

The Specific Shapes of Capillaries are Associated with Worse Prognosis in Patients with Invasive Breast Cancer

Hnin-Wint-Wint Swe¹, Masayoshi Fujisawa¹, Toshiaki Ohara¹, Yu Komatsubara¹, TeizoYoshimura¹, Tadahiko Shien², Akihiro Matsukawa¹

1. Department of Pathology and Experimental Medicine, Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University, Okayama, Japan

2. Department of Breast and Endocrine Surgery, Okayama University Hospital, Okayama, Japan

Corresponding Author: Masayoshi Fujisawa

mfujisawa@okayama-u-ac.jp

Department of Pathology and Experimental Medicine, Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University, 2-5-1 Shikata, Kita Ward, Okayama, 700-8558, Japan

Hnin Wint Wint Swe and Masayoshi Fujisawa contributed equally to this work.

Supplementary Table S1. Antibody and Staining Conditions

Antibody	Clone	Dilution	Pretreatment	Manufacturer
Collagen IV	CIV22	1:500	EDTA (pH 8.0), proteinase K	Cell Marque, Rocklin, CA
CD31 ¹	EP78	1:100	EDTA (pH 8.0)	Cell Marque, Rocklin, CA
CD31 ²	EPR3094	1:500	EDTA (pH 8.0)	Abcam, Cambridge, UK
Podoplanin (D2-40)	D2-40	1:50	EDTA (pH 8.0)	Dako, Carpinteria, CA
GATA 3	L50-823	1:250	EDTA (pH 8.0)	Cell Marque, Rocklin, CA

¹ Used for single (CD31) and double (CD31 and CollagenIV) staining on thin sections.

² Used for thick sections.

Supplementary Table S2. CD31 and collagen IV double staining procedure

Step	Process
1	Deparaffin slides in xylene, followed by 100%, 90% and 80% ethanol
2	Rinse slides with water
3	Enclose sections in a pressure cooker with 0.5 M EDTA (pH 8) and microwave (700W) for 20 minutes
4	Cool down at room temperature
5	Primary antibody incubation for 90 minutes ¹
6	Secondary antibody ² incubation for 60 minutes ¹
7	Color detection with ALP ³
8	Incubate slides in 0.004% proteinase K ⁴ for 3 minutes ¹
9	Incubate slides in 10% H ₂ O ₂ for 10 minutes ¹
10	Primary antibody incubation for 90 minutes ¹
11	Secondary antibody incubation for 60 minutes ¹
12	Histochemical reaction with DAB ⁵
13	Counterstaining with hematoxylin
14	Dry slides on a hot plate (50 °C) for 20 minutes
15	Mount sections by a conventional method

All the process was performed at room temperature.

¹ Followed by a rinse with Tris-buffered saline with Tween 20, three times.

² Simplestain MAX-PO, Nichirei, Tokyo, Japan

³ ImmPRESS Alkaline Phosphatase Polymer Reagent, Vector, Burlingame, CA

⁴ Cat.No.03115887001, Roche, Mennheim, Germany

⁵ Liquid DAB+ Substrate Chromogen system, Dako, Santa Clara, CA

Supplementary Table S3. CD31 single staining procedure

Step	Process
1	Deparaffin slides in xylene, followed by 100%, 90% and 80% ethanol
2	Rinse slides with water
3	Enclose sections in a pressure cooker with 0.5 M EDTA (pH 8) and microwave (700W) for 20 minutes
4	Cool down at room temperature
5	Incubate slides in 10% H ₂ O ₂ for 10 minutes ¹
6	Primary antibody incubation for 90 minutes ¹
7	Secondary antibody ² incubation for 60 minutes ¹
8	Histochemical reaction with DAB ³
9	Counterstaining with hematoxylin
10	Mount sections by a conventional method

All the process was performed in room temperature.

¹Followed by a rinse with Tris-buffered saline with Tween 20, three times

²Simplestain MAX-PO, Nichirei, Tokyo, Japan

³Liquid DAB+ Substrate Chromogen system, Dako, Santa Clara, CA

Supplementary Table S4. Staining procedure of thick FFPE sections (50µm)

Step	Process
1	Deparaffin sections in overnight xylene followed by 100%, 90% and 80% ethanol
2	Rinse sections with water
3	Enclose sections in autoclave machine with 0.5 M EDTA buffer (pH 8) and heat at 105°C for 20 minutes
4	Cool down at room temperature
5	Incubate slides in serum free blocking buffer for 60 minutes
6	Primary antibody (CD31 and GATA3) incubation at 4°C overnight ¹
7	Secondary antibody ² incubation at 4°C overnight ¹
8	Place sections on glasses and mount sections by aqueous mounting medium ³

¹ Followed by a rinse with Tris-buffered saline with Tween 20, three times

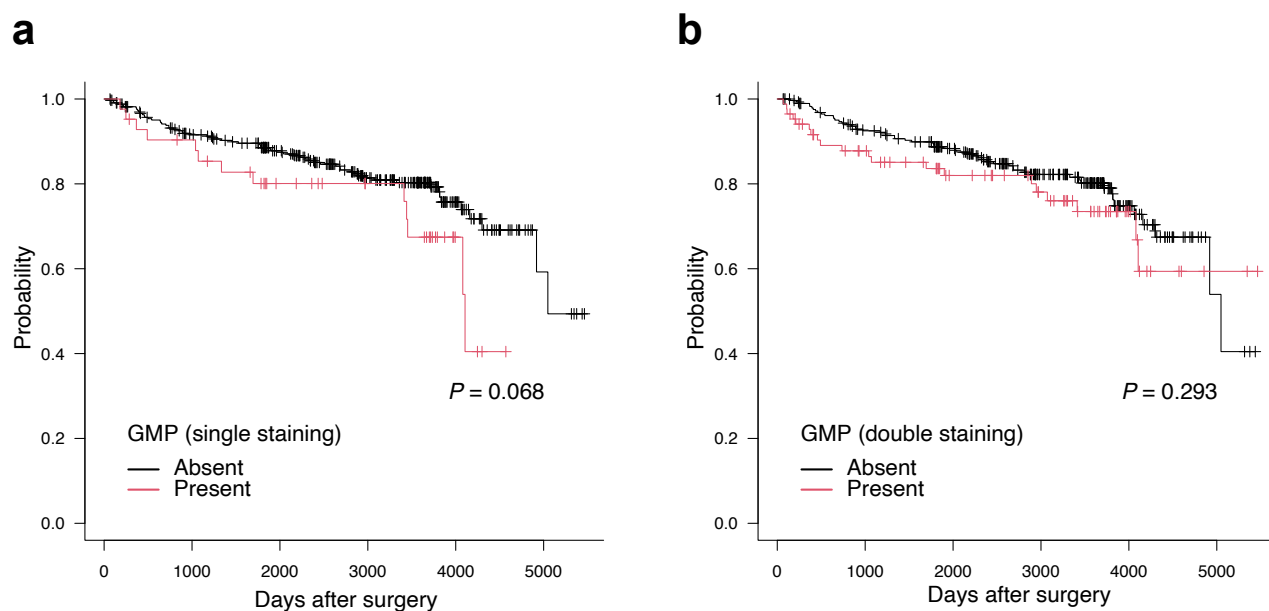
² Secondary antibodies: Alexa Fluor 488 goat anti mouse IgG and Alexa Fluor 647 goat anti rabbit IgG (1:250), Invitrogen, Carlsbad, CA

³ Aquatex mounting medium, Merck, Burlington, MA

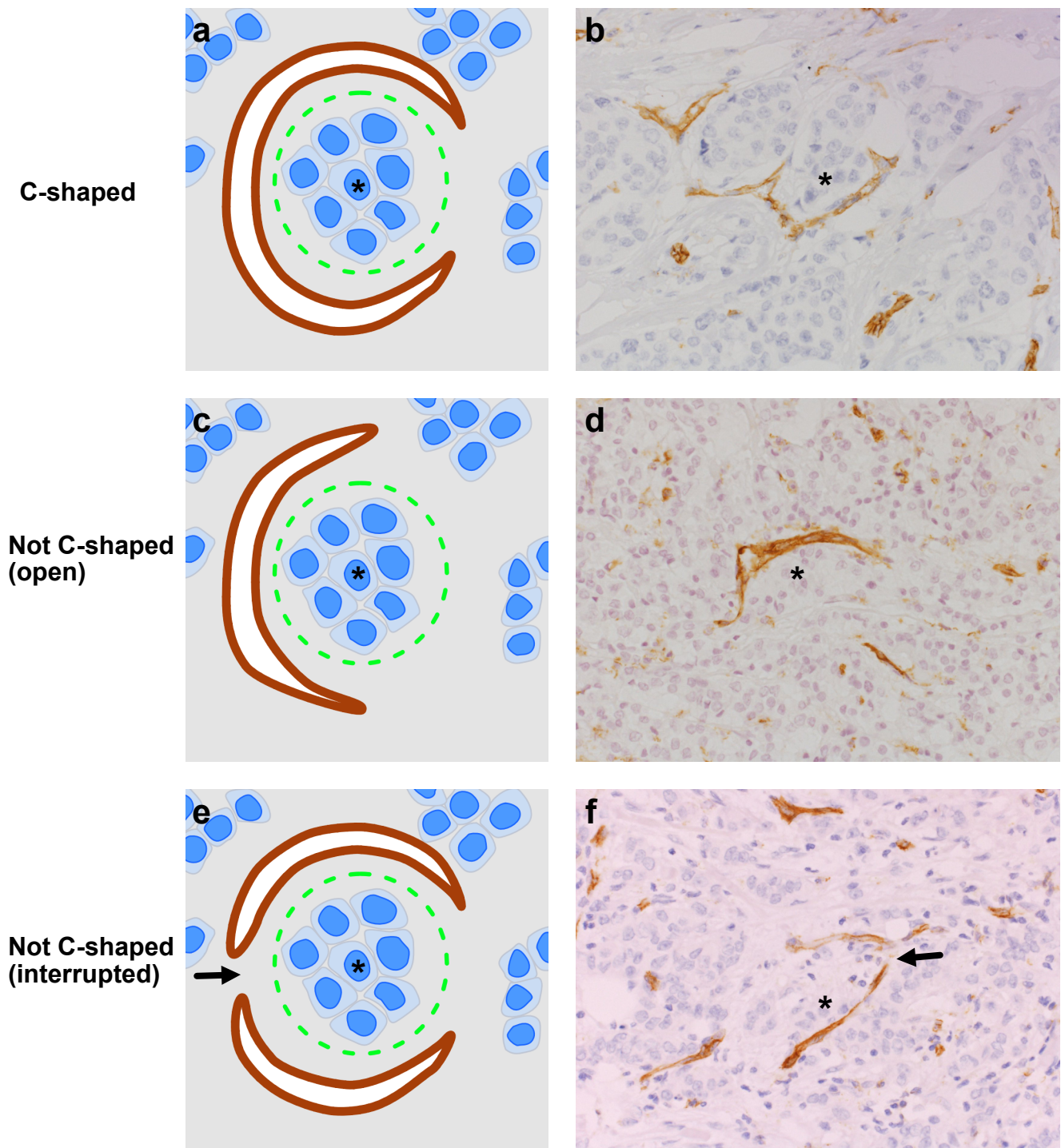
Supplementary Table S5. Correlation of glomeruloid microvascular proliferation and clinical parameters

Parameter	GMP (single staining)			GMP (double staining)		
	Absent n (%)	Present n (%)	P value	Absent n (%)	Present n (%)	P value
Total	366 (89.1)	45 (10.9)		320 (77.9)	91 (22.1)	
Tumor size			0.032*			0.002*
≤2cm	272 (74.3)	26 (57.8)		244 (76.2)	54 (59.3)	
>2cm	94 (25.7)	19 (42.2)		76 (23.8)	37 (40.7)	
Lymph node metastasis			0.861			0.595
Negative	248 (70.3)	32 (72.7)		221 (71.3)	59 (67.8)	
Positive	105 (29.7)	12 (27.3)		89 (28.7)	28 (32.2)	
Histological grade			< 0.001*			<0.001*
Grade 1-2	252 (68.9)	16 (35.6)		232 (72.5)	36 (39.6)	
Grade 3	114 (31.1)	29 (64.4)		88 (27.5)	55 (60.4)	
Estrogen receptor			< 0.001*			< 0.001*
Negative	70 (19.1)	23 (51.1)		50 (15.6)	43 (47.3)	
Positive	296 (80.9)	22 (48.9)		270 (84.4)	48 (52.7)	
Progesterone receptor			< 0.001*			< 0.001*
Negative	102 (27.9)	26 (57.8)		80 (25.0)	48 (52.7)	
Positive	264 (72.1)	19 (42.2)		240 (75.0)	43 (47.3)	
HER2 overexpression			1.000			0.245
Negative	311 (85.0)	39 (86.7)		276 (86.2)	74 (81.3)	
Positive	55 (15.0)	6 (13.3)		44 (13.8)	17 (18.7)	
Ki-67 index			< 0.001*			< 0.001*
≤20%	217 (59.3)	14 (31.1)		202 (63.1)	29 (31.9)	
>20%	149 (40.7)	31 (68.9)		118 (36.9)	62 (68.1)	
Subtype			< 0.001*			< 0.001*
ER+ and HER2-	269 (73.5)	22 (48.9)		246 (76.9)	45 (49.5)	
ER+ and HER2+	27 (7.4)	0 (0.0)		24 (7.5)	3 (3.3)	
ER- and HER2+	28 (7.7)	6 (13.3)		20 (6.2)	14 (15.4)	
ER- and HER2-	42 (11.5)	17 (37.8)		30 (9.4)	29 (31.9)	
Lymphatic vessel invasion						0.183
Negative	269 (73.5)	30 (66.7)	0.375	238 (74.4)	61 (67.0)	
Positive	97 (26.5)	15 (33.3)		82 (25.6)	30 (33.0)	
Blood vessel invasion			0.429			0.032
Negative	197 (53.8)	21 (46.7)		179 (55.9)	39 (42.9)	
Positive	169 (46.2)	24 (53.3)		141 (44.1)	52 (57.1)	

*P value < 0.05



Supplementary Fig S1 Kaplan Meier survival curves of patients with and without glomeruloid microvascular proliferation (GMP) for disease free survival
a:single staining, **b:**double staining



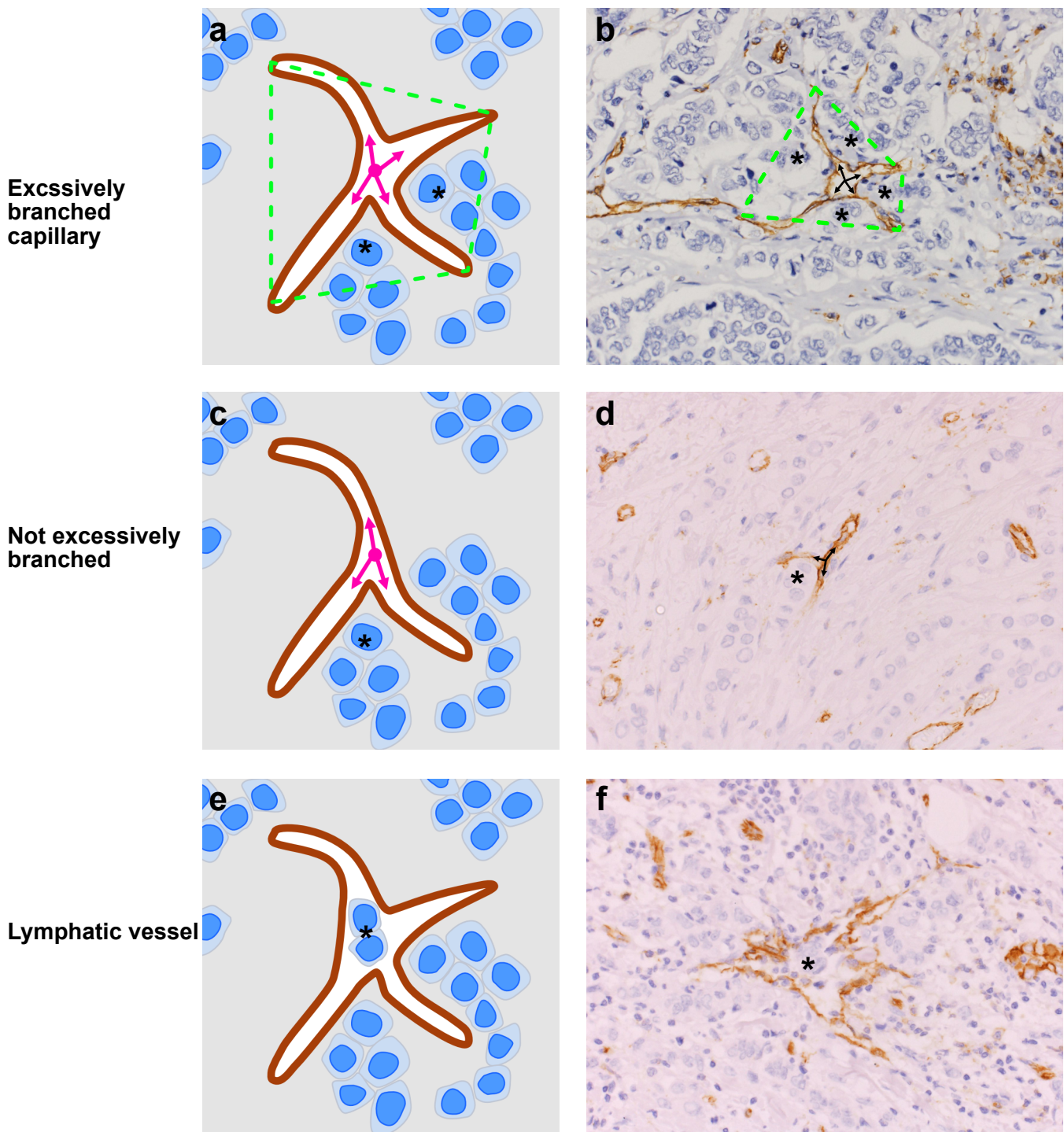
Supplementary Fig S2 Comparison of C-shaped and not C-shaped capillaries

a, b: C-shaped capillary encircles tumor cell nests (asterisk) more than 50%.

c, d: If the capillary's encirclement of tumor cell nests (asterisk) is less than 50%, the capillary is not regarded C-shaped.

e, f: If the capillary's encirclement of tumor cell nests (asterisk) is interrupted (arrow), the capillaries are not regarded C-shaped.

(**a, c, e:** Illustration. **b, d, f:** CD31 single staining)



Supplementary Fig S3 Comparison of excessively branched capillary and its mimics

a, b: Excesssively branched capillary has immediate four branches (arrows) and accompanies tumor cells (asterisks) nearby (within the green polygon).

c, d: If the capillary has only three branches (arrows), the capillary is not regarded excessively branched even if tumor cells (asterisk) are close to it.

e, f: Lymphatic vessel are excluded even if they have immediate four or more branches. Vessels which have tumor cells (asterisk) within the lumen are almost always lymphatic vessels (lymphatic vessel invasion of tumor cells).

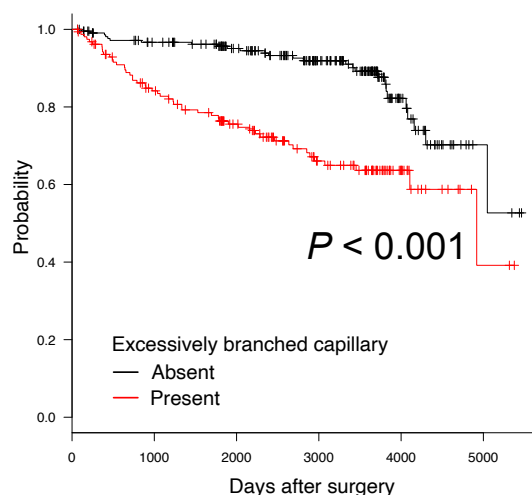
(**a, c, e:** Illustration. **b, d, f:** CD31 single staining)

Disease-free survival

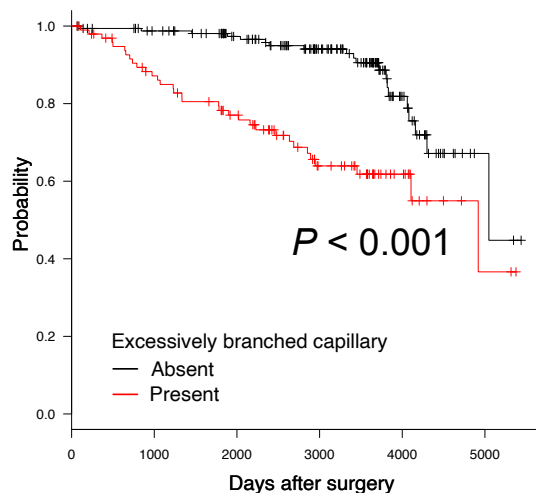
Excessively branched capillaries
(CD31 single staining)

— Absent
— Present

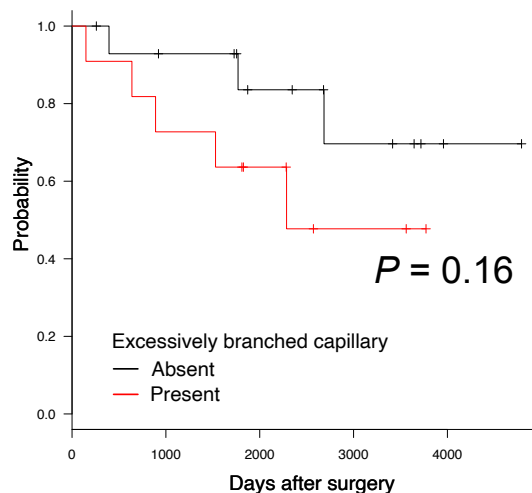
a All cases



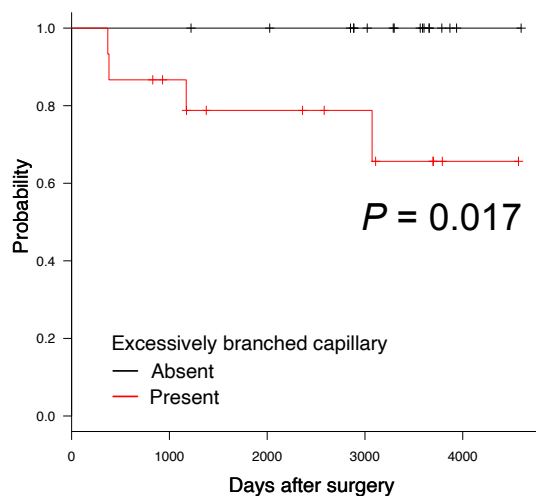
b ER+ and HER2-



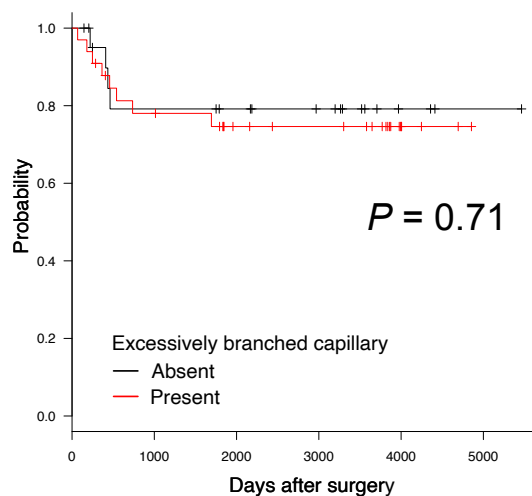
c ER+ and HER2+



d ER- and HER2+



e ER- and HER2-



Supplementary Fig S4 Kaplan Meier survival curves of patients with and without excessively branched capillaries for disease-free survival

a: All cases (n = 376)

b: ER-positive and HER2-negative group (n = 263)

c: ER-positive and HER2-positive group (n = 26)

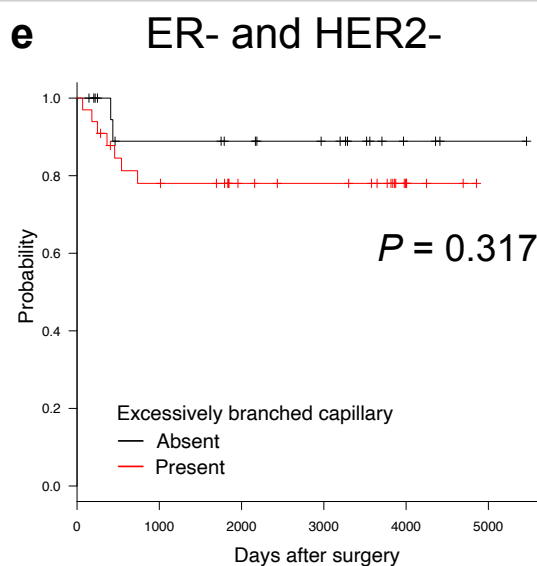
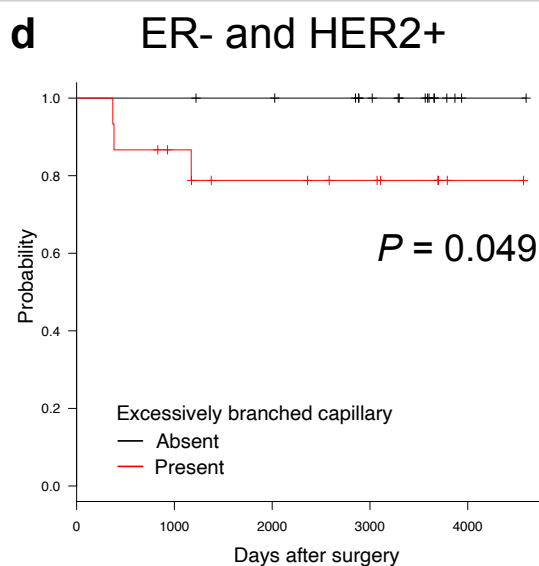
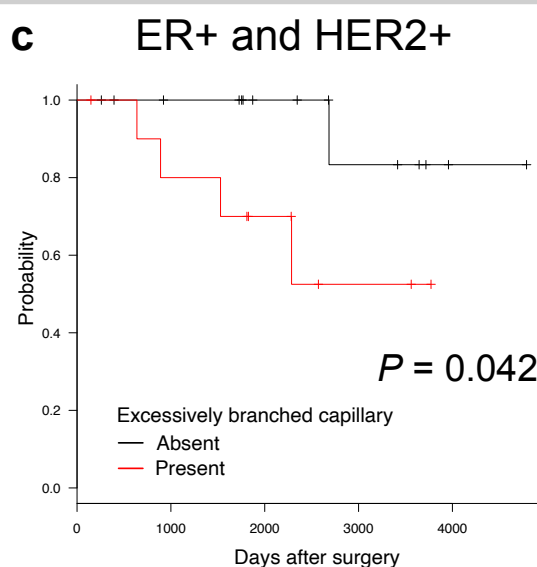
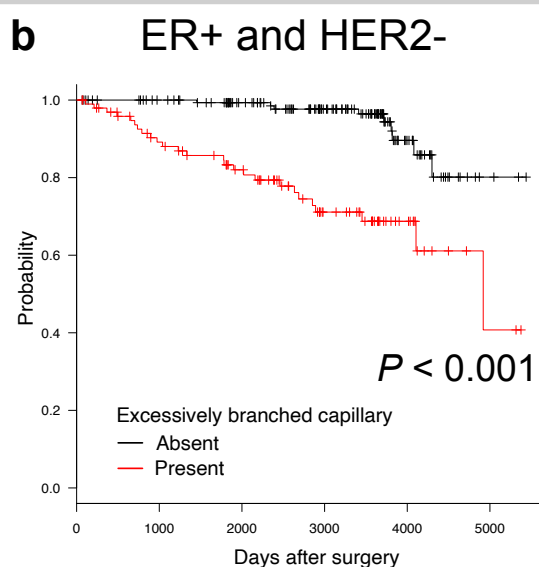
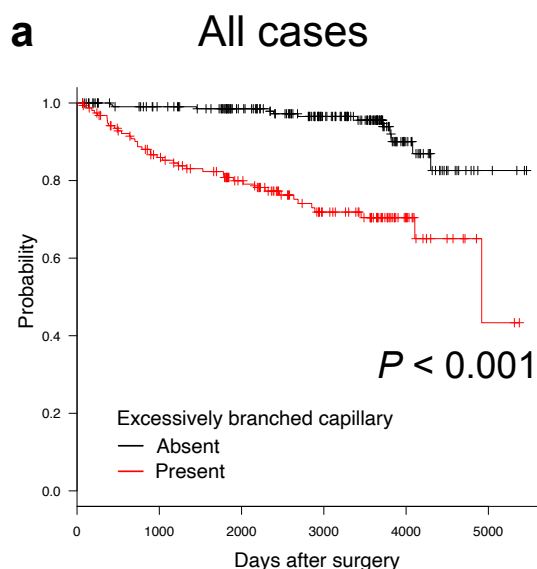
d: ER-negative and HER2-positive group (n = 32)

e: ER-negative and HER2-negative group (n = 55)

Cancer-specific survival

Excessively branched capillaries
(CD31 single staining)

— Absent
— Present



Supplementary Fig S5 Kaplan Meier survival curves of patients with and without excessively branched capillaries for cancer-specific survival

a: All cases (n = 376)

b: ER-positive and HER2-negative group (n = 263)

c: ER-positive and HER2-positive group (n = 26)

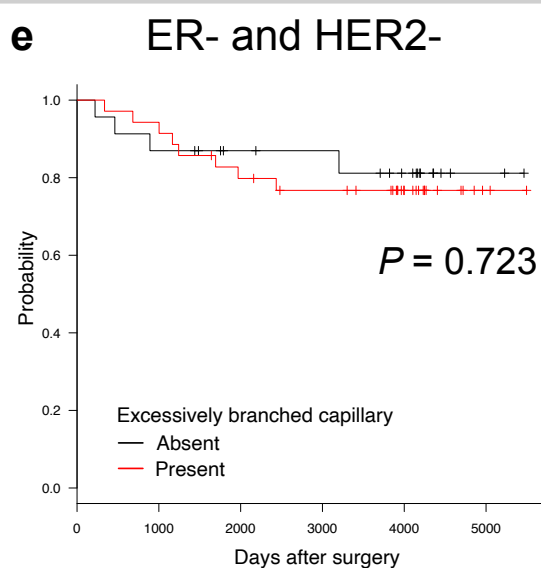
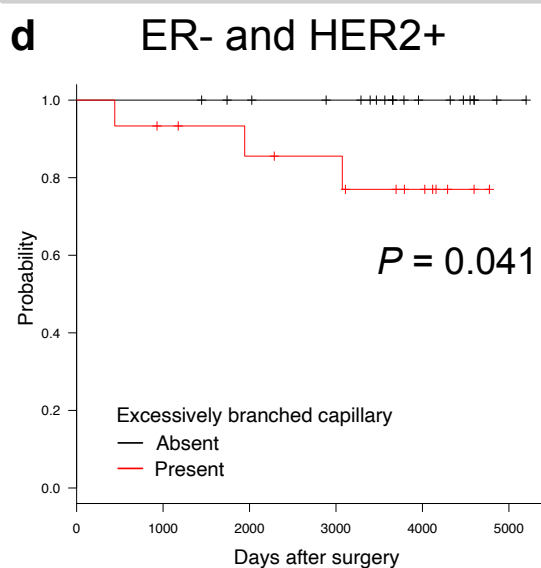
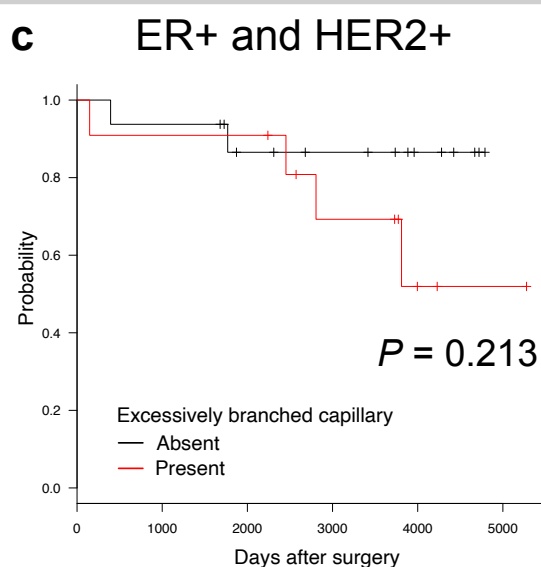
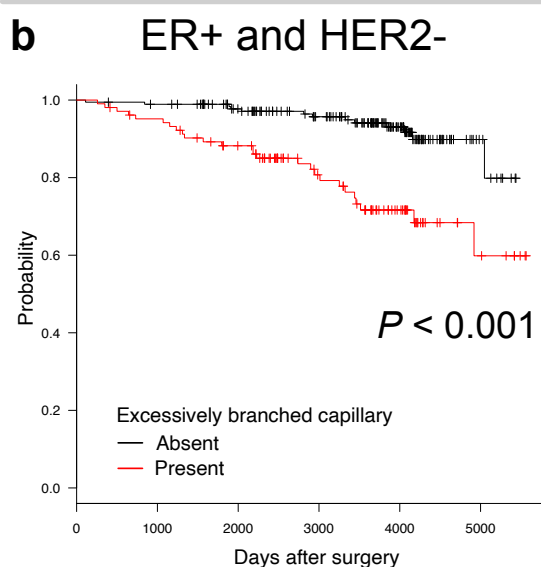
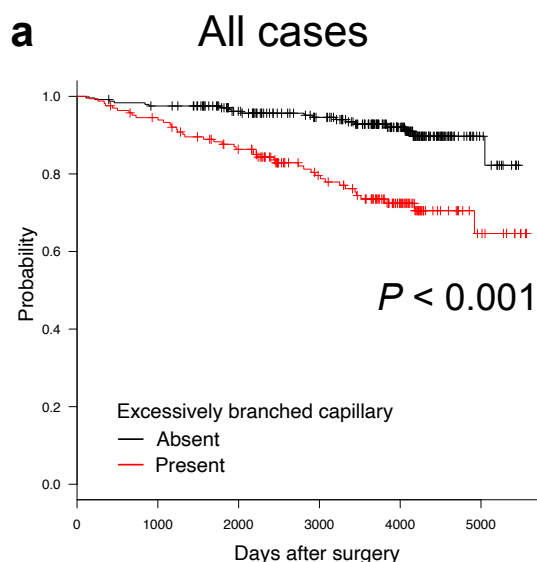
d: ER-negative and HER2-positive group (n = 32)

e: ER-negative and HER2-negative group (n = 55)

Overall survival

Excessively branched capillaries
(CD31 single staining)

— Absent
— Present



Supplementary Fig S6 Kaplan Meier survival curves of patients with and without excessively branched capillaries for overall survival

a: All cases (n = 407)

b: ER-positive and HER2-negative group (n = 288)

c: ER-positive and HER2-positive group (n = 27)

d: ER-negative and HER2-positive group (n = 34)

e: ER-negative and HER2-negative group (n = 58)