VASCULOGENIC MIMICRY IN PRIMARY BREAST CARCINOMA - A POTENTIAL ANTIANGIOGENIC TARGET: FREQUENCY AND MORPHOLOGICAL CHARACTERISTICS

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Summary

Several different mechanisms of tumor vascularisation including sprouting angiogenesis, intussusceptive angiogenesis, recruitment of endothelial progenitor cells, vessel co-option and vasculogenic mimicry (VM) are known to exist. VM is found to occur in different malignant tumors including breast carcinomas.

Aim of the present study was to establish the frequency and histo- morphological characteristics of VM in primary breast carcinomas.

Formalin-fixed, paraffin embedded tissues from 86 breast cancer patients were examined. Histological features of VM channels, histochemical and immunohistochemical staining characteristics were examined and described.

Vassculogenic mimicry was observed in 5 /86 (5.81%) of randomly selected breast carcinomas. The tumors were generally circumscribed, expansively growing masses with fine collagen septa separating the tumor in smaller nodules. VM channels were seen on H&E staining as well defined, round spaces in solid tumor areas, containing erythrocytes. Histochemical and immunohistochemical staining demonstrated lack of connective tissue elements supporting the structure of VM channels. The luminal surfaces of VM channels were PAS positive and EMA positive.

According to our observations introduction of anti VM drugs in breast cancer has its morphological basis. We suggest that VM should be recognised and included in pathology reports if present. Despite VM seems presently promising target for breast cancer treatment, it should be better understood both morphologically and functionally and studied in the context of breast cancer treatment.

Key words: vasculogenic mimicry, primary breast carcinoma, histomorphological features, morphological basis for targeted ani-vascularisation treatment

Introduction

Angiogenesis is a process essential for tumor progression [1]. Presently several different mechanisms of tumor vascularisation including sprouting angiogenesis, intussusceptive angiogenesis, recruitment of endothelial progenitor cells, vessel co-option and vasculogenic mimicry (VM) are known to exist [2]. While mechanisms of sprouting angiogenesis are presently well understood and strategies for its' inhibition are developed [3, 4], little is known about the so called VM. The "masking of tumor cells as endothelial" was initially described in human melanoma and was named vasculogenic mimicry [5]**.** The molecular mechanisms underlying this morphological phenomenon, observed in melanomas, were unveiled. They were found to involve molecular signaling, different from that observed in sprouting angiogenesis [6].

VM is found to occur in different malignant tumors including melanoma, synovial sarcoma, rhabdomyosarcoma, osteosarcoma, inflammatory and ductal breast carcinoma, ovarian carcinoma, prostatic carcinoma, hepatocellular carcinoma and squamous cell carcinoma [7] astrocytoma [8], pheochromocytoma [9] and Ewing sarcoma [10].

VM is observed if there is specific plasticity potential of malignant tumor cells, accompanied by remodeling of the extracellular matrix resulting in development of channels connected to the host microcirculation system [11, 12].

Presently, several approaches are suggested to block VM in tumors [13, 14], but a specific targeted "anti- vasculogenic mimicry" treatment is far from realistic in clinical practice, since VM is not component of nowadays pathology reports, and cases suitable for such treatment are not recognized.

The present article is an attempt to present the morphological appearance of VM in breast cancer in a way suitable to be used as basic reference for identification and quantification of VM in breast cancer in experimental practice on human breast carcinomas as well as in clinical practice (if useful).

The aim of the present study was to establish the frequency and histomorphological characteristics of VM in primary breast carcinomas.

Patients, materials and methods:

Formalin-fixed, paraffin embedded tissues from 86 breast cancer patients treated surgically between 2005 and 2007 were examined. Details concerning the analyzed tumors are presented on Table 1.

Table 1 Size at the time of operation, histological type, axillary node status, grade, ER status and HER2 status for the studied tumors.

tumor size (pT)	histological type according to WHO [15]	axillary node status (pN)	grade (G)	ER	HER2
T1 – 37 (43.02%) T2 – 35 (40.70%) T3 - 4 (4.65%) T4 – 10 (11.63%)	IDC 69 (80.23%) ILC 7 (8.14%) Mucinous 4 (4.65%) Cribriform 2 (2.33%) Papillary 1 (1.16%) Medullary 3 (3.49%)	N (–ve) – 38 (44.19%) N (+ ve) – 48 (55.81%)	G1 – 16 (18.60%) G2 – 61 (70.93%) G3 – 9 (10.47%)	(-ve) - 27 (31.40%) (+ve) - 59 (68.60%)	(0) - 49 (56.98%) (1+) - 15 (17.44%) (2+) - 9 (10.47%) (3+) - 13 (15.12%)

IDC – invasive ductal carcinoma; ILC – invasive lobular carcinoma; -ve – negative; +ve – positive

Hematoxylin and eosin- stained sections were initially reviewed and consecutive 4μm thick tissue samples from specimen containing VM cannels were prepared (one selected tissue block from each case). Materials were deparaffinised in xylene, and rehydrated using ethanol in a series of decreasing concentrations.

Immunohistochemical method:

For the purposes of the present study, immunostaining of the selected tumors was preformed with EMA; E-cadherin and CK AE1/AE3 antibodies (identifying epithelial differentiation), CD34 and D2-40 antibodies (identifying endothelial differentiation) as well as anti-Collagen type IV antibody for identification of basement membrane. Positivity of breast carcinomas for ER and HER2-overexpression was tested. Details concerning the used primary antibodies are presented on Table 2.

Table 2 General information concerning the used primary antibodies: clone, working concentration, manufacturer and antigen retrieval method used

Primary antibody	clone	dilution	Manufacturer	Antigen retrieval
Her2	c-erbB-2 oncoprotein	1:250	DAKO	Heat mediated - pressure cooking ; 16 psi; t= 124˚ C; Time 1 min
ER	SP1 α	Ready to use	DAKO	Heat mediated - pressure cooking ; 16psi; t= 124˚ C; Time 1 min
CK AE1/AE3	AE1/AE3	Ready to use	DAKO	Proteolysis - Proteinase k
CD34	1A4	Ready to use	DAKO	Heat mediated - pressure cooking ; 16psi; t= 124˚ C; Time 1 min
D2-40	D2-40	Ready to use	DAKO	Heat mediated - pressure cooking ; 16psi; t= 124˚ C; Time 1 min
anti-Collagen type IV	polyclonal	Ready to use	DAKO	Heat mediated - pressure cooking ; 16psi; t= 124˚ C; Time 1 min
E-cadherin	NCHr-38	1:70	DAKO	Heat mediated - pressure cooking ; 16psi; t= 124˚ C; Time 1 min
EMA	E29	Ready to use	DAKO	Heat mediated - pressure cooking ; 16psi; t= 124˚ C; Time 1 min

All immunostains were manually processed. The FLEX EnVISION (DAKO) method was used for the immunohistochemical staining. Immunohistochemical reactions were developed with 3-3` diaminobenzidine and sections counterstained with Mayer hematoxylin.

Histochemical method:

The presence and distribution of connective fibers were histochemmicaly studied using: Elastica staining - according to standard laboratory protocol [16]; van Giesone staining - according to standard laboratory protocol [16] and Gomory silver staining - according to laboratory protocol provided by the manufacturer (Emmonya Biotech Ltd.). The presence of aldehyde and mucosubstances was accessed by PAS methods according to standard laboratory protocol provided by the manufacturer (Merck). All histochemical staining procedures were manually processed.

Immunohistochemistry interpretation:

ER positivity and HER2 status were evaluated in accordance to criteria given in [17]. EMA was evaluated in vascular mimicry channels as well as in tumor cells as present (membranous) or absent. D2-40 and CD34 were evaluated in vascular mimicry channels and true vessels as present (membranous and cytoplasmic) or absent. Collagen IV expression was evaluated in vascular mimicry channels as present or absent. E-cadherin expression in tumor cells was evaluated as present (membranous) or absent. CK AE1/AE3 expression was evaluated in tumor cells as present (membranous, cytoplasmic) or absent.

Histochemistry interpretation

With Elastica staining cell nuclei are stained red, elastic fibers black-blue / dark-blue. With van Giesone staining cell nuclei are stained yellow to brown, collagen– red, muscle – yellow. With Gomory silver reticular fibers are stained black, and collagen fibers yellow to brown. With PAS staining cell nuclei are stained blue and mycosubstances are stained intensively pink [16].

Statistical analyses

The incidence of VM among all studied cases was calculated. Since the study is a descriptive one, no further statistical analysis was planned and preformed.

Results

VM was observed in 5 /86 (5.81%) of randomly selected breast carcinomas (details are presented on Table 3).

Table 3 Patients and tumors (breast carcinomas) in which VM was observed

Case	Age	Type	G	Size at presentation	N	Mitoses per 10 Hpf	E-cadherin	EMA	ER intensity /positivity %	Her2
1	58	IDC NOS	2	20mm	0	4	+	+	moderate/ 45%	2+
2	62	IDC NOS	2	15mm	0	5	+	+	moderate/ 50%	1+
3	51	IDC NOS	2	37mm	X	3	+	+	weak/ 60%	2+
4	58	IDC* NOS	2	25mm	1	2	+	+	weak / 35%	1+
5	67	IDC NOS	2	18mm	0	5	+	+	weak /70%	2+

* a multifocal lesion is present within the same breast (no vasculogenic mimicry was observed within the second lesion)

The average age of the patients with VM vascularisation was 59.2 (51-67) years. Three of the studied patients (case 2 /follow up for 19 months/; case 3 / follow up for 46 months/ and case 5 followed up for 51 months) were alive with no progression of the disease after surgery. Both case 1 and case 4 were lost from follow up several months after operation.

All tumors shared somewhat non specific features. The tumors were generally circumscribed, expansively growing masses with fine collagen septa separating the tumor in smaller nodules (Fig.1).

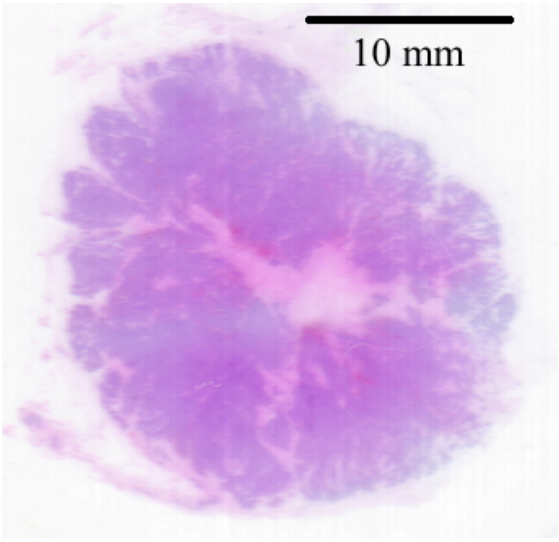


Fig.1. Typical appearance of tumor that exhibits VM. Solid areas of tumor cell predominate over relatively delicate fibrosis. No foci of necrosis were seen 1x, H&E.

The tumor cells were characterised with moderate atypia. The mitotic index in the observed cases was low, not overestimating 5 on 10 HPF. Many apoptosis were observed throughout the tumors. VM channels differed from simple tumor haemorrhages (Fig. 2)

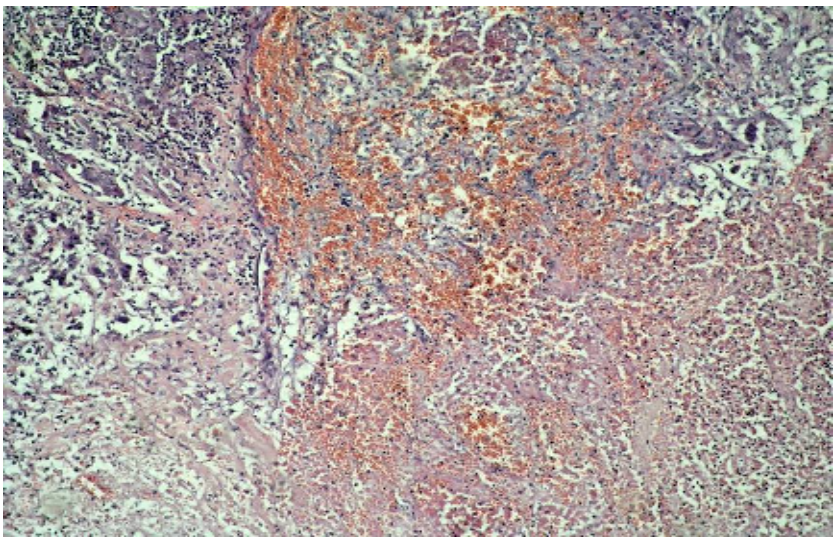


Fig. 2. Haemorrhage within breast cancer- erythrocytes are diffusely intermingled with tumor cells. Necrosis is present. H&E, 100x.

VM channels were seen on H&E staining as well defined, round spaces in solid tumor areas containing erythrocytes (Fig. 3A). CK AE1 / AE3 positivity observed in all tumor cells including in cells lining VM channels (Fig. 3B).

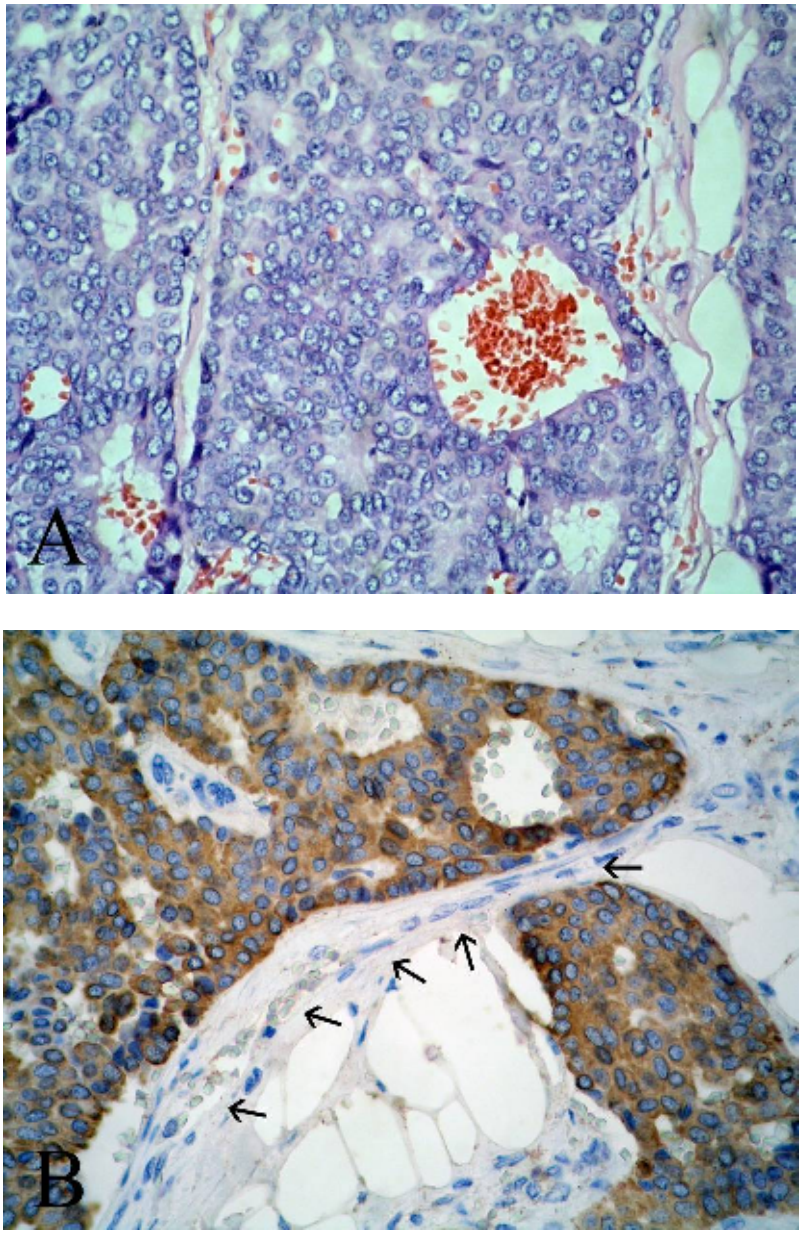


Fig 3.A) VM channels in breast carcinoma were found to be containing erythrocytes, non-endotheliated, round spaces lined by tumor cells. Necroses were not seen. H&E, 400x; **3B**) Endothelium-lined blood vessel negative, for CK AE1 /AE3 (pointed by arrows) and VM channels lined by CK AE1 /AE3 positive tumor cells were coexisting in some breast carcinomas. CK AE1 /AE3, 400x.

No endothelium (lumphovascular or vascular (hemangio) or developed basement membrane was observed in VM structures (Fig.4). D2-40 and Collagen IV staining was negative in all VM channels (not included as illustrations).

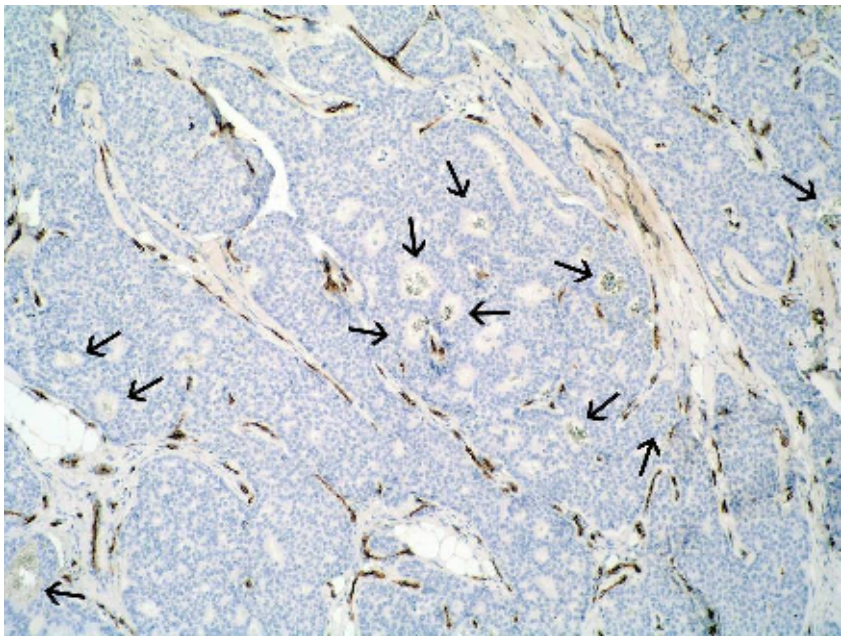
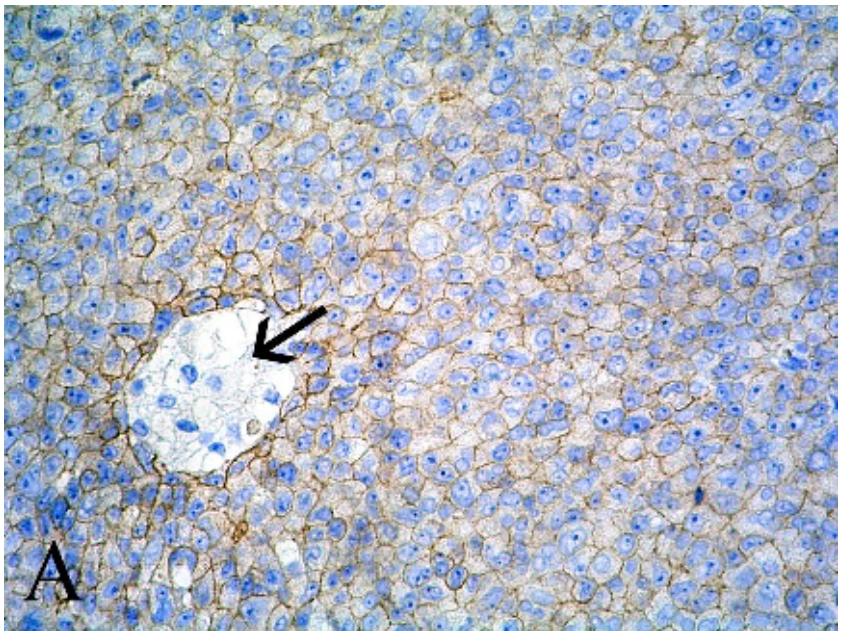


Fig 4. VM channels (pointed with arrows) and blood vessels (immunostaind in brown) were admixed within a tumor. While blood vessels were accompanied by connective tissue, VM channels were situated directly within the solid tumor nests. CD34, 100x.

The breast tumors expressing VM formation were E-cadherin expressing, HER2 equivocal or negative on immunohistochemistry and ER positive (Fig 5 A,B,C).



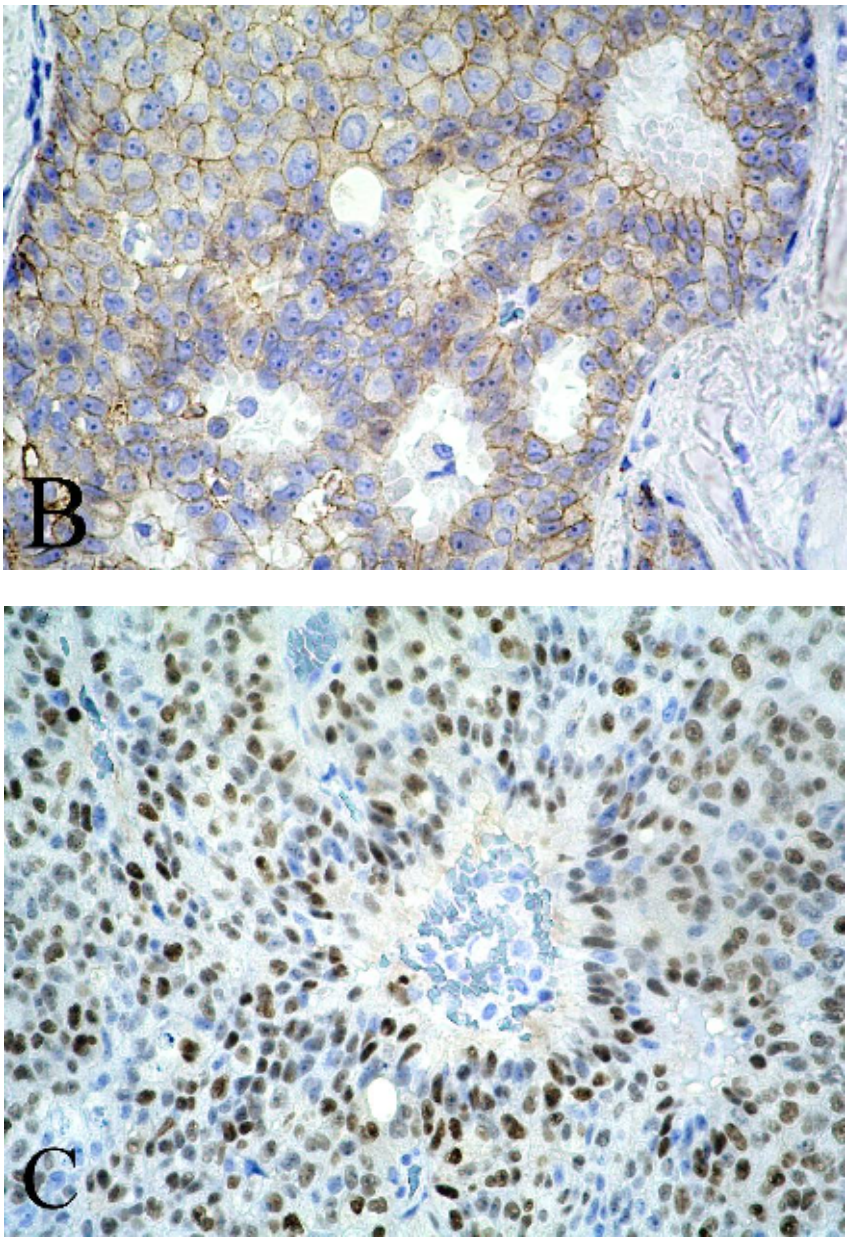


Fig 5 A Formation of empty space within solid area of the tumor (Arrow). Cells within the forming lumen have lost E-cadherin expression. E-cadherin, 400x; **5B** Complete, membranous moderately intensive staining. HER2 - 2+ HER2, 400x; **5C** Moderate nuclear staining for ER in 60% of the tumor cells. ER, 400x.

Unlike vessel VM channels were not supported by connective tissue. No reticular, collagen or elastic fibres were seen in association to VM channels (Fig.6 A,B,C).

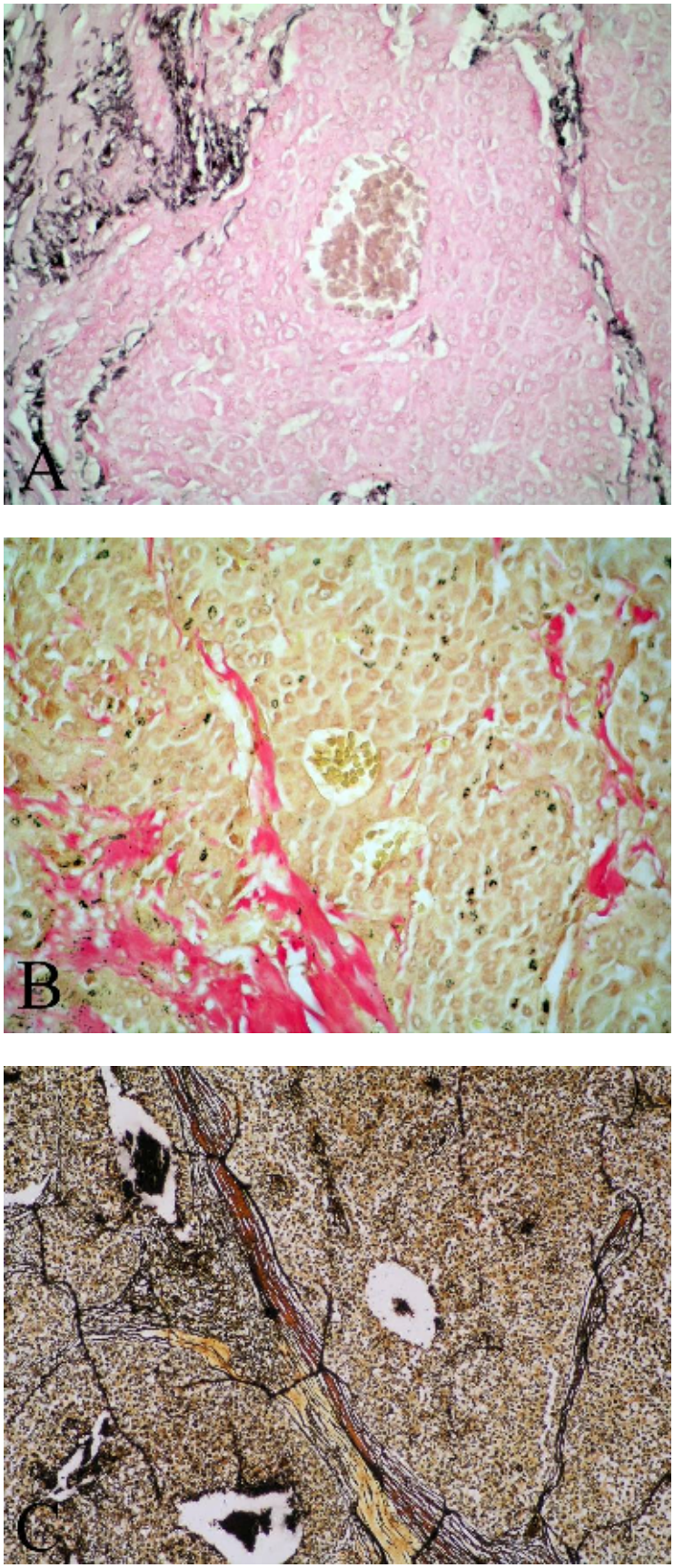


Fig.6 Neither elastic (A) nor collagen (B) or reticular (C) fibres were involved in the VM channel formation. VM channels were deprived of connective tissue support. **A)** Elastica, 400x; **B)** van Giesone, 400x; **C)** Gomori, 100x.

In VM potent tumors, the cells forming VM channel were found to have PAS positive (polysaccharides rich) apical (luminal) surface, while the remaining cells of the tumor, not involved in VM formation remained PAS negative (Fig7).

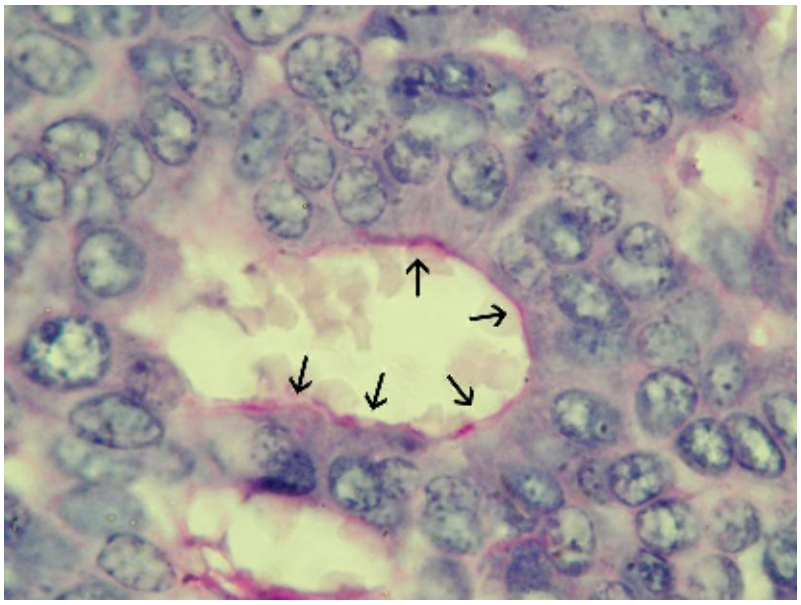


Fig. 7 PAS positive luminal surface of the tumor cells lining the VM channels (arrows). PAS, 400x.

The luminal surface of VM cannels forming tumor cells was EMA positive in all studied cases (Fig.8).

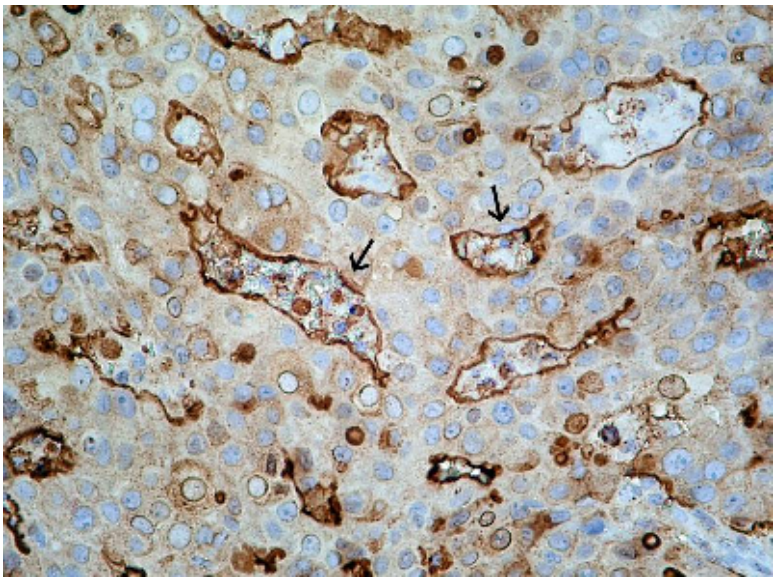


Fig 8 EMA positivity was observed on the luminal surface of tumor cells delineating VM spaces, some of which containing erythrocytes (arrows). Note that all cells of the solid component of the tumor remain weakly positive or negative. EMA, 400x.

On occasion connections between blood capillaries and VM channels were observed (Fig 9).

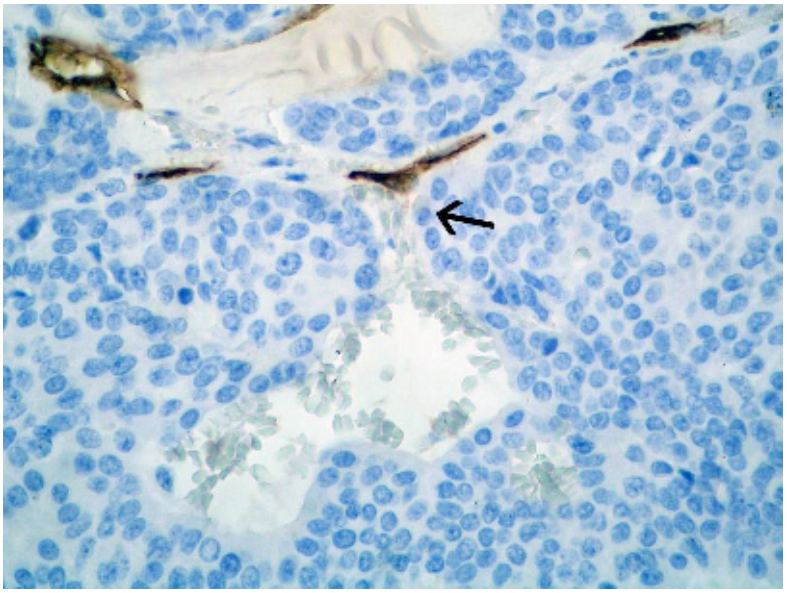


Fig 9 Communication between VM channel and blood vessel was seen (arrow). CD34, 400x.

Discussion

This study proved that VM in breast carcinomas is present and readily accessible during the routine microscopic examination of histological specimen and the used panel of markers proved that the observed structures were tumor –lined spaces [18,19] . The observed communications between blood capillaries and VM cannels supported previous observations and speculations on that topic [11, 18]**.** Probably tumors with certain phenotype were more likely to develop VM and eventually their metastasis also would develop VM vascularisation (we only suggest about metastasis). According to our observations VM is seen in expansively growing, moderately differentiated IDC that retained polar (apical) EMA expression in tumor VM channels, which expression is similar to the observed in normal glandular elements of the breast [20]. Furthermore it is known that expression of EMA is associated with decreased adhesion between cells and the extracellular matrix as well as between adjacent cells [21, 22]. Decreased adhesion between adjacent cells was found in the center of foci of VM formation, but the cells that lined the lumen of VM channels remained E-cadherin positive. EMA expression seemed to play important role in the development of VM in breast carcinomas by neutralising adhesion to the luminal parts of VM forming tumor cells and so retaining normal blood flow within VM channels.

In other tumors that are described to form VM channels, like malignant melanoma [7], EMA expression is not observed. This suggests that VM may eventually have more than one morphological type depending on the tumors tissues (tumors cell phenotype) in which it develops.

Evaluation of tumors vascularisation is presently based on marking and counting endothelial lined spaces [23-25]. Because of the lack of endothelial cell lining, VM (as well as its' impact on the metabolism of tumors is likely to be underestimated by the presently available methods for angiogenesis evaluation. Because of the relatively high frequency of VM, experiments on angiogenesis inhibition in breast cancer shall always estimate the presence and extend of VM.

Unfortunately up to date there is not a single marker or algorithm for identification of VM in breast cancer. There is limited information on the evolution of VM in breast cancer. Based on

our observations we suggest a stepwise hypothesis for the development and evolution of VM in breast cancer and description of the morphological substrate that can be obtained by light microscopy at each step (Table 4).

Table 4 VM: formation, evolution and histomorphological hallmarks

Evolution	Morphological appearance of VM
A) At the beginning there is expansively growing tumor, that does not co-opt host vessels, and so its' vascularization from outside falls behind oxygen requirements of the tumor.	none
B) Some cells lose E-cadherin positivity and "wither" due to metabolic impairment and micro channels are developed. The developed channels are lined with glycoprotein, that probably avoid blood coagulation and solid growth and preserved E- cadherin expression on the cells lining VM channels prevents haemorrhages to occur. In the mean time, blood capillaries growing at the edge of the breast carcinoma become leaky due to hypoxia [26] and start to proliferate and extravasations of blood in the developed channels occurs.	Empty spaces containing debris from apoptotic cells; single erythrocytes filled spaces may be seen. The luminal surface, lined with tumor cells is EMA and PAS positive.
C) Some "immature" capillaries drain into the tumor lined channels, establishing slow, but sufficient to maintain tumor cell vitality and circulation.	Luminal surface lined with tumor cells is EMA and PAS positive; erythrocytes filled spaces are present within the tumor nests, no connective tissue adjacent to VM is normally seen.
D) The initial vasculogenic mimicry may become true vessels as endothelial cells proliferate, migrate and line the primitive clefts.	VM channels may be endothelised with the progression of sprouting angiogenesis.

The relatively high incidence of the phenomenon VM suggests its potential implication in clinical practice.

The prognostic value of this phenomenon is presently uncertain [27]. For instance Folberg R suggested that VM in malignant melanoma serves not only to supply oxygen and nutrients required for tumor growth, but also to enhance metastasis [28]. Our study suggested that VM is not associated with death within the first year after surgery. Because of the limited number of cases in the present study – only three cases with over one year of follow up, no reliable conclusions on the impact of VM on survival can be drown.

Strangely, VM as tumor associated vascular phenomenon has been recognized since 1948, when Willis stated that "in rapidly growing tumors, vessels consist of little more than

irregular channels lined by endothelium only or by naked tumor cells." [29]. Up to date VM observed in some cases of breast carcinoma is poorly understood. It seems that the presence of VM in primary (probably rapidly growing tumors as stated by Willis RA) and / or in distant metastasise of certain phenotype of breast carcinomas may indicate resistance to presently used, antiangiogenic drugs [10].

According to our observations introduction of anti-VM drugs in breast cancer has its morphological basis. Despite VM seems presently promising target for breast cancer treatment, especially with affordable substances like Doxycycline (experimented on microcirculation in melanoma) [13], it should be better understood both morphologically and functionally and studied in the context of breast cancer treatment.

Finally we suggest that VM should be recognised and included in pathology reports if present.

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