Microvascular Proliferation in Luminal A and Basal-like Breast Cancer Subtypes

Maria Ryssdal Kraby[1], Kristi Krüger[2], Signe Opdahl[3,4], Lars J. Vatten[4], Lars A. Akslen[2,5], Anna M. Bofin[1]

[1]Department of Laboratory Medicine, Children's and Women's Health, Norwegian University of Science and Technology, Trondheim, Norway

[2] Centre for Cancer Biomarkers, University of Bergen, Bergen, Norway

[3]Central Norway Regional Health Authority, Norway

[4]Department of Public Health and General Practice, Norwegian University of Science and Technology, Trondheim, Norway

[5]Department of Pathology, Haukeland University Hospital, Bergen, Norway

E-mail corresponding author: kraby@stud.ntnu.no

Department of Laboratory Medicine, Children's and Women's Health, Norwegian University of Science and Technology, Erling Skjalgssons gate, 7030 Trondheim, Norway

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Abstract

Purpose

The aim of this study was to examine Microvessel Density (MVD), Proliferating Microvessel Density (pMVD) and Vascular Proliferation Index (VPI) in Basal-like phenotype (BP) and Luminal A subtypes of breast cancer and to study their prognostic value.

Methods

Dual-colour immunohistochemistry for von Willebrand factor and Ki67 was done on sections from 62 Luminal A and 62 BP tumours matched for grade and selected from 909 breast cancers previously reclassified into molecular subtypes. Associations between MVD, pMVD and VPI, molecular subtypes and breast cancer prognosis were estimated using linear regression and survival analyses.

Results

Both pMVD (difference 1.9 microvessels/mm^2 ($p=0.002$)) and VPI (difference 1.7 percentage points ($p=0.014$)) were higher in BP tumours compared to Luminal A. No clear difference between subtypes was found for MVD. However, only MVD was associated with prognosis. Hazard ratio (HR) for breast cancer death for all cases was 1.10 (95% CI 1.02-1.18)/10 vessels increase. Among Luminal A tumours, HR was 1.22/10 vessels increase/mm^2 ($p<0.001$) and in BP it was 1.04 ($p=0.37$).

Conclusions

High MVD was associated with poor prognosis in Luminal A, but not in BP cancers. Vascular proliferation was higher in BP, indicating a more active angiogenesis than in Luminal A tumours. The Luminal A subgroup comprised mostly histopathological grade 3 cancers in this selected series, and further studies are needed to clarify whether MVD provides additional prognostic information for Luminal A tumours irrespective of grade. This may contribute to stratification of this large group of patients and may aid in identifying tumours with a particularly good prognosis.

INTRODUCTION

Angiogenesis is a necessity for tumour growth,[1]. Without it, tumour size does not exceed 1-2mm^3,[1-4]. After vascularization, tumour growth rate quickly increases and becomes exponential,[2 4]. A quantitative measure of angiogenesis within a tumour could therefore provide important prognostic information in cancer patients.

Microvessel Density (MVD) is an established method for quantifying the number of vessels within a tumour. However, the method does not reflect ongoing angiogenesis,[5]. Proliferating Microvessel Density (pMVD) and Vascular Proliferation Index (VPI) describe endothelial cell proliferation within the tumour and may provide better estimates of ongoing angiogenesis,[6-10]. Recent studies have indicated that VPI is an independent prognostic factor in endometrial cancer,[6], prostate cancer,[10] and breast cancer,[8 9]. Furthermore, high VPI has been associated with aggressive breast cancer features and with the basal-like phenotype,[7-9].

Gene expression studies have led to the identification of five biologically distinct breast cancer subtypes: Luminal A, Luminal B, Basal-like phenotype (BP), HER2 overexpressing and Normal breast-like,[11 12]. These subtypes are characterized by different clinical outcomes. While the Luminal A subtype has the best prognosis, the poorest outcomes are found in the BP and HER2 overexpressing subtypes,[13].

Immunohistochemical (IHC) and in situ hybridisation (ISH) biomarkers have been used as surrogates for gene expression analysis in studies of breast cancer subtypes,[7 9 14-21]. Although not capable of identifying the intrinsic subtypes with complete consistency,[22 23], IHC and ISH markers provide prognostic information similar to that of gene expression analyses,[17 19 24]. This is now an established approach in studies of large patient cohorts,[17 19 21 24].

The aims of this study were to compare MVD, pMVD and VPI in the Luminal A and BP breast cancer subtypes, and to study the effect of these factors on prognosis in breast cancer patients with long-term follow-up.

MATERIALS AND METHODS

Study population

Between 1956 and 1959, 25 897 women born between 1886 and 1928 in Nord-Trøndelag county, Norway, were invited to participate in a breast cancer survey organized by the Norwegian Cancer Society. Through information linkage with the Cancer Registry of Norway, these women were followed up for breast cancer occurrence from 1961 to 2008. Information on date and cause of death was available from the Cause of Death Registry,[25-27]. A total of 1393 primary breast cancers were diagnosed during the follow-up period. Of these, 909 tumours were retrieved from the archives of the Department of Pathology and Medical Genetics, St. Olav's Hospital, Trondheim, and reclassified into molecular breast cancer subtypes,[21]. Sixty-three tumours were classified as BP and 433 tumours were classified as Luminal A. Of the BP tumours, 62 were suitable for inclusion in the present study. A Luminal A case was selected from the same patient cohort for each BP case, matched for tumour grade if possible. Among the BP tumours, 52 were grade 3, 7 were grade 2 and 3 were grade 1. There were 42 grade 3, 17 grade 2 and three grade 1 Luminal A tumours. The average grade for Luminal A tumours was 2.6, compared with 2.8 for BP cancers.

Specimen characteristics

All cases were classified according to histopathological type and grade, and then reclassified into molecular subtypes using IHC and ISH as surrogates for gene expression analyses. Full-face sections were cut at 4μm from formalin-fixed, paraffin-embedded (FFPE) tissue blocks, stained with haematoxylin-erythrosine-saffron (HES). Classification into histopathological type and grade was done by two experienced pathologists, according to World Health Organization Classification of Tumours of the Breast,[28] and the Nottingham Grading System,[29 30]. In cases of disagreement, consensus was reached after discussion. Next, three 1mm in diameter tissue cores were extracted from peripheral areas of each tumour and assembled in tissue microarrays (TMA). Immunohistochemical (IHC) staining was done on sections from the TMA blocks for the following markers:

oestrogen receptor (ER), progesterone receptor (PR), Ki67, human epidermal growth factor receptor 2 (HER2), cytokeratin 5 (CK5) and epithelial growth factor receptor 1 (EGFR). In addition, *HER2* gene copy number status was estimated using chromogenic in situ hybridization (CISH) . Tumours were reclassified into molecular subtypes according to the algorithm in Fig1,[21].

Immunohistochemical staining

For the present study, 4μm full-face sections were retrieved from the freezer at -20°C. Pretreatment was carried out in a Pre-Treatment Link (PT link) (DAKO). The sections were immersed in Dako Target Retrieval Solution buffer pH 6 (DAKO). The temperature of the solution was raised to 97°C and maintained for 20 minutes before cooling. Immunostaining was performed using Dako Autostainer Plus (DAKO). The process commenced with eight minutes of enzyme blocking using Dual Endogenous Enzyme Block (DAKO). Primary antibodies, rabbit von Willebrand factor antibody (3.8μg/L) (Polyclonal rabbit, A0082, Dako) and mouse Ki67 antibody (160μg/L) (Clone MIB1, M7240, Dako), were mixed with Dako antibody diluent (DAKO) and incubated for 60 minutes at room temperature. Secondary antibodies, SouthernBiotech Alkaline Phosphatase (AP) /Goat Anti-Mouse and Dako EnVision Detection System-Peroxidase/rabbit, diluted 1:100, were incubated for 30 minutes (room temperature). Ki67 was visualised by applying Ferangi Blue (BIOCARE medical) for 15 minutes. After rinsing with dH_2O, Amino-Ethyl-Carbasol (AEC) Substrate Chromogen (Dako) was applied for 15 minutes to visualize von Willebrand factor. Coverslipping with Dako Faramount Aqueous Medium (DAKO). von Willebrand factor positive cells showed reddish-brown cytoplasmic staining and Ki67 positive nuclei stained blue as seen in Fig2.

Scoring and reporting

The most vascularized areas of the tumours were identified in the IHC-stained sections by two of the authors (MRK, AMB) prior to vessel counting. The number of microvessels was then counted in 10 high-power fields (400x) within the preselected areas. Each field comprised at least 50% tumour tissue. Sclerotic areas, fibrotic scars and areas close to normal breast tissue or necrosis were avoided. A countable microvessel was defined as a von Willebrand factor positive endothelial cell or cell cluster. A visible lumen was not necessary to fulfil the requirements of a vessel. However, in areas with long, twisted branches of endothelium or glomeruloid microvascular proliferations (vascular nests), each lumen was counted as a separate vascular unit.

Proliferating microvessels were counted in the same ten fields. A proliferating vessel was defined as a microvessel containing at least one endothelial cell nucleus with positive Ki67 staining. MVD and pMVD values were given as number of vessels per mm^2, estimated as the average number of vessels per visual field divided by the visual field area of the microscope. VPI was defined as the ratio between pMVD and MVD, given in percent.

All cases were assessed by one observer (MRK) who had undergone a period of training prior to the study. Using a test series of 24 colon cancer sections, intra- and interobserver (MRK, KK) agreement were estimated using Kappa (κ) and Spearman rho (ρ). Training was ended when the desired level of agreement, consistent over time, was achieved (κ>0,6 and ρ>0,8). This study complies with the REMARK reporting recommendations for tumour marker studies,[31].

Statistical analyses

Linear regression was used to estimate the association between breast cancer subtype and MVD, pMVD and VPI. Age (<65, 65-79, ≥80 years), grade (1, 2 and 3) and stage (I, II and III/IV) at diagnosis were adjusted for. Breast cancer prognosis was assessed using survival analyses, where patients were followed until death from breast cancer, death from other causes or until December 31, 2010, whichever came first,[21]. Using Kaplan-Meier plots with log-rank tests, breast cancer specific survival (BCSS) was compared for patients with MVD, pMVD and VPI below and above the median value. Cox proportional hazards models were used to estimate risk of death from breast cancer per unit increase in MVD, pMVD and VPI, as well as for patients with values below and above the median value. Hazard ratios (HR) were calculated with 95% confidence intervals (CIs) adjusting for age, stage, grade and subtype (if applicable). Survival analyses were performed for all cases combined and for the two subtypes separately.

To evaluate the robustness of the results, the analyses were repeated with adjustment for time period of diagnosis (10-year categories), narrower age categories, restriction to grade 3 tumours and with the cut-off value set at the 75th percentile for MVD, pMVD and VPI instead of the median. Proportionality between hazards was checked by comparing log minus log plots of survival and by performing tests based on Schoenfeld residuals and assumptions were met. All analyses were performed using Stata 13.1 (StataCorp LP, College Station, TX, USA).

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 women and their tumours are presented in Table 1. A total of 38.7% of the patients with Luminal A tumours and 45.2% of the patients with BP tumours died from breast cancer during follow-up. The median age at diagnosis was 74 years for Luminal A cases and 72 years for BP cases.

Table 1: Descriptive characteristics for the 124 breast cancer cases

	Luminal A	**Basal-like phenotype**	**Total**
Number of cases	62	62	124
Median age at diagnosis, years (IQR)	74.0 (68.0-79.0)	72.0 (64.0-80.0)	73.0 (64.5-79.5)
Median follow-up time, years (IQR)	5.7 (2.6-11.6)	4.9 (2.3-11.2)	5.3 (2.4-11.4)
Median time to breast cancer death, years (IQR)	3.2 (1.8-8.3)	2.5 (1.2-3.7)	2.5 (1.5-5.0)
Death from breast cancer, n (%)			
Yes	24 (38.7)	28 (45.2)	52 (41.9)
No	38 (61.3)	34 (54.8)	72 (58.1)
Grade, n (%)			
1	3 (4.8)	3 (4.8)	6 (4.8)
2	17 (27.4)	7 (11.3)	24 (19.4)
3	42 (67.7)	52 (83.9)	94 (75.8)
Tumour diameter, n (%)			
<2 cm	13 (21.0)	5 (8.1)	18 (14.5)
≥2 cm	39 (62.9)	40 (64.5)	79 (63.7)
Unknown	10 (16.1)	17 (27.4)	27 (21.8)
Lymph node status, n (%)			
Negative	22 (35.5)	19 (30.7)	41 (33.1)
Negative, less than 5	7 (11.3)	5 (8.1)	12 (9.7)
Positive	22 (35.5)	27 (43.6)	49 (39.5)
Unknown (not examined)	11 (17.7)	11 (17.7)	22 (17.7)
Stage, n (%)			
I	30 (48.4)	25 (40.3)	55 (44.4)
II	27 (43.6)	30 (48.4)	57 (46.0)
III-IV	5 (8.1)	7 (11.3)	12 (9.7)
Ki67, n (%)			
<15 %	62 (100.0)	10 (16.1)	72 (58.1)
≥15 %	0 (0.0)	52 (83.9)	52 (41.9)
Ck 5, n (%)			
Negative	56 (90.3)	8 (12.9)	64 (51.6)
Positive	6 (9.7)	54 (87.1)	60 (48.4)
EGFR, n (%)			
Negative	62 (100.0)	22 (35.5)	84 (67.7)
Positive	0 (0.0)	40 (64.5)	40 (32.3)
Median MVD, microvessels/mm^2 (IQR)	63.4 (49.0-81.3)	72.9 (49.6-103.2)	66.9 (49.6-94.6)
Median pMVD, microvessels/mm^2 (IQR)	1.2 (0.6-2.9)	2.3 (1.2-4.6)	1.7 (0.6-3.5)

Median VPI, percentage points (IQR)	1.8 (0.8-4.3)	3.2 (1.3-6.8)	2.3 (1.1-5.9)

IQR=interquartile range, MVD=Microvessel Density, pMVD=Proliferating Microvessel density, VPI=Vascular Proliferation Index

For all tumours combined, the median MVD was 66.9 microvessels/mm^2(Inter Quartile Range (IQR) 49.6-94.6). The median pMVD was 1.7 microvessels/mm^2 (IQR 0.6-3.5). The median VPI was 2.3% (IQR 1.1-5.9). The scatterplot in Fig3 shows the distribution of MVD and pMVD in Luminal A and BP tumours.

Table 2 shows unadjusted and adjusted differences in mean MVD, pMVD and VPI according to tumour subtype. BP tumours had higher pMVD (*Δ* 1.9 proliferating microvessels/mm², 95% CI 0.7-3.1, p=0.002) and VPI (*Δ* 1.7 percentage points, 95% CI 0.3-3.0, p=0.014) than Luminal A tumours. No clear associations were found between subtypes and MVD score. There was little variation between unadjusted and adjusted estimates.

Table 2. Mean differences in MVD, pMVD and VPI according to tumour subtype based on linear regression.

	***Δ* Unadjusted**	95% CI	***Δ* Adjusted***	95% CI
MVD, microvessels/mm^2				
Luminal A		Ref		Ref
Basal-like phenotype	9.6	-4.1-23.2	7.5	-6.1-21.2
pMVD, microvessels/mm^2				
Luminal A		Ref		Ref
Basal-like phenotype	1.9	0.6- 3.1	1.9	0.7-3.1
VPI, percentage points				
Luminal A		Ref		Ref
Basal-like phenotype	1.5	0.2-2.8	1.7	0.3-3.0

*Adjusted for age, grade, subtype and stage

Patients with MVD above the median showed a trend towards poorer BCSS than those with MVD below the median (p=0.066)(Fig4). HR for death from breast cancer was 1.10 (95% CI 1.02-1.18, p=0.008) per 10 vessels increase in MVD (Table 3), adjusted for age, grade, stage and subtype. Neither pMVD nor VPI showed any clear prognostic association (Fig5a and 5b). HR was 1.04 (95% CI 0.96-1.13, p=0.28) for each single vessel increase in pMVD and 0.99 (95% CI 0.91-1.08, p=0.90) for every percentage point increase in VPI. Adjusting for age, grade, stage and subtype had little influence on the estimates.

Table 3. Risk of death from breast cancer according to MVD, pMVD and VPI

	Unadjusted HR	95% CI	Adjusted HR*	95% CI
MVD, microvessels/mm^2				
<66.9	1	Ref	1	Ref
≥66.9	1.68	0.96-2.95	1.44	0.79-2.65
Per 10 vessels increase	1.09	1.03-1.16	1.10	1.02-1.18
pMVD, microvessels/mm^2				
<1.7	1	Ref	1	Ref
≥1.7	0.98	0.57-1.69	0.86	0.48-1.54
Per 1 vessel increase	1.05	0.99-1.13	1.04	0.96-1.13
VPI, percentage points				
<2.3	1	Ref	1	Ref

≥2.3	0.74	0.43-1.29	0.61	0.33-1.11
Per percentage point increase	1.01	0.94-1.09	0.99	0.91-1.08

* Adjusted for age, grade, subtype and stage

In separate analysis of each subtype, high MVD was associated with poorer survival for patients with the Luminal A subtype (p=0.038)(Fig6), but not for patients with the BP subtype (p=0.7)(Fig6). After adjusting for age, stage and grade, HR was 1.22 (95% CI 1.09-1.37, p<0.001) per 10 vessels increase in the Luminal A and 1.04 (95% CI 0.95-1.15, p=0.37) per 10 vessels increase in the BP subtype (Table 4). No association was found between pMVD or VPI and prognosis in either subtype.

Table 4. Risk of death from breast cancer according to MVD, pMVD and VPI by tumour subtype

	Unadjusted HR	95% CI	Adjusted HR*	95% CI
MVD, microvessels/mm^2				
Luminal A, per 10 vessels increase	1.11	1.02-1.21	1.22	1.09-1.37
BP**, per 10 vessels increase	1.07	0.98-1.17	1.04	0.95-1.15
pMVD, microvessels/mm^2				
Luminal A, per 1 vessels increase	0.97	0.78-1.21	1.11	0.86-1.43
BP**, per 1 vessels increase	1.07	1.00-1.15	1.04	0.96-1.14
VPI, percentage points				
Luminal A, per percentage point increase	0.93	0.80-1.08	0.98	0.83-1.16
BP**, per percentage point increase	1.06	0.97-1.17	1.02	0.91-1.13

*Adjusted for age, grade and stage

**Basal-like phenotype

The results were similar when the analyses were carried out with cut-off at the 75th percentile, with adjustments for time period at diagnosis, using different methods to adjust for age and in analyses restricted to grade 3 cases.

DISCUSSION

In this study there was no clear difference in MVD between BP and Luminal A tumours. However, there was a clear difference in BCSS between Luminal A tumours with high and low MVD, suggesting that MVD may be of prognostic significance in Luminal A tumours. A higher number of proliferating vessels was found among BP tumours compared to Luminal A tumours, although neither pMVD nor VPI was associated with BCSS.

Tumour capillaries are different from normal vessels of the body. Tumour vessel diameter is typically three times larger, mural cell coverage is sporadic, and the vessel can suddenly end blindly or anastomose with itself[32 33]. The capillary walls of tumour vasculature are about ten times leakier than normal capillaries,[34], and they are difficult to identify in HES-stained sections. In 1991, Microvessel Density (MVD) was introduced by Weidner et al., who showed that risk of metastasis increased with increasing microvessel count. However, without an estimation of the proliferating fraction, it is not a measure of ongoing angiogenesis within a tumour [6]. Some studies have found MVD to be a prognostic factor in cancer [35-37], while others have not [6 8 9].It has been suggested that measuring vascular proliferation may be a better estimate of actively ongoing angiogenesis within a tumour,[6-10 32 38]. In the present study, significantly more proliferating vessels were found in the BP compared to Luminal A tumours. This finding is in accordance with those of other studies,[7 9], and is also in line with studies suggesting that the BP has more aggressive features than the Luminal A subtype,[12 20 39].

However, while pMVD and VPI have been associated with poor prognosis in previous studies,[6-10], no such associations were found in the present study. It should be recognized that pMVD is generally low in breast cancer,[8], and therefore prone to variations in estimation leading to a similar variation in VPI. Also, the two tumour categories in our study were matched for histological grade, and this could influence the results.

When analysing all cases in this study, MVD was found to be a borderline prognostic factor. This is in accordance with previous studies,[5 40]. However, the results suggest that MVD is a prognostic factor in the Luminal A subtype, but not in BP tumours. Cases were matched according to grade whenever possible, and therefore the Luminal A cases in this study represent a high-grade subpopulation of the Luminal A subtype comprising mostly grade 3 tumours. Despite this, a clear difference in survival was found between Luminal A patients with high MVD and low MVD. This finding has, to the best of our knowledge, not been presented in any previous studies. To further explore and validate this finding, MVD should be investigated in a larger population of Luminal A breast cancers including all grades.

The majority of women in this cohort lived in an era when birth control pills, hormone replacement therapy and mammography screening were largely unavailable,[25 26]. Furthermore, they were diagnosed with breast cancer in a time period or at an age when adjuvant therapy was not an option, thus providing an opportunity to study the near natural course of the disease after surgery. However, it must be recognised that there may be differences between the tumours arising in this cohort and tumours occurring among women who have had access to mammography screening and menopausal hormonal treatment.

Today, breast cancer treatment is based on histological type, histopathological grade, stage, proliferation rate and expression of ER, PR and HER2,[41]. In addition, breast cancer can be classified according to molecular subtypes,[11]. While the main focus has been to identify breast cancer patients with poor prognosis, it is equally important to identify women with a particularly good prognosis to avoid unnecessary treatment. The results of this study underline the need to further study the prognostic and predictive value of MVD, pMVD and VPI. Vascular proliferation was higher in the BP, indicating that this subtype might be more susceptible to anti-angiogenic treatment. Studies are needed to further explore whether pMVD and VPI could serve as predictive markers in the BP category.

The Luminal A subtype has the best prognosis of all subtypes,[12 13 19 21]. High MVD was significantly associated with poorer prognosis among high grade Luminal A tumours. If MVD provides additional prognostic information for these tumours, this might be helpful in further stratifying this large group of patients according to prognosis and may thus contribute to identifying breast cancer patients with a particularly good prognosis that may receive unnecessary treatment today.

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Conflicts of interest: None declared.

Take home messages

MVD is a prognostic factor in high grade Luminal A breast cancer. Vascular proliferation is higher in the Basal-like subtype of breast cancer, but is not associated with BCSS in this series.

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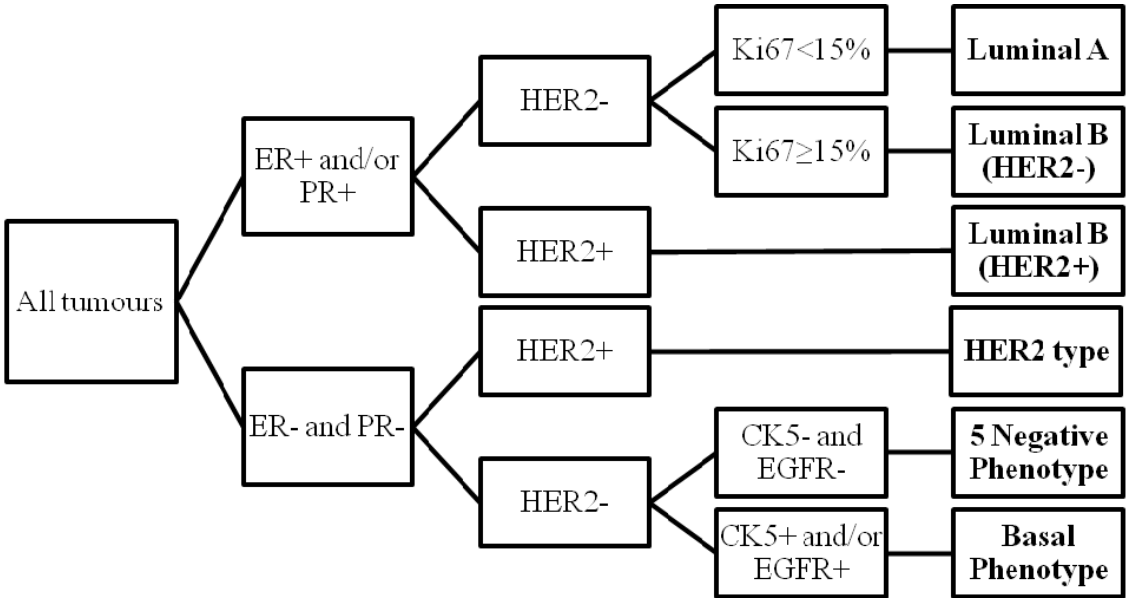

Classification algorithm for molecular subtyping. Ref Engstrom MJ, et al. Molecular subtypes, histopathological grade and survival in a historic cohort of breast cancer patients. Breast cancer research and treatment. 2013 Aug;140(3):463-73

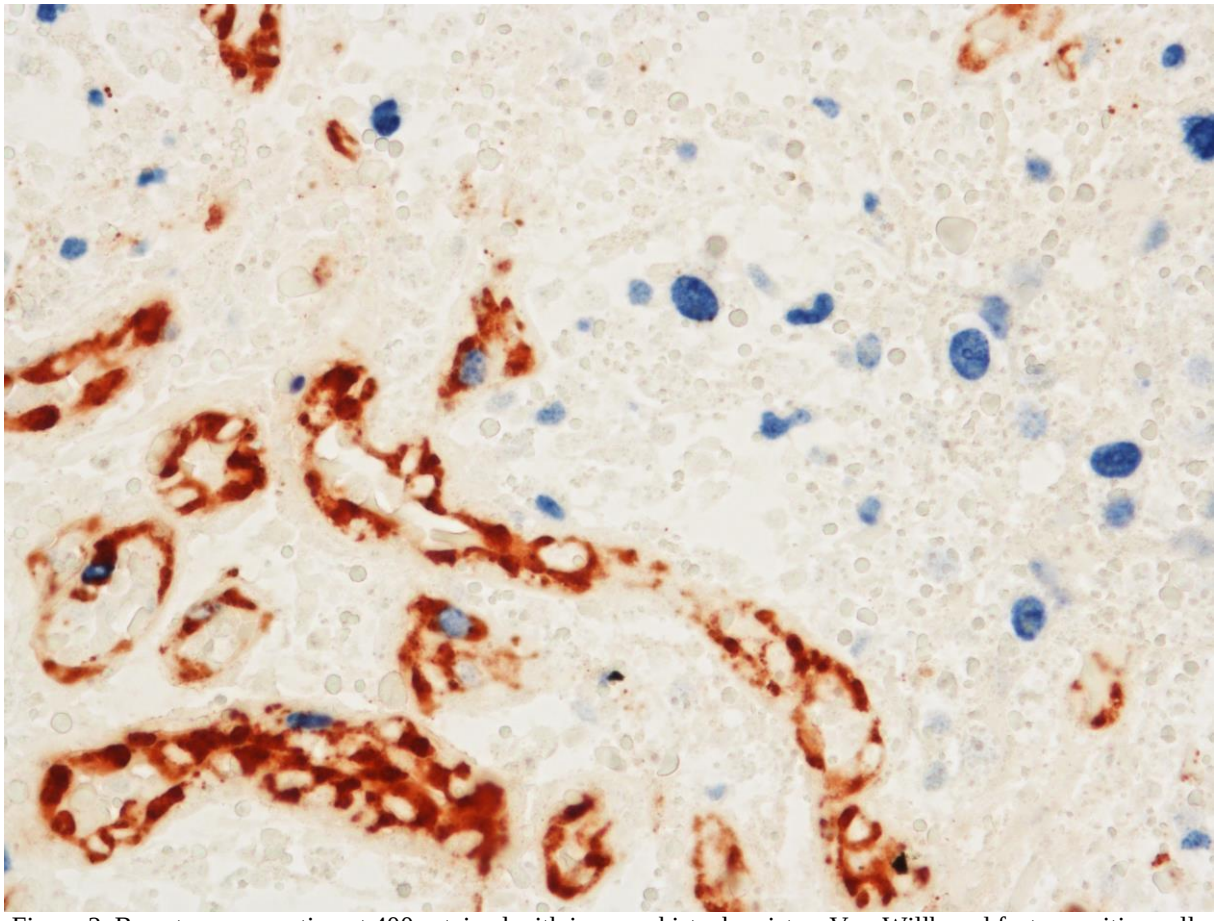

Figure 2. Breast cancer section at 400x stained with immunohistochemistry. Von Willbrand factor positive cells display reddish-brown cytoplasm and Ki67 positive cells display blue nuclei.

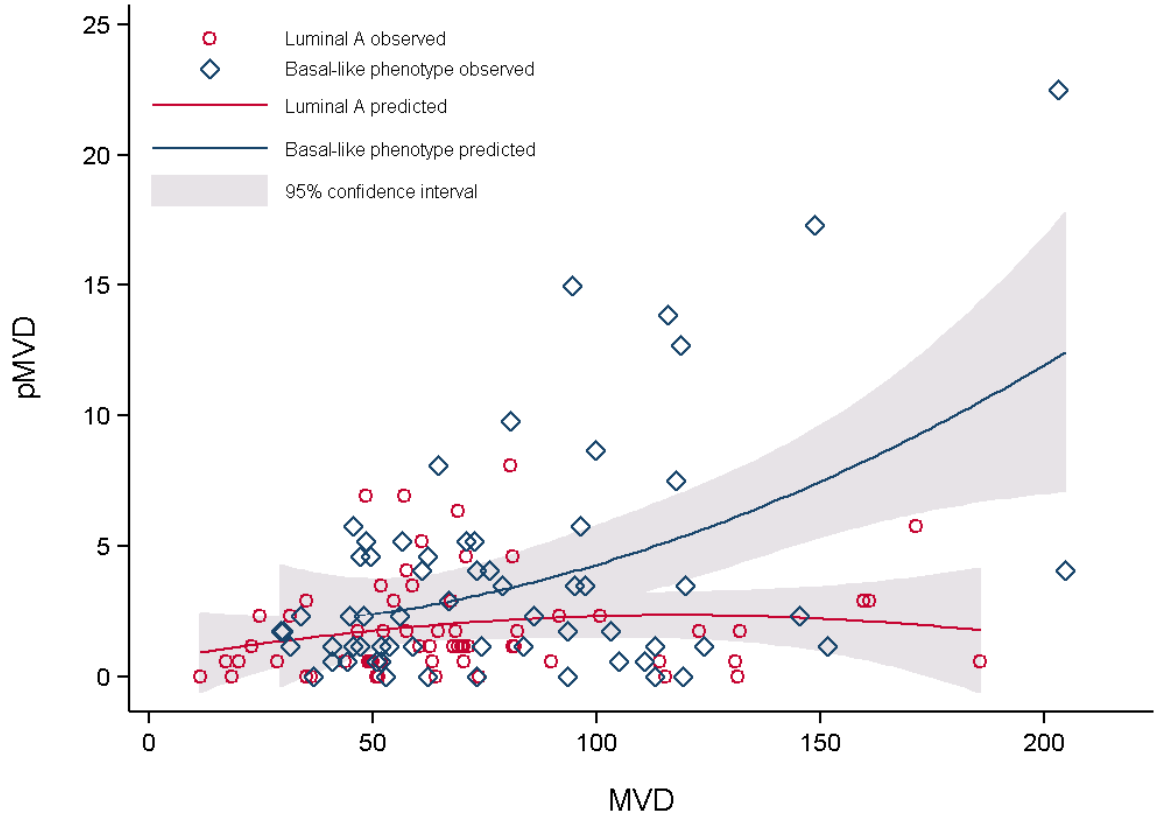

Figure 3. Distribution of Luminal A and Basal-like phenotype subtypes according to MVD and pMVD. Predicted values were estimated by quadratic functions.

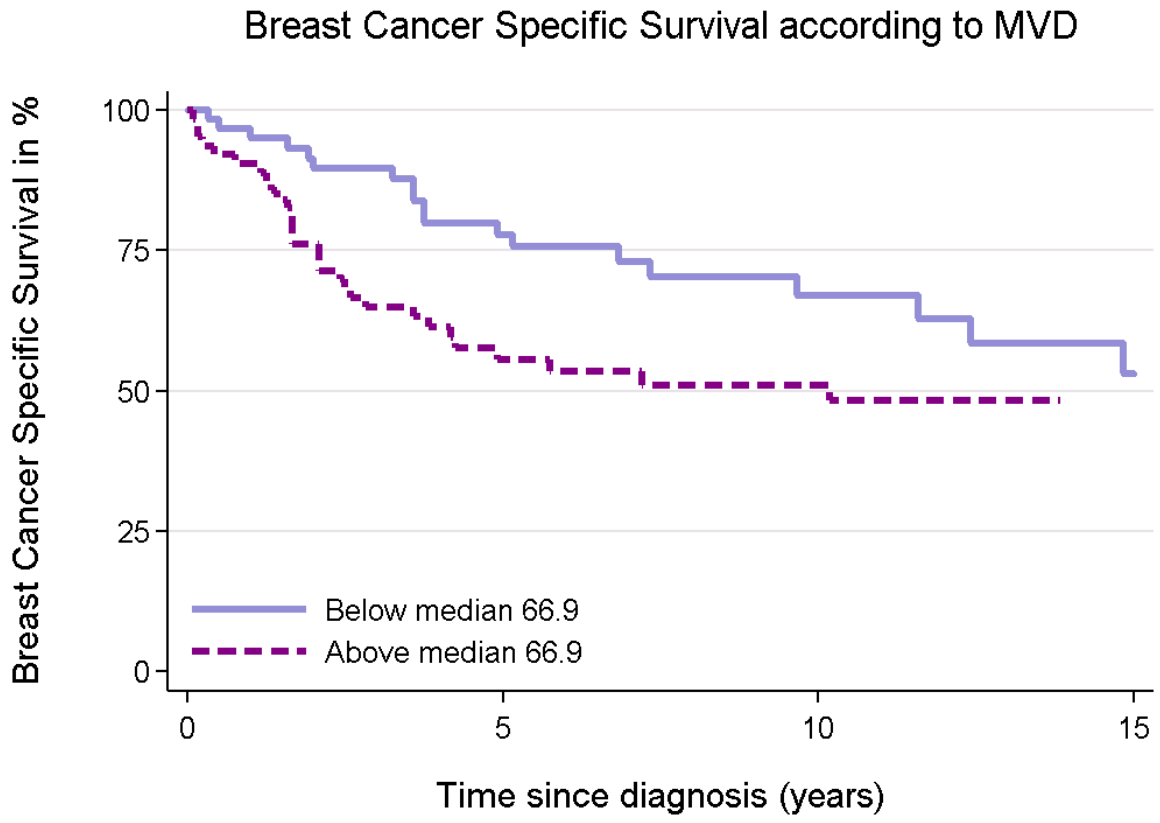

Figure 4. Breast cancer specific survival according to MVD. Log rank test: p=0.066

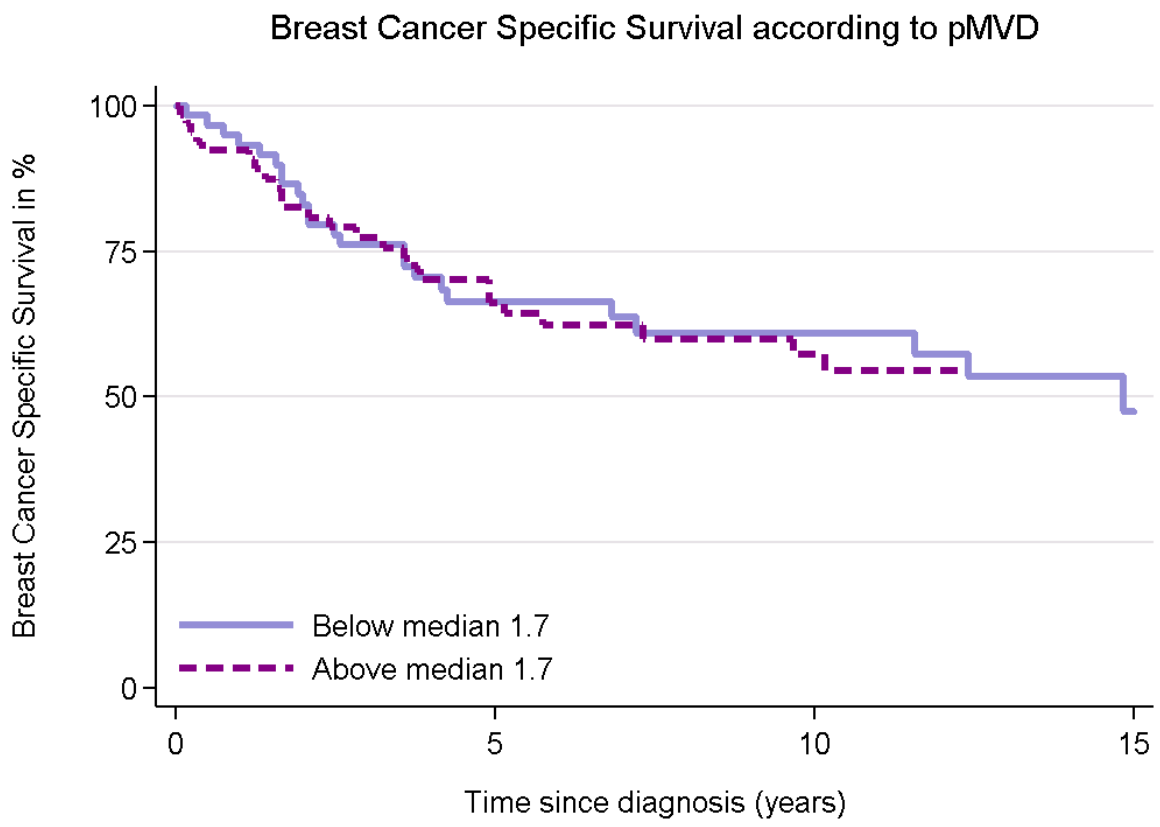

Figure 5a. Breast cancer specific survival according to pMVD. Log rank test: p=0.73

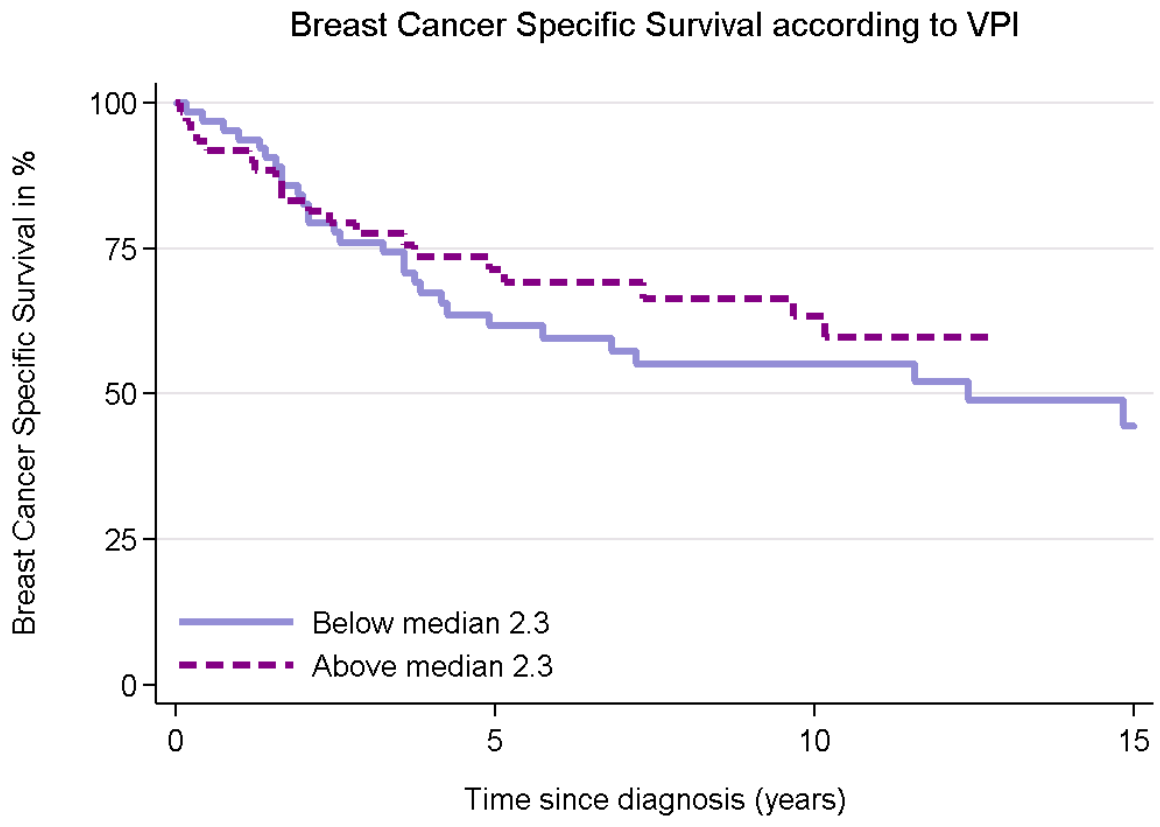

Figure 5b. Breast cancer specific survival according to VPI. Log rank test: p=0.24

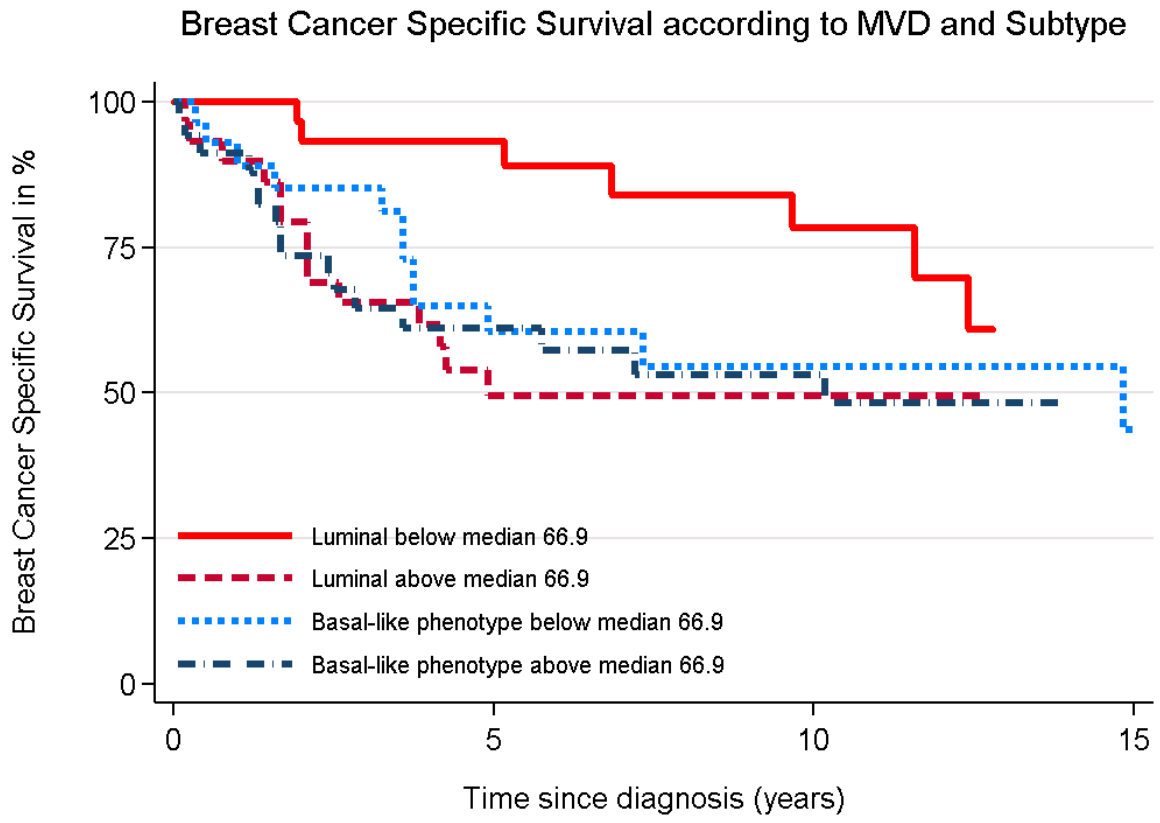

Figure 6. Breast cancer specific survival according to MVD and subtype. Log rank test for Luminal A cases: p=0.038. Log-rank test for Basal-like phenotype cases: p=0.7

Figure texts

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Figure 5b Breast cancer specific survival according to VPI. Log rank test: p=0.24

Figure 6. Breast cancer specific survival according to MVD and subtype. Log rank test for Luminal A cases: p=0.038. Log-rank test for Basal-like phenotype cases: p=0.7