Original paper

Correlation between blood and lymphatic vessel density and results of contrast-enhanced spectral mammography

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Contrast-enhanced spectral mammography (CESM) is a novel technique used for detection of tumour vascularity by imaging the moment in which contrast, delivered to the lesion by blood vessels, leaks out of them, and flows out through lymphatic vessels.

In our study, we included 174 women for whom spectral mammography was performed for diagnostic purposes. The relationship between enhancement in CESM and blood vessel density (BVD), lymphatic vessel density (LVD) or the percentage of fields with at least one lymphatic vessel (distribution of podoplanin-positive vessels – DPV) and other related parameters was assessed in 55 cases. BVD, LVD and DPV were assessed immunohistochemically, applying podoplanin and CD31/CD34 as markers of lymphatic and blood vessels, respectively.

The sensitivity (in detection of malignant lesions) of CESM was 100%, while its specificity – 39%. We found a significant positive correlation between the intensity of enhancement in CESM and BVD ($p = 0.007$, $r = 0.357$) and a negative correlation between the intensity of enhancement in CESM and DPV ($p = 0.003$, $r = -0.390$). Lesions with the highest enhancement in CESM showed a high number of blood vessels and a low number of lymphatics.

Conclusions: 1) CESM is a method characterized by high sensitivity and acceptable specificity; 2) the correlation between CESM results and blood/lymphatic vessel density confirms its utility in detection of tissue angiogenesis and/or lymphangiogenesis.

Key words: contrast-enhanced spectral mammography, tissue lymphangiogenesis and/or angiogenesis, normal breast tissue, benign breast disease, invasive breast carcinoma.

Introduction

Angiogenesis, which is a common feature of invasive carcinomas, is a process of new blood vessel formation [1, 2]. Prognostic significance of blood vessel density (BVD) in breast cancer was reported by some authors [1, 3, 4, 5], and questioned by others [6, 7]. Not only the number of blood vessels but also their permeability is different in malignancies as compared to normal tissues [8, 9, 10, 11]. Contrary to blood vessels, not all carcinomas elicit formation of a new lymphatic drainage system [1]. Nevertheless, a relationship between the number of lymphatic vessels and patient survival rate has been reported by some researchers [3, 4, 6, 12, 13, 14, 15].

Utilizing the knowledge about tumour angio- and lymphangiogenesis, the following methods have been recently developed for *in vivo* vascularity imaging: ultrasound (US) performed after contrast administration, breast magnetic resonance imaging (MRI) and contrast-enhanced spectral mammography (CESM). Contrast-enhanced US was first used in breast imaging to increase the diagnostic accuracy of colour or power Doppler imaging [16, 17, 18, 19]. This was mainly due to improved visualization of the intratumoural vascular architecture after contrast agent administration. Different time intensity curves were found for malignant and benign breast masses [20]. Further study revealed that breast mass contrast-enhanced ultrasonography findings correlated with histological features [21]. Despite good results of the studies presented above, the cost of contrast agent used in US examination was too high; therefore, the application of this method in breast cancer diagnostics is limited. The last of the above-mentioned methods is MRI. Although breast MRI is thought to be the most sensitive imaging technique for breast cancer detection and assessment of the extent of the disease, it is often not available to women because of financial reasons, mainly the lack of insurance coverage. Moreover, the quality of breast MRI can differ significantly across practices [22, 23, 24, 25].

Contrast-enhanced spectral mammography is a novel technique accepted by the Food and Drug Administration (FDA) for clinical use in the U.S. in 2011 and intensively developed in the last few years. The main appliance of this method is the assessment of tumour neoangiogenesis by administration of a chelated iodine-based X-ray contrast agent. Contrast-enhanced spectral mammography can be performed by using a current digital mammography system with some specific software and hardware adaptations for image acquisition and processing. Contrast-enhanced spectral mammography is of interest in the non-screening setting and has the potential to increase cancer detection rate and to improve cancer staging, particularly the assessment of the extent of disease.

Imaging both breasts in two views is possible due to the dual-energy technique (for example craniocaudal [CC] and mediolateral oblique [MLO] view) after a single injection of contrast medium and bilateral CESM examination. Contrast-enhanced spectral mammography could be particularly useful in case of dense breasts, if the sensitivity of mammography is lower. Another clinical recommendation for CESM could be equivocal results of previous mammography and ultrasound, mainly because it has the advantage of being a fast imaging technique with immediate availability in the mammography suite without a new appointment and consequently without loss of time.

Contrast-enhanced spectral mammography is used for detection of tumour vascularity by imaging the moment in which contrast, delivered to the lesion by blood vessels, leaks out of them, and flows out through lymphatic vessels. Therefore, hypothetically, the number of blood vessels and lymphatic vessels in the lesion, assessed in tissue sections, should correlate with the results of CESM. This prompted us to study the relationship between enhancement in CESM and blood vessel density (BVD) or lymphatic vessel density (LVD). In order to gain a better insight into the problem, we analysed vessels (blood and lymphatic) without a visible lumen, with a lumen, and all of them, separately. Additionally, in the case of lymphatic vessels, we determined the percentage of fields with at least one lymphatic vessel.

Material and methods

Patients

Between 2011 and 2013, 174 women (mean age 55.7 $\pm$0.8 [SE]) with glandular breast anatomy reported in the previous mammography examination or with a lesion requiring further diagnostics underwent spectral mammography for diagnostic purposes. In this group 191 contrast-enhancing foci (suspected lesions) were found. Histological examination of lesions enhancing in CESM was performed after core biopsy or vacuum-assisted core biopsy guided by ultrasonography or mammography. This verification confirmed the presence of 191 lesions. In 157 patients there was a single lesion, while in 17 women there were 2 lesions. There were 95 invasive carcinomas, 19 *in situ* carcinomas and 77 benign lesions (details are presented in Table I). Additionally, as a control, we included 11 sections of normal breast tissue from Madden's modified mastectomy specimens, from quadrants of breast in which cancer tissue was absent.

This study was performed in compliance with the Declaration of Helsinki and it received the approval of the Ethical Committee at the Regional Medical Chamber in Krakow.

Table I. Histological characteristics of lesions diagnosed with contrast-enhanced spectral mammography

Type of lesion	Parameter	Category	N
benign lesion			77
	histology	chronic inflammatory lesion	2
		regressive changes (fibrosclerosis/fibrosis)	26
		fibroadenoma	31
		usual ductal hyperplasia	17
atypical hyperplasia	–	–	1
carcinoma *in situ*	–	–	19
invasive carcinoma			95
	histology	ductal	77
		lobular	10
		ciribiform	2
		papillary	2
		apocrine	1
		mucinous	1
		not assessed	2
	grade of differentiation	G1	15
		G2	50
		G3	20
		not assessed	10
	pT stage	pT1	67
		pT2	25
		pT3	1
		pT4	1
		not assessed	1
	pN stage	pN1	68
		pN2	19
		pN3	5
		pN4	2
		not assessed	1

Methodology

Contrast-enhanced spectral mammography

All CESM examinations were performed with a digital mammography device developed by GE Healthcare (SenoBright) allowing dual-energy CESM acquisitions. Before exposure patients were given an intravenous injection of non-ionic contrast agent (Ultravist 370, 1.5 ml/kg of body weight), using a power injector (Covidien, Optistar Elite Injector) at a rate of 3 ml/s with a bolus chaser of saline. Two minutes and 4 minutes (for MLO and CC, respectively) after the initiation of contrast agent administration, a pair of exposures (low- and high-energy) in each view (MLO and CC) was performed automatically. Proper image processing allowed for combination of low-energy and high-energy images to generate subtracted images with contrast agent uptake information in each view.

All images were evaluated by one radiologist, blind to patients' history. The following data were recorded for each tumour: presence or absence of con-

trast agent uptake, intensity of contrast agent uptake (assessed qualitatively as being strong medium or weak), the pattern of enhancement in CESM (homogeneous, non-homogeneous and ring-like), as well as size and location of enhancement in the breast.

Immunohistochemistry

Immunohistochemistry was performed in 55 lesions (12 benign lesions: 4 regressive changes, 5 fibroadenomas, 1 hamartoma, 2 usual ductal hyperplasia; 6 *in situ* carcinomas; 37 invasive carcinomas: 34 ductal carcinomas, 1 lobular carcinoma, 1 invasive papillary carcinoma, 1 Paget disease of the nipple), on formalin-fixed, paraffin-embedded tissue sections (4 μm thick), which were mounted on SuperFrost slides (Menzel-Gläser, Braunschweig, Germany), dewaxed in xylene and rehydrated through a series of alcohols. Antigen retrieval was performed with Target Retrieval Solution (TRS, pH = 6.1, 96°C for 50 min, Agilent Technologies Dako Denmark A/S, Glostrup, Denmark); quenching the activity of endogenous peroxidases was performed with 0.3% H_2O_2 in 100% methanol (30 min incubation), while blocking of unspecific antibody binding was performed with UltraVision Protein Block (5 min, Thermo Scientific, Fremont, USA). We used anti-podoplanin antibody (clone D2-40, dilution 1 : 100; Cell Marque, Rocklin, California, USA) for visualization of lymphatic vessels, anti-CD34 antibody (clone QBEnd 10, dilution 1 : 50; Agilent Technologies Dako Denmark A/S, Glostrup, Denmark) and anti-CD31 antibody (clone 1A10, dilution 1 : 50, Leica-Biosystems, Buffalo Grove, USA) for visualization of blood vessels. BrightVision (30 min, room temperature; Immunologic, Duiven, Netherlands) was used to visualize the presence of vessels. In the double-staining procedure VIP (violet colour) was used as a peroxidase substrate (Vector Laboratories, Burnligame, USA) for visualization of CD34 (Fig. 1A, B; arrow) or CD31 (Fig. 1C, E, G; arrow), while DAB (brown colour) was used for podoplanin (Fig. 1A, C, E, G; arrowhead). Eventually, slides were counterstained with Mayer's hematoxylin.

Evaluation of immunohistochemistry

Distribution of podoplanin-positive vessels (DPV) was assessed in the whole examined specimen (over 4 microscopic fields were required for its assessment). In each field (10× objective) the absence or presence of lymphatics (with lumen, without lumen or with emboli) was recorded. Based on the aforementioned assessment, each field was classified as positive (with at least one podoplanin-positive vessel) or negative (without lymphatics). Eventually, the percentage of fields with at least one lymphatic vessel was calculated (DPV).

Additionally, we assessed (in more than 4 microscopic fields) the density of CD34-positive or CD31-positive blood vessels and podoplanin-positive lymphatic vessels (CD34BVD or CD31BVD and LVD, respectively). Blood vessel density and LVD were expressed as mean number of vessels (blood and lymphatic, respectively) per microscopic field (10× objective magnification, area 0.848 mm^2 of the specimen field). In the case of invasive malignancies, BVD and LVD were assessed in the intratumoural and peripheral area of the lesion. We calculated BVD and LVD separately for small vessels without a lumen (BVDsmall [Fig. 1B; arrowhead] and LVDsmall), vessels with a lumen (BVDlumen [Fig. 1B; arrow] and LVDlumen [Fig. 1C; arrowhead]) and all types of vessels (with and without a lumen) (BVD and LVD).

The analysis was performed with digital image analysis using a CX41 microscope, SC30 camera and Cell D software (Olympus Europa GmbH, Hamburg, Germany). Vessels were marked manually and then counted automatically. All evaluations were determined without the knowledge of CESM results.

Because in some non-invasive lesions and normal breast tissue CD34 immunopositivity of myofibroblasts (surrounding normal ducts, lobules, and intraductal carcinomas) hindered calculation of BVD (Fig. 1D, star), we decided to apply CD31 instead. Therefore, we compared BVDsmall, BVDlumen and BVD, obtained using CD31 and CD34, to test whether these markers can be used interchangeably. For this purpose, serial sections from 6 malignant lesions were stained with: (1) CD31 (VIP)/podoplanin (DAB) (Fig. 1C, E, G) and (2) CD34 (VIP)/podoplanin (DAB) (Fig. 1D, F, H). Then we selected microscopic fields with the same fragment of tissue from CD31- and CD34-stained slides. Next, four paired fields (one for CD31, the other for CD34) for each of six tumours were taken (in sum 24 fields for CD31 and for CD34). Finally, we assessed BVDsmall, BVDlumen and BVD both for CD31 and for CD34: CD31BVDsmall, CD34BVDsmall, CD31BVDlumen, CD34BVDlumen, CD31BVD and CD34BVD.

Statistical analysis

The STATISTICA v. 10 software (StatSoft, Inc. Tulsa, OK, USA) was used for all calculations. The p value < 0.05 was considered significant. Differences between groups were estimated using one-way analysis of variance (ANOVA) or Kruskal-Wallis ANOVA. After one-way analysis of variance post-hoc Tukey's HSD test was performed. The Wilcoxon signed-rank test (a paired difference test) was used to compare two matched samples and to assess whether their population mean ranks differ (repeated measurements using different antibodies). The Pearson correlation coefficient was used to test for a correlation between two continuous variables. Spearman's rank correlation coefficient was applied to measure statistical dependence between two variables. Pear-

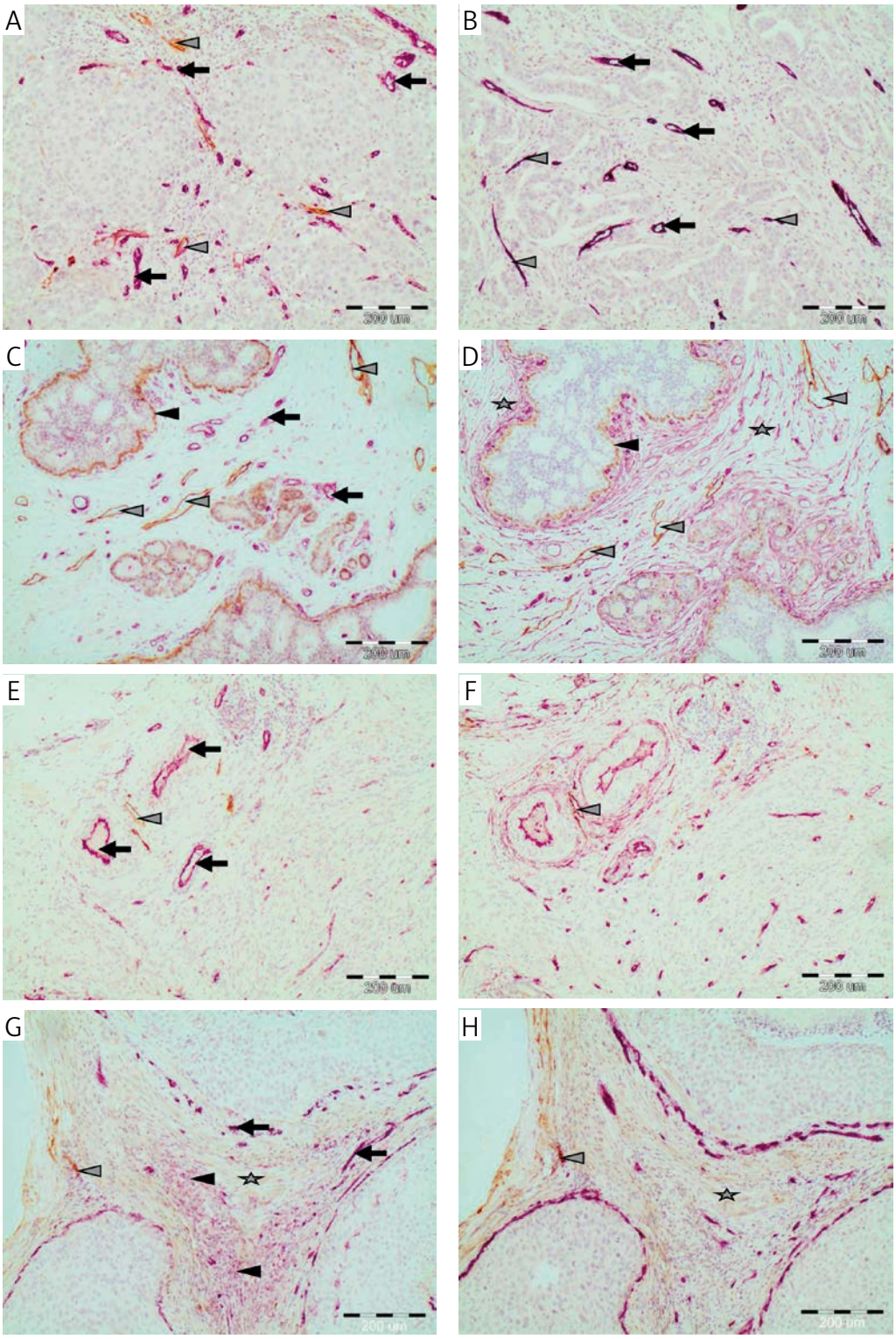

Fig. 1. Staining pattern of CD34 (violet colour; A, B, D, F, H), CD31 (violet colour; C, E, G) and podoplanin (brown colour; A-H) in invasive ductal carcinomas (A, B, E, F) and *in situ* carcinomas (C, D, G, H). **A**) Invasive ductal carcinoma with CD34-stained blood vessels (arrow) and podoplanin-stained lymphatic vessel (arrowhead); **B**) carcinoma without lymphatic vessels in intratumoural area, and with CD34-stained blood vessels with lumen (arrow) and without lumen (arrowhead); **C, D**) two serial sections from *in situ* carcinoma stained with anti-CD31/podoplanin (C) and anti-CD-34/podoplanin (D): blood vessels (C, arrow) podoplanin-positive lymphatic vessels (C, D; grey arrowhead), podoplanin-positive myoepithelial cells (C, D; black arrowhead), CD34-stained myofibroblasts (D; asterisk); **E, F**) two serial sections from invasive ductal carcinoma stained with anti-CD31/podoplanin (E) and anti-CD-34/podoplanin (F): blood vessels (E, arrow), podoplanin-positive lymphatic vessels (E, F; grey arrowhead); **G, H**) two serial sections from *in situ* carcinoma: blood vessels (G, arrow) podoplanin-positive lymphatic vessels (G, H; grey arrowhead), podoplanin-positive stromal fibroblasts (G, H; asterisk). All microphotographs were taken at 10× (objective)

son's χ^2 was applied to test the independence of categorical variables expressed in a cross-tab.

Results

Expression of podoplanin, CD34 and CD31

Podoplanin expression was found in endothelial cells of lymphatic vessels (Fig. 1A, C-H; arrowhead), cancer-associated stromal fibroblasts (Fig. 1G-H; asterisk represents weak staining), myoepithelial cells surrounding ducts, lobules or *in situ* carcinomas (Fig. 1C, D; black arrowhead).

CD34 expression was found in endothelial cells of blood vessels (Fig. 1A, arrow; 1B arrow and arrowhead) and myofibroblasts surrounding ducts, lobules or *in situ* carcinomas (Fig. 1D, asterisk). Because CD34-positive myofibroblasts (observed in benign lesions) hindered obtaining reliable results for BVD, in some cases we applied double staining with CD31 (violet colour) and podoplanin (brown colour).

CD31 was present in endothelial cells of blood vessels (Fig. 1C, E, G; arrow) and absent in myofibroblasts surrounding ducts, lobules or *in situ* carcinomas (Fig. 1C) that were immunopositive for CD34 (Fig. 1D, asterisk). Additionally, CD31 expression was found in leukocytes infiltrating tumour stroma (Fig. 1G; black arrowhead).

Blood and lymphatic vessel density in normal breast tissue, benign lesions, *in situ* carcinomas and invasive carcinomas

Some invasive carcinomas (18/37 cases, 48.6%) presented no lymphatics in the intratumoural or peripheral area (Fig. 1B). However, all of them presented CD34 (or CD31)-stained blood vessels.

In the whole group the mean values of BVDsmall, BVDlumen and BVD were: 48.0 ±31.8 (SD), 15.4 ±10.7, 63.4 ±34, respectively. On the other hand, for LVDsmall, LVDlumen, LVD and DPV we found the following mean values: 1.4 ±1.8, 3.2 ±4.6, 4.8 ±6.0 and 54.1 ±43, respectively.

Invasive carcinomas presented lower values of LVDsmall and DPV than other lesion types (Fig. 2A), while normal breast tissue presented higher values of LVDlumen and LVD than all other lesions (invasive and *in situ* carcinomas and benign lesions) (Fig. 2C, E). Invasive and *in situ* carcinomas, as compared to benign lesions and normal breast tissue, were characterized by significantly higher BVDsmall and BVD (Fig. 2B, F).

We found a negative correlation between BVDsmall and: (a) DPV [$R = -0.443$, $p < 0.001$], (b) LVDlumen [$R = -0.445$, $p < 0.001$] and (c) LVD [$R = -0.378$, $p = 0.002$]; as well as between BVD and: (a) DPV [$R = -0.408$, $p = 0.001$], (b) LVDlumen [$R = -0.375$, $p = 0.002$] and (c) LVD [$R = -0.316$, $p = 0.010$]. No correlation ($p > 0.05$) was observed between BVDsmall or BVD and LVDlumen. Moreover, BVDlumen was not correlated with DPV, LVDsmall, LVDlumen or LVD ($p > 0.05$).

In the group of invasive carcinomas BVD was not correlated with LVD ($p > 0.05$ for studied parameters).

Comparison of blood vessel density assessed based on CD31 and CD34 immunopositivity in endothelial cells

In all analysed cases CD31 immunopositivity of endothelium (Fig. 1C, E, G) was weaker than CD34 immunopositivity (Fig. 1D, F, H). Nevertheless, no difference was found between: (1) CD34BVDsmall (50.5 ±13.2; mean ± SD), and CD31BVDsmall (51.4 ±12.5) ($p = 0.683$); (2) CD34BVDlumen (7.6 ±7.6) and CD31BVDlumen (7.9 ±3.5) ($p = 0.683$) as well as (3) CD34BVD (68.1 ±10.5) and CD31BVD (69.3 ±11.7) ($p = 0.683$). This convinced us that we can use CD31 in cases where myofibroblast CD34 immunopositivity hindered obtaining reliable results of BVD (Fig. 1D; asterisk).

Contrast enhancement in contrast-enhanced spectral mammography and histological parameters

The sensitivity of CESM was 100%: all carcinomas (invasive or *in situ*) (114 cases) presented enhancement (weak, medium or strong) in CESM. On the other hand, specificity of CESM was 39.0%: no enhancement in CESM was observed in 30/77 benign lesions (Table II). We found a significant relationship between enhancement in CESM and lesion type ($p < 0.001$, from Pearson's χ^2 test; Table II). Moreover, in the group of carcinomas, strong enhancement was significantly more frequent in invasive than in *in situ* carcinomas ($p < 0.001$, from Pearson's χ^2 test; Table II).

In the group of invasive carcinomas, there was a significant positive correlation between enhancement in CESM and: pT, pN ($p = 0.003$, $r = 0.314$; $p = 0.002$, $r = 0.333$, respectively; Spearman correlation coefficient). Moreover, we found a significant positive correlation between lesion size in CESM and both pT and pN ($p = 0.001$, $r = 0.362$; $p = 0.001$, $r = 0.340$).

Patients with benign lesions were significantly younger (53.3 ±9.9: mean age ± SD) than these with *in situ* (56.5 ±9.3) or invasive carcinomas (57.5 ±11.3) ($p = 0.021$ from Kruskal-Wallis test).

Correlations between contrast enhancement in contrast-enhanced spectral mammography and parameters related to blood and lymphatic vessel density

With Spearman's rank correlation, we found a significant positive correlation between the intensity of

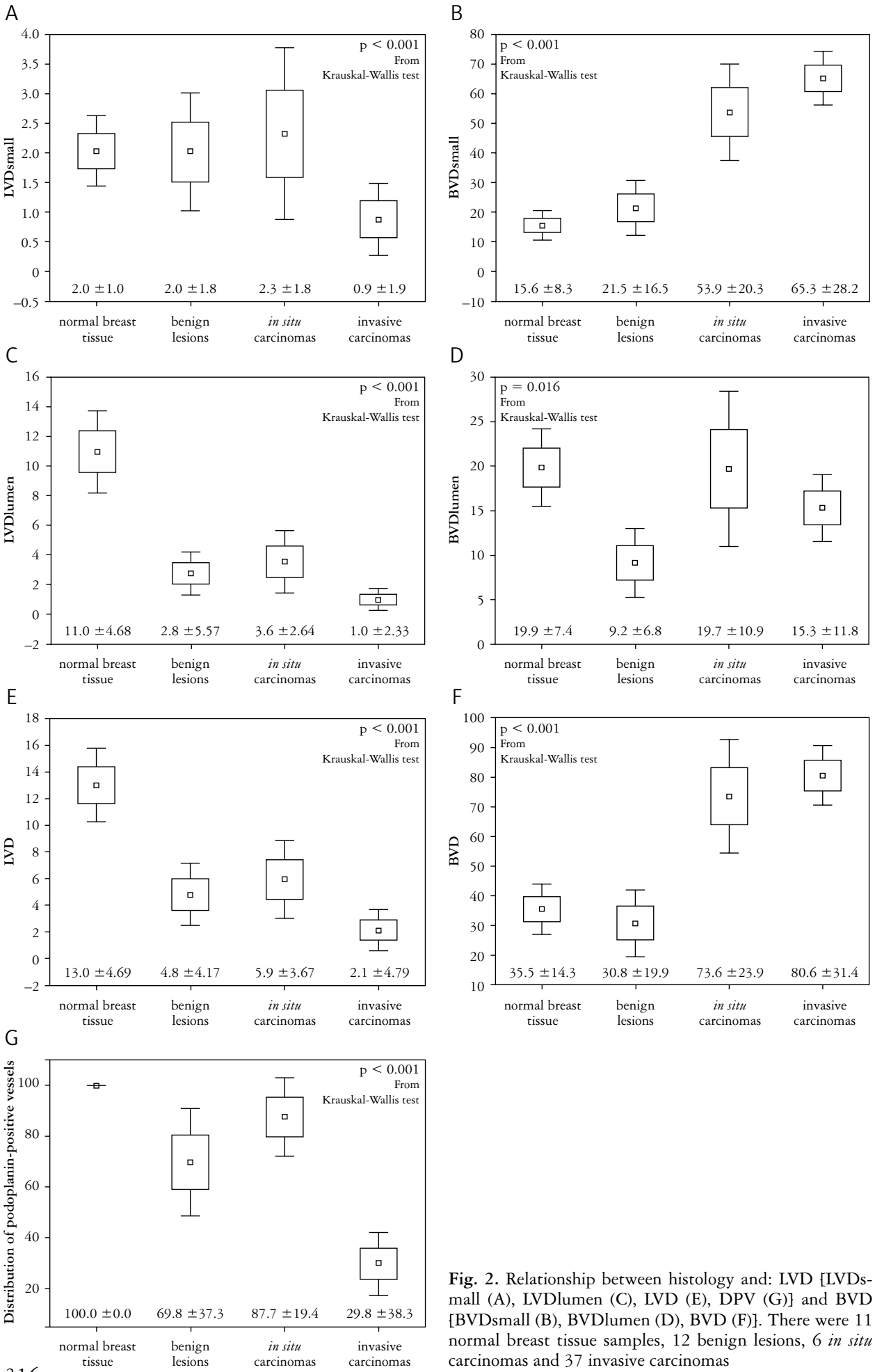

Fig. 2. Relationship between histology and: LVD [LVDsmall (A), LVDlumen (C), LVD (E), DPV (G)] and BVD [BVDsmall (B), BVDlumen (D), BVD (F)]. There were 11 normal breast tissue samples, 12 benign lesions, 6 *in situ* carcinomas and 37 invasive carcinomas

Table II. Relationship between contrast enhancement in contrast-enhanced spectral mammography and histology of lesions

Histological type of lesions	Enhancement in CESM			
	Absent (n = 30)	Weak (n = 47)	Medium (n = 45)	Strong (n = 69)
	n (%)*	n (%)	n (%)	n (%)
benign lesion (n = 77)	30 (39.0)	23 (29.9)	12 (15.6)	12 (15.6)
in situ carcinoma (n = 19)	0	11 (57.9)	6 (31.6)	2 (10.5)
invasive carcinoma (n = 95)	0	13 (13.7)	27 (28.4)	55 (57.9)

enhancement in CESM and density of blood vessels: BVDsmall ($p < 0.002$, $r = 0.403$) and BVD ($p = = 0.007$, $r = 0.357$). The same relation was confirmed by Kruskal-Wallis ANOVA: BVDsmall, BVDlumen and BVD (Fig. 3B, D, F, Fig. 4) were significantly higher in lesions with medium or strong enhancement in CESM than in lesions with weak or no enhancement.

Moreover, with Spearman's rank correlation, there was a significant negative correlation between the intensity of enhancement in CESM and density of lymphatic vessels: DPV ($p = 0.003$, $r = –0.390$), LVDsmall ($p = 0.038$, $r = –0.281$), LVDlumen ($p = 0.003$, $r = –0.393$) and LVD ($p = 0.008$, $r = –0.352$). With Kruskal-Wallis ANOVA we found significantly lower values of LVDlumen, LVD and DPV (Fig. 3C, E, G) in cases with strong enhancement in CESM, as compared to those with no enhancement in CESM.

There was a statistically significant relationship between enhancement in CESM and presence or absence of lymphatic vessels ($p = 0.047$). In all lesions (5 cases, 100%) with no enhancement in CESM, lymphatic vessels were present. However, in 50% (13/26 cases) of lesions with strong enhancement in CESM no lymphatics were found. In lesions with weak and medium enhancement in CESM there were 71.4% (5/7 cases) and 76.5% (13/17 cases) of cases with lymphatic vessels, respectively.

Benign lesions with false-positive results in CESM (the presence of enhancement in CESM, n = 5) had a higher number of blood vessels (BVD) and a lower number of lymphatic vessels (DPV) as compared to benign lesions with true-negative results (lack of enhancement in CESM, n = 7). In benign lesions with false-positive results in CESM the mean value of BVD and DPV was 40.8 ±18.8 and 53.8 ±42.3, respectively, while in benign lesions with true-negative results in CESM the mean value of BVD and DPV was 16.8 ±11.9 and 92.3 ±7.8, respectively ($p = 0.018$ for BVD and $p = 0.072$ for DPV). All invasive and *in situ* carcinomas were true-positive in CESM; therefore, we did not analyse the lack of enhancement vs. its presence. However, in the above-mentioned group we observed significantly lower DPV (24.9 ±36.3) in carcinomas with strong enhancement in CESM than in carcinomas with medium or weak enhancement (55.9 ±42.3) ($p = 0.015$). On the other hand, in carcinomas with weak enhancement in CESM, significantly lower values of BVDlumen and BVD (8.5 ±4.7 and 60.8 ±18.8, respectively) were found as compared to carcinomas with medium (23.6 ±15.5 and 95.9 ±42.9, respectively) or strong enhancement (13.4 ±7.9 and 75.0 ±19.8, respectively) ($p = 0.042$, $p = 0.057$, respectively).

The pattern of enhancement in CESM (homogeneous, non-homogeneous and ring-like) was related to neither BVD (BVDsmall, BVDlumen, BVD) nor LVD (LVDsmall, LVDlumen, LVD, DPV) ($p > 0.05$).

In the group of invasive carcinomas, there was no significant correlation between parameters describing lymphatics and BVD (LVDsmall, LVDlumen, LVD, DPV, BVDsmall, BVDlumen) and pT, pN stage and grade ($p > 0.05$).

Discussion

To the best of our knowledge, for the first time we found that the strongest enhancement in CESM characterizes lesions with a high number of blood vessels and a low number of lymphatics (or their poor distribution: DPV). This result confirms that the CESM technique detects and/or measures angio-/lymphangiogenesis of the lesion, after intravenous injection of a non-ionic contrast agent (Ultravist 370) and during a pair of breast exposures (low- and high-energy) in each view [26, 27]. As suggested by our results, the enhancement in CESM is present when non-ionic contrast agent is profusely delivered to breast tissues through a dense network of blood vessels, leaks out from the capillaries and "gets stuck" in the tissue because of the low number of lymphatics, hindering its outflow. In contrast, in tissues with a high number of lymphatic vessels and a relatively low number of blood vessels (benign lesions and normal breast tissue, as confirmed by our results) the outflow of contrast is not perturbed. Therefore, as we speculate, no enhancement is observed during breast exposure (no enhancement in CESM). The relationship between high number of blood vessels and intensity of enhancement in CESM might be additionally related to lesser selectivity and higher permeability of tumour

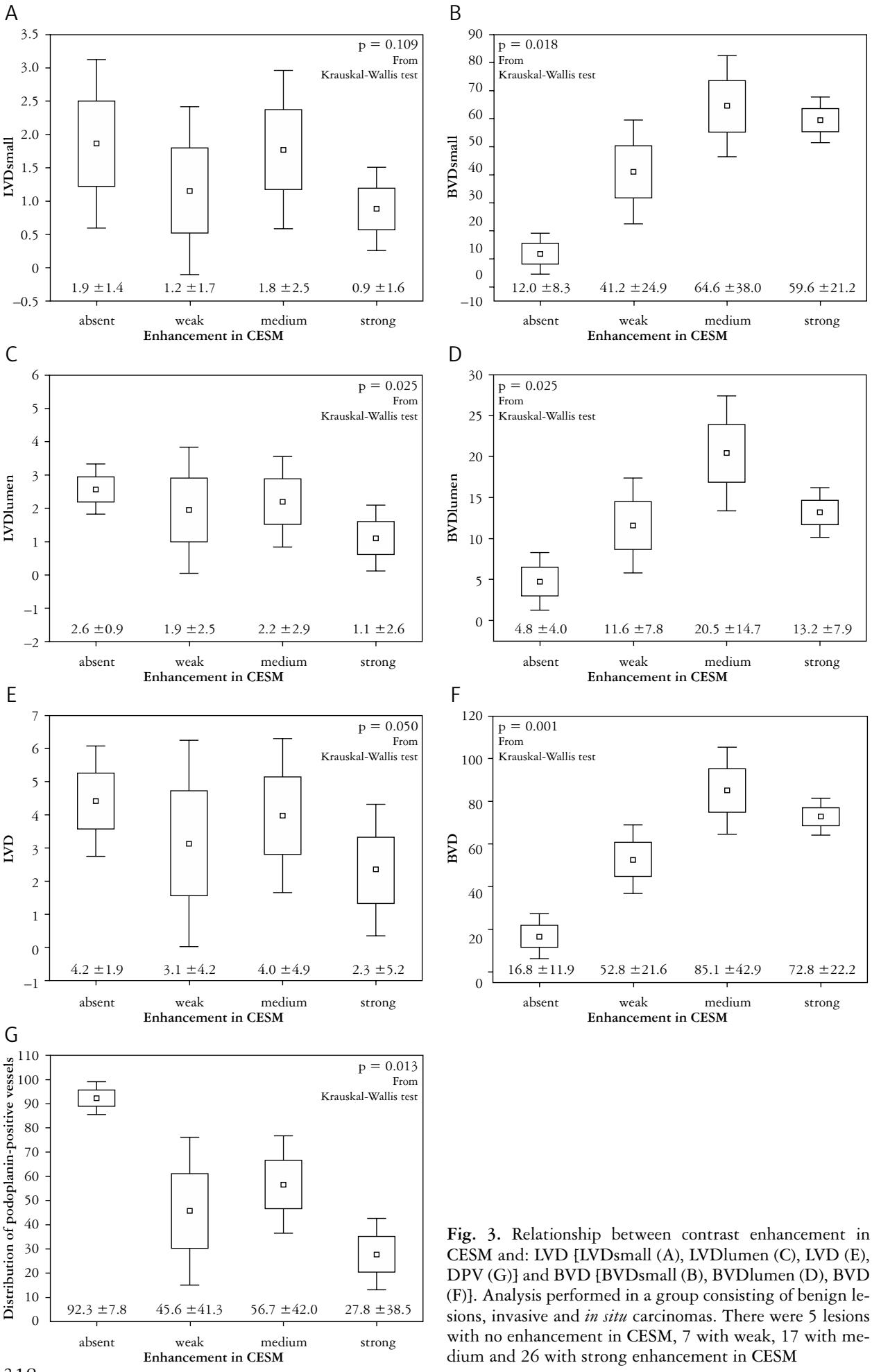

Fig. 3. Relationship between contrast enhancement in CESM and: LVD [LVDsmall (A), LVDlumen (C), LVD (E), DPV (G)] and BVD [BVDsmall (B), BVDlumen (D), BVD (F)]. Analysis performed in a group consisting of benign lesions, invasive and *in situ* carcinomas. There were 5 lesions with no enhancement in CESM, 7 with weak, 17 with medium and 26 with strong enhancement in CESM

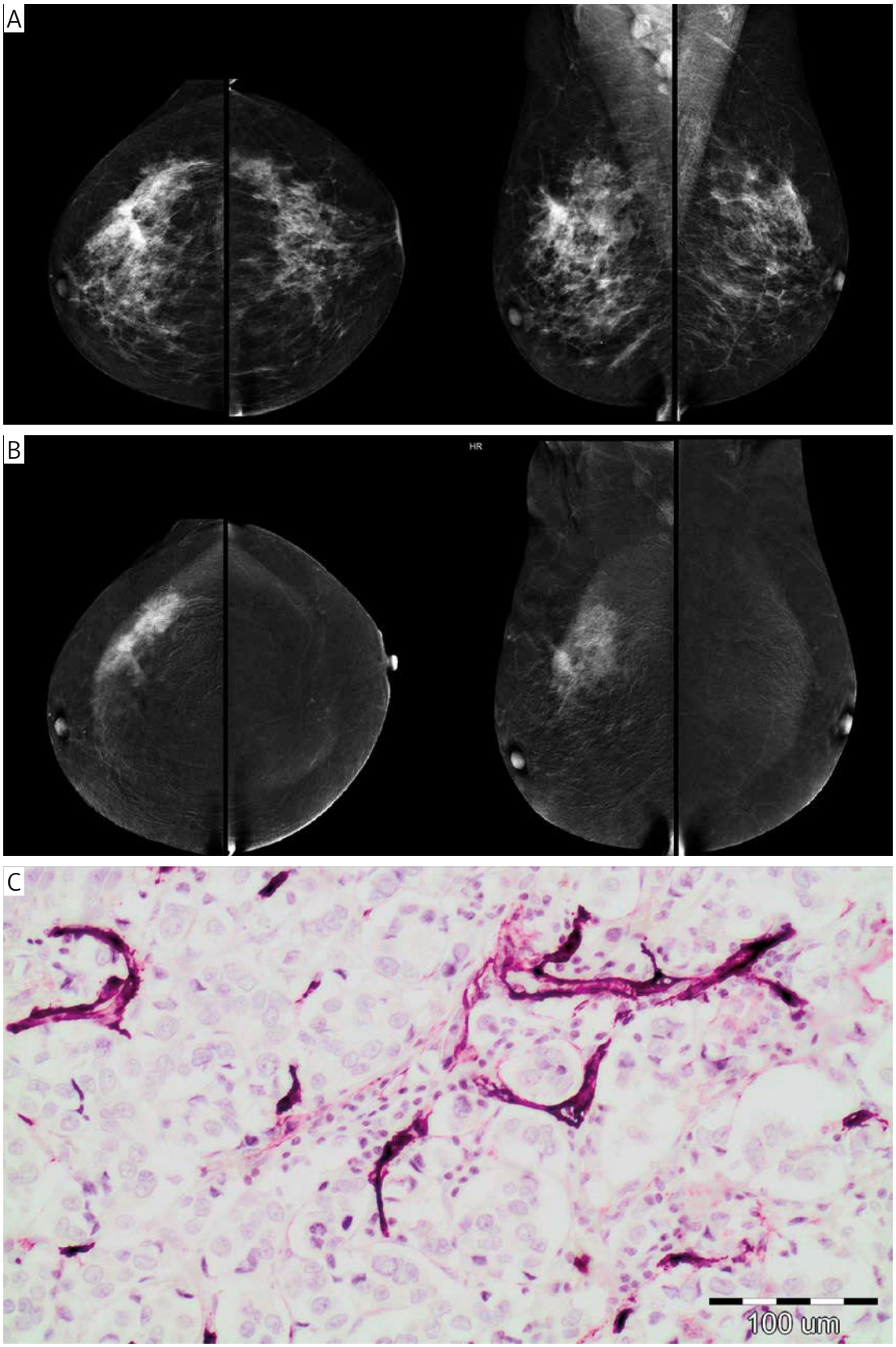

Fig. 4. Infiltrating ductal carcinoma (arrows) of the right breast in 66-year-old woman with dense fibroglandular breast tissue: **A**) Digital conventional mammography (MG) with mediolateral oblique (MLO) view of the right breast reveals architectural distortion 4 × 3 cm. This mass is not visible in MG craniocaudal (CC) projection. **B**) Contrast-enhanced spectral mammography (CESM) in the same patient shows poorly demarcated focus of enhancement 6 × 4 cm visible in both MLO and CC projections. **C**) In this case, histopathological examination revealed infiltrating carcinoma with a high number of CD34-stained blood vessels (violet colour) and no podoplanin-stained lymphatic vessels. Microphotograph was taken at 20× objective

vessels as compared to normal vessels (presumably due to large "gaps" between endothelial cells and discontinuity of the vascular basement membrane) [8, 9, 10, 11].

These speculations are clearly confirmed by an analysis performed by us, separately for benign lesions and carcinomas. Benign lesions with false-positive results (presence of enhancement in CESM), as compared to benign lesions with true-negative results (lack of enhancement in CESM), were characterized by a higher number of blood vessels (expressed as BVD) and a low number of lymphatic vessels (expressed as DPV). A similar tendency was observed in the group of invasive/*in situ* carcinomas, in which the strong enhancement in CESM (all carcinomas were true-positive) was related to a lower number of lymphatics and a high number of blood vessels.

The relationship between breast cancer vascularity and results of imaging techniques detecting tumour angiogenesis has also been studied by other researchers [26, 28, 29, 30]. For the CESM technique, Dormain *et al.* [27] reported a statistically insignificant trend toward higher intratumoural BVD in breast cancer patients with true-positive results as compared to false-negatives. This result is similar to ours: for true-positive cancer patients (we did not find false-negatives) we found significantly higher BVD in carcinomas with medium/strong enhancement in CESM. The discrepancy between the aforementioned results might be the effect of the smaller number of cases reported by Dormain *et al.* [27] and a different method of BVD assessment. A significant positive correlation between BVD and lesion enhancement in magnetic resonance was also reported [28, 29, 30]. However, no correlation was found between enhancement and LVD [28].

In the present study, 51.4% of invasive carcinomas presented lymphatics in the intratumoural or peripheral area. This is within the range of our previous results in female breast cancer (79.3% [31, 32] and male breast cancer (77% [33]) and within the range of other authors' results (50% [5], 85% [34]). Moreover, invasive carcinomas presented lower LVD (LVDsmall and DPV) than other lesion types, while normal breast tissue presented higher values than all other lesions (benign lesions, *in situ*/invasive carcinomas). Our results confirm the statement of Vermeulen *et al.* [1] that not all carcinomas elicit formation of a new lymphatic drainage system and some of them use pre-existing lymphatic vessels from normal tissue.

An inverse relation was observed for blood vessels (which were present in all analysed tissues): invasive and *in situ* carcinomas, as compared to benign lesions and normal breast tissue, were characterized by significantly higher BVD. This result confirms the existence of tumour angiogenesis [1, 2].

The negative correlation between BVD and LVD found by us, for all analysed tissues (except for invasive carcinomas), as well as previously described results, confirms that malignant breast lesions elicit angiogenesis [1, 2], but only some of them are in a position to activate lymphangiogenesis [1]. This feature of tissues can be exploited by imaging techniques using contrast, which is delivered with efficiency proportional to BVD, partially leaks out from the capillaries (with higher intensity in tumour vessels because of their permeability) and is drained proportionally to LVD. In cases with an abnormal (as compared to normal tissue) proportion of both the above-mentioned vessel types, contrast accumulation in tissue may take place, which, on the other hand, might be visualized during exposure.

Interestingly, two parameters describing LVD – distribution of podoplanin-positive vessels (DPV) and number of lymphatics with a lumen (LVDlumen) – correlated the most significantly (with the lowest p-values) with histology and enhancement in CESM. This result suggests that normal breast tissue, benign lesions and carcinomas differ in the number of lymphatic vessels with a lumen and in distribution of all lymphatics. This, on the other hand, suggests the need for studies on the prognostic significance of the two above-mentioned parameters (DPV and LVDlumen). In the case of blood vessels, the most significant correlation with histology was found in the case of blood vessels without a lumen (BVDsmall), suggesting intensive angiogenesis [1] in carcinomas as compared to benign lesions and normal tissue.

The utility of contrast-enhanced spectral mammography might be confirmed by strong correlation between the size of the lesion measured on CESM and: the size of the lesion measured on histological section (pT) (which was found by us and other researchers [27] and pN status (found in our study).

In our study, the sensitivity of CESM was 100% and is comparable to the results of other researchers, where it ranged from 63.5 to 100% [35, 36, 37, 38]. Moreover, in the group of carcinomas (*in situ* and invasive) we found a significant relationship between the degree of enhancement in CESM and histology (strong enhancement characterized invasive carcinomas). Specificity of CESM reported by us was relatively high (39%) and might suggest that some features of neoplastic angiogenesis might begin in selected benign lesions, before the development of malignant phenotype.

We plan to enlarge the group of cancer patients and study the relationship between histological characteristics of tumours (other than lymphatic/blood vessel density) and their degree of enhancement in CESM as well as the prognostic significance of the aforementioned parameters.

Contrast-enhanced spectral mammography, similarly to other diagnostic methods, has the following limitations: (1) enhancement of benign lesions (it is worth mentioning, however, that benign lesions may enhance not only in CESM but also in MRI); (2) lack of possibility of drawing an enhancement curve: the shape of the curve usually indicates the character of the lesion, which in many cases can reduce the number of unnecessary surgical procedures; (3) 20% higher radiation dose of CESM in comparison to MG; (4) lack of unambiguous classification describing the type and grade of breast lesions in CESM: so far, lesion assessment in CESM has been subjective, and other imaging methods have to be used for verification of the lesion; (5) inability to perform biopsy under CESM guidance: lesion enhancement in CESM is transient and lesions not visible in other methods require exposing patients to another contrast administration and marker placement under CESM control; (6) examination not possible in patients with renal failure if renal clearance is lower than [< 40 ml/min/1.73 m^2]; (7) small availability: at present, there are only three CESM installations in Poland. Presented values are not representative for the whole population, which results from focusing on patients with suspected cancers.

To recapitulate, the existence of a correlation between results of CESM and lymphatic/blood vessel density, which are potential prognostic factors [1, 3, 4, 5, 6, 12, 13, 14, 15, 39, 40], found in the present study, suggests that, in the future, CESM might bring some prognostic information for breast cancer patients. However, further studies are needed.

Additionally to studying the correlation between tumour vascularity and enhancement in CESM, we compared the staining pattern of anti-CD34 and anti-CD31. The immunoreactivity of CD34–, podoplanin was the same as described and discussed in our previous studies [31, 32, 33, 39] or as reported by other authors [41].

In some cases we decided to apply CD31 for calculation of BVD, because in some non-invasive lesions and normal breast tissue CD34 immunopositivity of myofibroblasts hindered calculation of this parameter. Therefore, we compared BVDsmall, BVDlumen and BVD, obtained using CD31 and CD34. We found very high accordance between the results in the case of all types of blood vessels: with a lumen, without a lumen, and all of them. This finding confirmed previous reports suggesting the same staining pattern and intensity of CD31 and CD34 staining in blood vessels of the skin [41, 42], bone marrow, kidney and lung [41]. In our study, occasional and irregular expression of CD31 and CD34 in lymphatic vessels [41, 42] did not influence assessment of BVD because of application of podoplanin as a marker of lymphatic vessels, in the double-staining procedure.

Conclusions

Contrast-enhanced spectral mammography might be recommended as a method with high sensitivity for breast cancer diagnostics.

The correlation between intensity of enhancement in CESM and blood/lymphatic vessel density confirms that CESM is the method of detection of tissue's blood and lymphatic vessel density.

Existence of a correlation between CESM results and lymphatic/blood vessel density (which are potential prognostic factors) suggests that, in the future, CESM might bring some prognostic information for breast cancer patients.

The authors declare no conflict of interest.

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