#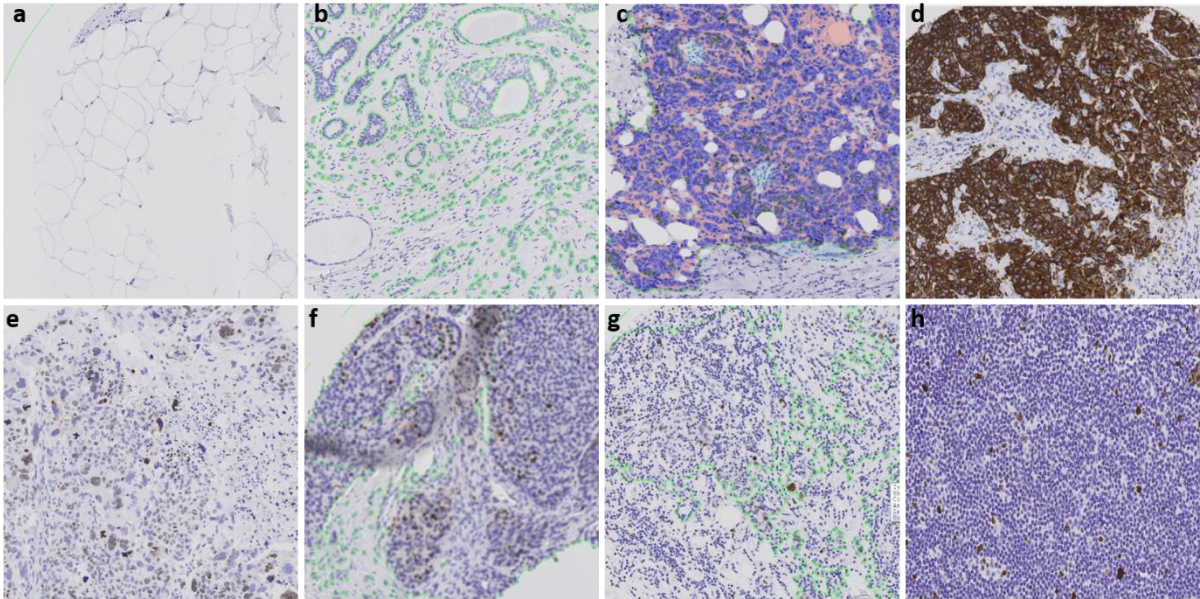 Supplemental Material to *Digital image analysis of Ki-67 stained tissue microarrays and recurrence in tamoxifen-treated breast cancer patients*

Immunohistochemistry for Ki-67

Histology sections of 4 μm were cut from each TMA block and mounted onto silanized slides (#S3003; Dako, Glostrup, Denmark) and dried overnight at 37°C followed by 1 hour at 60°C. Sections were deparaffinised in xylene and rehydrated in decreasing concentrations of alcohol. Antigen was retrieved with a highly stabilized retrieval system (ImmunoPrep, Instrumec, Oslo, Norway) using 10 mM TRIS/1 mM EDTA (pH 9.0) as the retrieval buffer. Sections were heated for 3 min at 110°C followed by 10 min at 95°C, then cooled to 20°C. Ki-67 was detected using clone MIB-1 (DAKO, Glostrup, Denmark) at 1:100 dilution (Dako antibody diluent S0809), before the sections were incubated for 30 min at 22°C. For visualization, the EnVisionTM Flex detection system (Dako, K8000) was used. Sections were incubated for 5 min with peroxidase-blocking reagent (SM801), 20 min with the EnVisionTM FLEX/HRPDetection Reagent (SM802), 10 min with EnVisionTM FLEX DAB+ Chromogen (DM827)/EnVisionTM FLEX Substrate Buffer (SM803) mix and 5 min with EnVisionTM FLEX Hematoxylin (K8008). The slides were then dehydrated and mounted. All immunohistochemical stainings were performed using a Dako Autostainer Link 48 instrument and EnVisionTM FLEX Wash Buffer (DM831) and were controlled in the NordiQC external quality programme.

Supplemental Figures

S1 Figure. Examples of cores that were excluded due to the following reasons: a) stroma/empty core, b) too few tumor cells present, c) excessive blue HE-staining, d) excessive membranous/cytoplasmic brown DAB-staining , e) poor morphology/too weak staining f) poor imaging/artefacts, g) and h) tumor-infiltrating lymphocytes.

Supplemental Tables

S1 Table. Labelling of image pixels and subsequent classification of cells in Visiopharm®.

Assigned label	Color	Detection of pixels	Classification
Label 001	Green	Brown/DAB	Ki-67 positive
Label 002	Blue	Cells/HE	Ki-67 negative
Label 003	Red	Stroma	Background

DAB: 3,3'diaminobenzidine. HE: hematoxylin- and eosin.

S2 Table. Summary of the TMA-DIA set-up.

Patient IDs on the 35 TMAs	Excluded (%)	Ki-67 hotspot Range (%)	Patients with Ki-67 score ≥15%	Patients with Ki-67 score ≥30%	Cores remaining after DIA		
					3	2	1
1456	149 (10)	0 - 92	451	196	830 (57%)	302 (22%)	174 (12%)

S3 Table. Patient characteristics with Ki-67 median=low.

Patient characteristics Ki-67 median=low	ER/TAM type							
	ER+/TAM+				ER-/TAM-			
	Case status				Case status			
	Cases		Controls		Cases		Controls	
	N	%	N	%	N	%	N	%
Total	245	100.0	276	100.0	76	100.0	79	100.0
Year of diagnosis								
85-93	100	40.8	106	38.4	19	25.0	20	25.3
94-96	55	22.4	62	22.5	25	32.9	21	26.6
97-01	90	36.7	108	39.1	32	42.1	38	48.1
Age at diagnosis								
35-44	10	4.1	<= 5		12	15.8	12	15.2
45-54	50	20.4	62	22.5	32	42.1	28	35.4
55-64	134	54.7	<= 142		21	27.6	26	32.9
65-70	51	20.8	71	25.7	11	14.5	13	16.5
Menopausal status								
Premenopausal	17	6.9	15	5.4	29	38.2	34	43.0
Postmenopausal	228	93.1	261	94.6	47	61.8	45	57.0

Patient characteristics Ki-67 median=low	ER/TAM type							
	ER+/TAM+				ER-/TAM-			
	Case status				Case status			
	Cases		Controls		Cases		Controls	
	N	%	N	%	N	%	N	%
UICC tumor stage								
I	<=5		<= 5		7	9.2	<= 5	
II	109	44.5	117	42.4	38	50.0	36	45.6
III	<= 135		<= 158		31	40.8	<= 42	
Histological grade								
I	61	24.9	95	34.4	11	14.5	13	16.5
II	95	38.8	101	36.6	36	47.4	28	35.4
III	33	13.5	17	6.2	22	28.9	19	24.1
Missing	56	22.9	63	22.8	7	9.2	19	24.1
Surgery type								
Breast conserving	20	8.2	42	15.2	11	14.5	12	15.2
Mastectomy	225	91.8	234	84.8	65	85.5	67	84.8
Systemic adjuvant chemotherapy					<= 5			
No	211	86.1	251	90.9			26	32.9
Yes	34	13.9	25	9.1	<= 75		53	67.1
Radiation therapy								
No	171	69.8	179	64.9	43	56.6	36	45.6
Yes	74	30.2	97	35.1	32	42.1	34	43.0
Missing	0	0	0	0	1	1.3	9	11.4
Tamoxifen protocol, years								
N/A	0	0	0	0	76	100.0	79	100.0
1	116	47.3	126	45.7	0	0	0	0
2	40	16.3	46	16.7	0	0	0	0
5	89	36.3	104	37.7	0	0	0	0

S4 Table. Patient characteristics with Ki-67 median=high.

Patient characteristics Ki-67 median=high	ER/TAM type							
	ER+/TAM+				ER-/TAM-			
	Case status				Case status			
	Cases		Controls		Cases		Controls	
	N	%	N	%	N	%	N	%
Total	188	100.0	160	100.0	170	100.0	157	100.0
Year of diagnosis								
85-93	78	41.5	72	45.0	64	37.6	55	35.0
94-96	33	17.6	29	18.1	43	25.3	46	29.3
97-01	77	41.0	59	36.9	63	37.1	56	35.7

Patient characteristics Ki-67 median=high	ER/TAM type							
	ER+/TAM+				ER-/TAM-			
	Case status				Case status			
	Cases		Controls		Cases		Controls	
	N	%	N	%	N	%	N	%
Age at diagnosis								
35-44	<= 5		9	5.6	38	22.4	32	20.4
45-54	44	23.4	30	18.8	66	38.8	59	37.6
55-64	<= 97		83	51.9	50	29.4	46	29.3
65-70	46	24.5	38	23.8	16	9.4	20	12.7
Menopausal status								
Premenopausal	13	6.9	15	9.4	65	38.2	59	37.6
Postmenopausal	175	93.1	145	90.6	105	61.8	98	62.4
UICC tumor stage								
I	<= 5		<= 5		8	4.7	13	8.3
II	85	45.2	<= 84		95	55.9	85	54.1
III	<= 102		75	46.9	67	39.4	59	37.6
Histological grade								
I	23	12.2	23	14.4	7	4.1	<= 5	
II	93	49.5	73	45.6	72	42.4	50	31.8
III	44	23.4	32	20.0	66	38.8	<= 70	
Missing	28	14.9	32	20.0	25	14.7	36	22.9
Surgery type								
Breast conserving	23	12.2	14	8.8	24	14.1	35	22.3
Mastectomy	165	87.8	146	91.3	145	85.3	122	77.7
Missing	0	0	0	0	1	0.6	0	0
Systemic adjuvant chemotherapy								
No	166	88.3	138	86.3	33	19.4	68	43.3
Yes	22	11.7	22	13.8	137	80.6	89	56.7
Radiation therapy								
No	112	59.6	102	63.8	96	56.5	70	44.6
Yes	76	40.4	58	36.3	70	41.2	62	39.5
Missing	0	0	0	0	4	2.4	25	15.9
Tamoxifen protocol, years								
N/A	0	0	0	0	170	100.0	157	100.0
1	86	45.7	75	46.9	0	0	0	0
2	29	15.4	27	16.9	0	0	0	0
5	73	38.8	58	36.3	0	0	0	0

S5 Table. Distributions of the mean and hotspot Ki-67 score across ER/TAM-strata.

	ER+/TAM+		ER−/TAM−	
	No. (%)		No. (%)	
	Cases	Controls	Cases	Controls
Mean Ki-67 (3 cores)				
<30%	420	424	198	188
30% or above	13	13	45	47
Mean Ki-67 (3 cores)				
< median*	245	277	74	79
Median or above	188	160	169	156
Hotspot				
<30%	408	412	181	172
30% or above	25	25	62	63
Hotspot				
<median*	242	273	79	80
Median or above	191	164	164	155

*medians are full sample medians

S6 Table. Descriptives; age at surgery, Ki-67-mean and hotspot by ER/TAM group.

	ER⁺/TAM⁺				ER⁻/TAM⁻				OVERALL MEAN
	N	Mean (Std Deviation)	Median	Range	N	Mean (Std Deviation)	Median	Range	
AGE AT SURGERY	962	59.1 (6.7)		34 (35-69)	539	52.7 (8.9)		34 (35-69)	56.8
KI-67 MEAN	870	7.5 (9.1)		79 (0-79)	478	17.2 (17.3)		90 (0.01-90	10.9
HOTSPOT	870	9.7 (10.8)	6.7	79 (0-79)	478	21.4 (20.1)	15.2	92 (0.03-92)	13.8

S7 Table. Adjusted ORs by ER-specific medians.

ER⁺/Tam⁺	ER⁻/Tam⁻
Adjusted OR (95%)	Adjusted OR (95%)
0.79 (0.60,1.06)	1.18 (0.78,1.80)

S8 Table. Associations between Ki-67-expression score and breast cancer recurrence within strata, adjusted also for grade.

DIA Ki-67 expression	ER⁺/Tam⁺				ER⁻/Tam⁻			
	Recurrent cases/controls (n)	Matched OR (95% CI)	Adjusted OR (95%)ᵃ	Adjusted OR2 (95%)ᵇ	Recurrent cases/controls or means	Matched OR (95% CI)	Adjusted OR (95%)ᵃ	Adjusted OR2 (95%)ᵇ
All cores								
<median	245/276	1	1	1	76/79	1	1	1
≥median	188/160	0.75 (0.55,1.03)	0.72 (0.54,0.96)	**0.74 (0.52,1.04)**	170/157	1.00 (0.66,1.53)	0.85 (0.54,1.34)	**0.89 (0.53,1.50)**
Hotspot core								
<median	243/272	1	1	1	80/80	1	1	1
≥median	190/164	0.78 (0.57,1.07)	0.73 (0.55,0.98)	**0.80 (0.57,1.13)**	166/156	1.05 (0.69,1.59)	0.86 (0.55,1.35)	**0.87 (0.52,1.49)**

ᵃ Adjusted for year of diagnosis, menopausal status, county of residence, UICC stage, receipt of chemotherapy, type of surgery, age category, receipt of radiotherapy and comorbidity. Line number two includes grade (high number of missing reduced sample size).
ᵇ Adjusted also for grade.

S9 Table. Association between DIA-Ki-67 score and breast cancer recurrence, by median time to recurrence.

	Time to recurrence (yrs post diagnosis)	Median time to recurrence (yrs)ᶻ	Cases/ controls	Matched OR (95% CI)ᵃ	Adjusted OR (95% CI)ᵇ
ER+/Tam+					
	1-5	2.8	329/125	0.75 (0.48,1.17)	0.74 (0.47,1.18)
	6-10	6.7	104/311	0.86 (0.53,1.40)	0.83 (0.50,1.37)
ER-/Tam-					
	1-5	2.1	223/70	0.69 (0.37,1.27)	0.68 (0.34,1.39)
	6-10	6.9	23/166	1.85 (0.68,5.04)	1.40 (0.40,4.90)

ᶻ Median time to recurrence based on recurrent cases.
ᵃ Estimated using conditional logistic regression with conditioning on the matched factors (diagnosis year, county of residence, menopausal status and UICC stage).
ᵇ Estimated using logistic regression with additional adjustment for chemotherapy, receipt of radiotherapy, type of surgery, age category at diagnosis and comorbidity. Due to missing values, adjusted analyses were calculated in lower number of cases/controls than the matched analysis (For ER+/Tam+: 2.8 yrs=320/121, 6.6 yrs=100/304, and for ER-/Tam-: 2.1 yrs=210/60, 6.9 yrs=22/136).

S10 Table. Adjusted ORs by chemo - overall median.

	ER$^+$/Tam$^+$	ER$^-$/Tam$^-$
	Adjusted OR (95%)	Adjusted OR (95%)
No Chemotherapy	0.64 (0.47,0.88)	0.19 (0.04,0.94)
Chemotherapy	1.38 (0.55,3.47)	1.02 (0.62,1.69)

S11 Table. Adjusted ORs by chemo and ER-specific medians.

	ER$^+$/Tam$^+$	ER$^-$/Tam$^-$
	Adjusted OR (95%)	Adjusted OR (95%)
No Chemotherapy	0.73 (0.54,0.99)	0.75 (0.24,2.31)
Chemotherapy	1.43 (0.52,3.92)	1.40 (0.87,2.26)