0.96-1.08

[1] Adjusted for age. [2]Adjusted for age, stage, subtype, grade. *Subtype-specific median value.

When each molecular subtype was studied separately, high MVD was associated with poor prognosis in the HER2 type in both unadjusted and adjusted analyses (Figure 5, Tables 3, 5 and 6). HR was 1.07 (95% CI 1.02-1.12) per 10 vessel increase in the adjusted analyses, and 2.51 (95% CI 1.25-5.02) for those with MVD above the HER2-median compared to those with values below. High MVD was not associated with prognosis in BP tumours (HR 1.04 (95% CI 0.94-1.14) per 10 vessel increase). For 5NP, high MVD was associated with increased risk of death with adjusted HR 1.13 (95% CI 1.03-1.23) per 10 vessel increase.

For all cases combined, neither high pMVD nor high VPI was associated with prognosis in the unadjusted analyses (Figure 6, Tables 4 and 7). In adjusted analyses, HR was 1.04 (95% CI 1.01-1.07) per single vessel increase for pMVD and 1.02 (95% CI 0.96-1.08) per percentage point increase for VPI. In analyses stratified for subtype, HR was 1.05 (95% CI 1.01-1.08) per single vessel increase in the HER2 subtype. Vascular proliferation was not associated with prognosis in the BP or 5NP.

Table 5 Relative risk of death from breast cancer according to MVD, pMVD and VPI by tumour subtype. HER2 type, basal phenotype (BP) and five negative phenotype (5NP)

	Unadjusted HR	95% CI	Adjusted[1] HR	95% CI	Adjusted[2] HR	95% CI
MVD, microvessels/mm²						
BP, per 10 vessel increase	1.06	0.96-1.16	1.05	0.95-1.15	1.04	0.94-1.14
HER2 type, per 10 vessel increase	1.07	1.03-1.12	1.07	1.02-1.12	1.07	1.02-1.12
5NP per 10 vessel increase	1.10	1.03-1.18	1.10	1.02-1.18	1.13	1.03-1.23
pMVD, microvessels/mm²						
BP, per single vessel increase	1.08	1.00-1.16	1.09	1.00-1.18	1.06	0.97- 1.16
HER2 type, per single vessel increase	1.03	1.00-1.07	1.03	1.00-1.07	1.05	1.01- 1.08
5NP, per single vessel increase	0.94	0.83-1.06	0.96	0.85-1.10	0.96	0.79- 1.16
VPI, percentage points						
BP, per 1 percentage point increase	1.08	0.99-1.19	1.08	0.98-1.19	1.06	0.95- 1.18
HER2 type, per 1 percentage point increase	0.99	0.92-1.07	0.99	0.91-1.07	1.05	0.97- 1.15
5NP, per 1 percentage point increase	0.87	0.75-1.02	0.89	0.74-1.06	0.81	0.63-1.04

[1]Adjusted for age. [2]Adjusted for age, grade, stage

Table 6 Relative risk of death from breast cancer according to MVD by basal phenotype (BP) and HER2 type. Cut-off for each subtype is the median MVD value for the individual subtype.

	Unadjusted HR	95% CI	Adjusted[1] HR	95% CI	Adjusted[2] HR	95% CI
MVD, microvessels/mm²						
BP						
≤72.7	1	Ref	1	Ref	1	Ref
>72.7	1.15	0.54-2.46	1.10	0.51-2.36	1.04	0.48- 2.22
HER2 type						
≤83.6	1	Ref	1	Ref	1	Ref
>83.6	2.48	1.27-4.85	2.45	1.24- 4.84	2.51	1.25- 5.02

[1]Adjusted for age. [2]Adjusted for age, grade, stage

Table 7 Risk of death from breast cancer (cumulative incidence) for all cases combined according to pMVD and VPI. Median as cut-off.

		5 years after diagnosis		15 years after diagnosis	
	Total (*n*)	Breast cancer deaths (*n*)	Risk (%), (95% CI)	Breast cancer deaths (*n*)	Risk (%), (95% CI)
pMVD					
≤2.3*	77	37	48.1 (37.6-59.7)	40	52.4 (41.7-64.0)
>2.3*	74	31	41.9 (31.6-53.9)	39	53.2 (42.3-65.0)
VPI					
≤3.6*	77	36	46.8 (36.4-58.5)	41	53.7 (42.9-65.2)
>3.6*	74	32	43.2 (32.9-55.3)	38	51.9 (41.0-63.8)

*Median value for all cases.

DISCUSSION

In the present study of non-luminal breast cancer, high MVD was associated with poorer survival. Furthermore, MVD appears to be an independent prognostic factor in the 5NP and HER2 subtypes of breast cancer, but not in the BP.

While some studies have found MVD to be of prognostic value in breast cancer (6-9), others have not (27, 28). Varying cohort profiles and lack of consensus with regard to staining methods, antibodies, area of the tumour selected for vessel counting and counting procedures may, in part, explain this discrepancy (6, 8, 27-29). Furthermore, previous studies have not taken the heterogeneity of breast cancers into account when studying the prognostic value of MVD (6-8, 28). To the best of our knowledge, the prognostic value of MVD across the three non-luminal molecular subtypes of breast cancer has not previously been reported. The finding that MVD is a prognostic marker in the HER2 type and 5NP, but not in the BP, further supports the suggestion that molecular subtypes have different biology and prognostic markers. Our findings indicate that if studying breast cancers as one disease entity rather than each subtype specifically, important information may be lost.

In this study, MVD appears to be of prognostic value in the 5NP. However, there were only 30 cases of this subtype and the 95% CIs were wide. Further studies on a larger number of cases are needed to determine the true prognostic value of MVD in 5NP tumours.

Neither pMVD nor VPI differed significantly between the three subtypes. There are several possible explanations for this. Firstly, the sample size may not have been sufficiently large to detect the differences in question. Secondly, the number of proliferating microvessels in breast cancer is low and therefore particularly vulnerable to random variation. Thirdly, all tumour subtypes in the present study are known to have aggressive features. The lack of difference in pMVD and VPI between these subtypes might be due to a generally higher vascular proliferation in all non-luminal subtypes.

Some studies have found vascular proliferation markers to have independent prognostic value in endometrial cancer, prostate cancer and breast cancer (10, 27, 28, 30), while others have not (9). VPI was not associated with prognosis in the present study. When the median was set as cut-off, high pMVD was not associated with poor prognosis. However, there was an increased risk of breast cancer death per single proliferating vessel increase. The significance of this finding is uncertain and could be due to random variation or non-linearity of the underlying association. Furthermore, it should be noted that low pMVD is vulnerable to observer variation and intratumoural heterogeneity.

This study comprises only non-luminal tumours, which would be expected to have high tumour cell proliferation rates. At total 73.5 % of all cases were Ki67 high. The causal relationship between tumour cell proliferation and vessel proliferation, including the possibility that MVD and pMVD might be a consequence of tumour cell proliferation, is not well understood and would require a

different study design than the present study. Others have reported that the process of tumour angiogenesis occurs independent of tumour cell proliferation and oncogenic stimulation (31, 32), and is regulated by a set of factors referred to as the angiogenic switch (31).

According to present guidelines, breast carcinomas are classified into subgroups using ER, PR, HER2 and Ki67. As a result, BP and 5NP tumours are grouped together as TN breast cancers, and receive the same therapy (19). In the present study, linear regression analyses were also performed with 5NP and BP tumours grouped together as one TN subgroup, and compared to the HER2 group. There were no differences between the TN and HER2 groups in MVD, pMVD or VPI (data not shown). Thus, the observed differences in tumour vascularity in the present study would not have been discovered if TN cancers had been studied as one group. Importantly, the greatest differences in MVD were found between the BP and 5NP. Based on our findings, grouping 5NP and BP together as a common TN group may camouflage important information about tumour biology.

The 5NP appears to be a distinct molecular subtype with poor prognosis (14, 15). Some have even found 5NP to have the second poorest prognosis of all subtypes in patients who did not receive systemic adjuvant therapy (14). Still, very little is known about the biological characteristics of this subtype, and patients today are only treated with chemotherapy in addition to surgery and radiation (19). To ensure 5NP patients the most appropriate treatment, more information about the biology of these tumours is needed. In the present study, 5NP tumours had higher MVD than both BP and HER2, despite the low sample size. Vascular proliferation is generally low in breast cancer (28), implying that ongoing angiogenesis is low. It is possible for tumour cells to gain access to vasculature by means of other processes than angiogenesis (33-36). One example is co-option, where tumour cells hijack existing vasculature and migrate along the vessels of the host organ (35, 37). Another is intussusception, where vessels that already exist are split into daughter vessels (38). A third is vasculogenic mimicry, where tumour cells with high plasticity gain endothelial cell-like characteristics and form tubules (36). There is a need to investigate whether some molecular subtypes of breast cancer avail themselves of mechanisms other than angiogenesis for gaining tumour vasculature. Based on our results, this might be particularly interesting in the 5NP subtype, which has considerably increased MVD compared to other subtypes, but no corresponding increase in vascular proliferation.

Conclusions

MVD is an independent prognostic factor in the HER2 and 5NP subtypes of breast cancer, where high MVD is associated with poor survival. MVD is not associated with poor survival among BP cases. The 5NP subtype has significantly higher MVD than both the BP and HER2 subtypes.

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Competing interests: None declared

Licence for Publication

Key messages

Microvessel density is higher in the five negative phenotype compared to the HER2 type and the basal phenotype.

High microvessel density is associated with poor survival in the HER2 type and the five negative phenotype, but does not have prognostic value in the basal phenotype.

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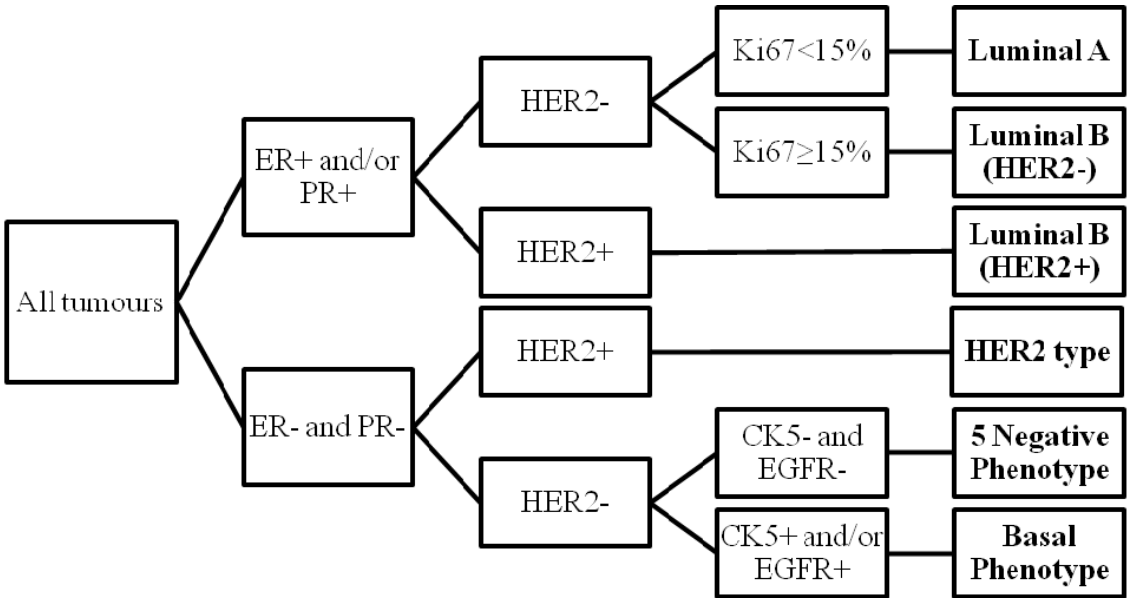

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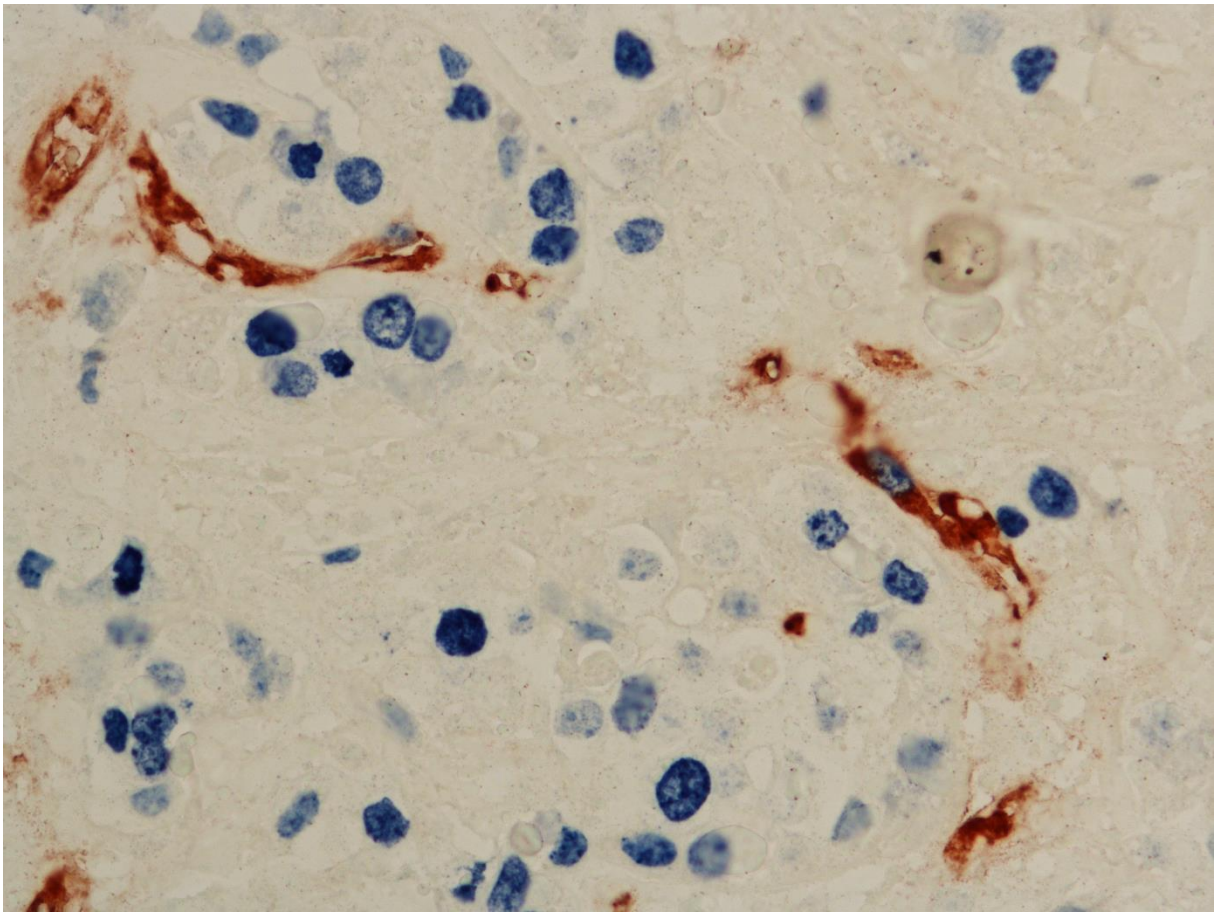

Figure 2. Breast cancer section at x400 magnification, stained with immunohistochemistry. Case number 538, HER2 type. Von Willebrand factor-positive endothelial cells display reddish-brown cytoplasm and Ki67 positive proliferating cells display blue nuclei. Photo: MRK/AMB.

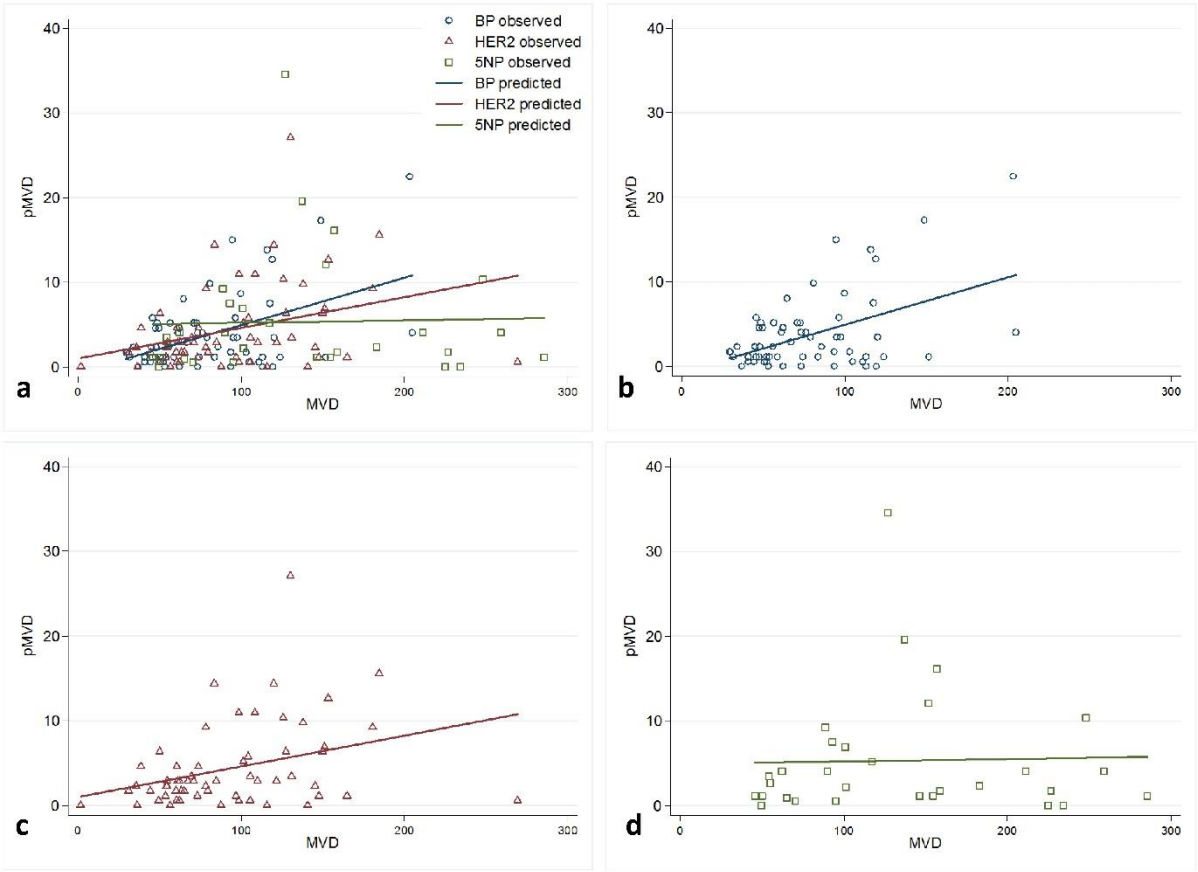

Figure 3 Distribution of non-luminal subtypes according to microvessel density (MVD) and proliferating MVD (pMVD). Predicted values were estimated by linear functions. One extreme outlier (HER2 type) is not displayed in the scatterplot (MVD 470.6/mm^2, pMVD 67.5/mm^2). a: All cases. b: Basal phenotype. c: HER2 type. d: Five negative phenotype.

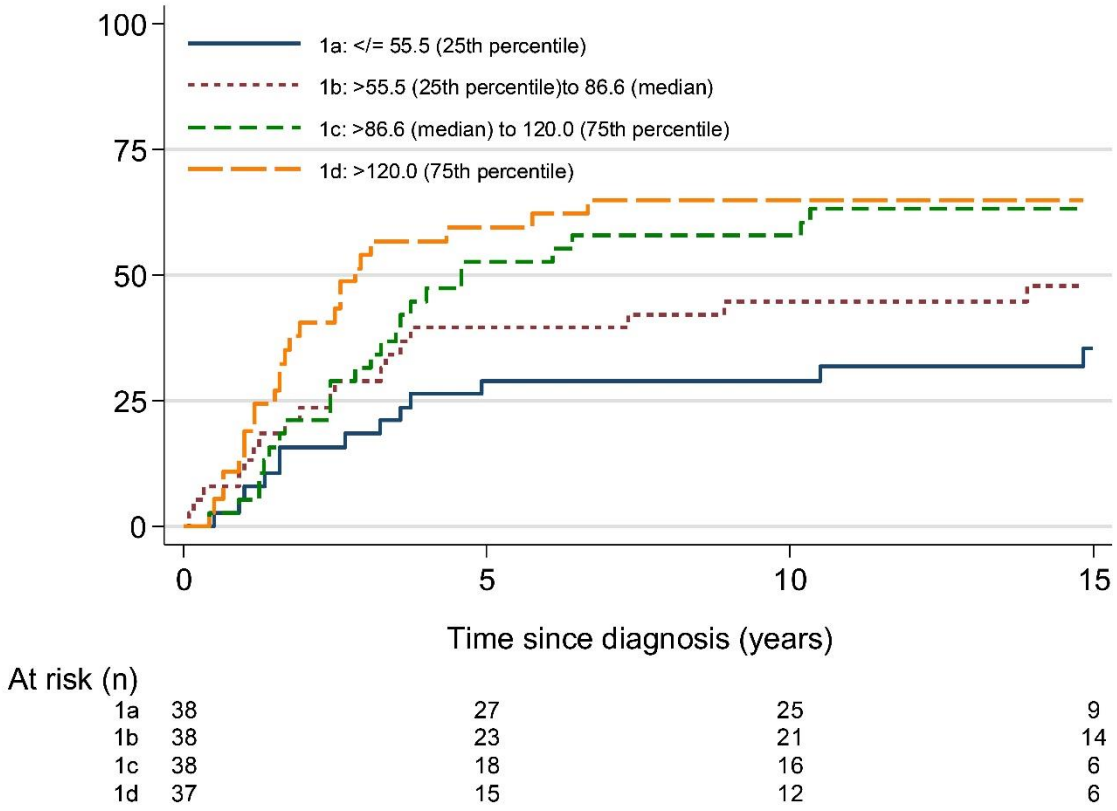

Figure 4. Risk of death from breast cancer (cumulative incidence) according to quartiles of microvessel density. Gray's test: p=0.027.

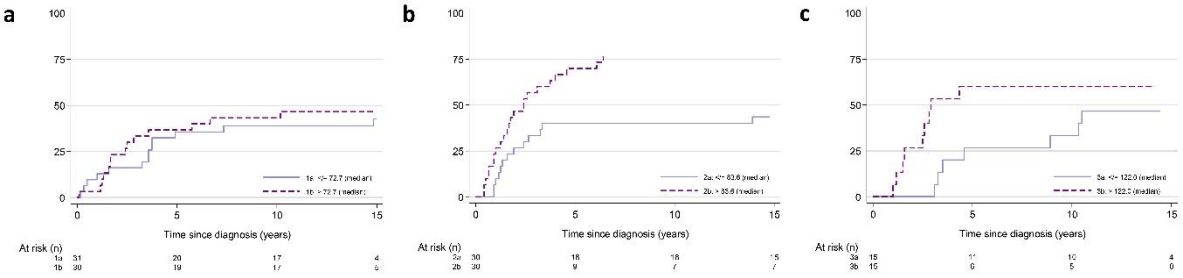

Figure 5. Risk of death from breast cancer (cumulative incidence) according to microvessel density. The cut-off for each subtype is the median MVD value for the individual subtype. a: Basal phenotype. Gray's test: p=0.671. b: HER2 type. Gray's test: p=0.0135. c: 5 negative phenotype. Gray's test: p=0.139.

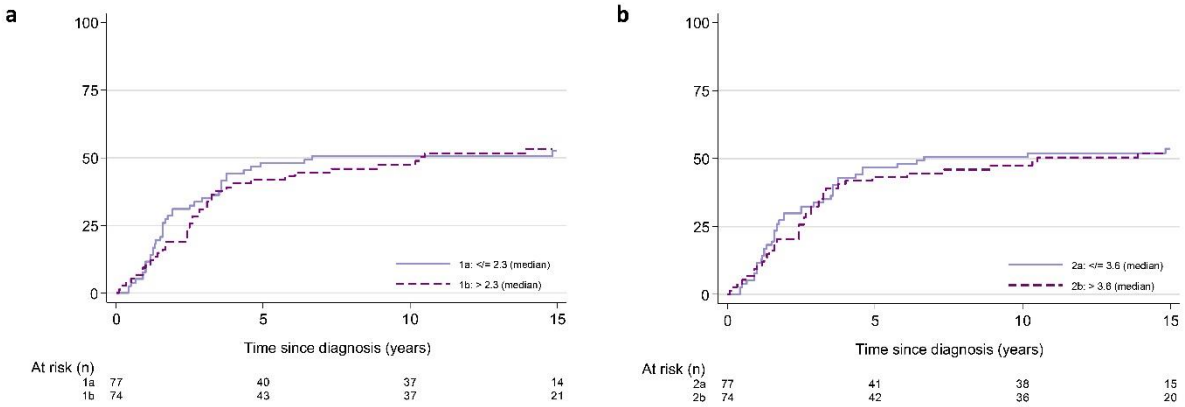

Figure 6. Risk of death from breast cancer (cumulative incidence) according to vascular proliferation above and below the median. a: Proliferating microvessel density. Gray's test: p=0.947. b: Vascular proliferation index. Gray's test: p=0.855.

Figure legends

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