ELECTRONIC SUPPLEMENRARY MATERIAL

To

Prognostic potential of automated Ki67 evaluation in breast cancer: different hot spot definitions versus true global score

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Table S1 List of features used in the random trees' classifier in QuPath

Nucleus: Area
Nucleus: Perimeter
Nucleus: Circularity
Nucleus: Max caliper
Nucleus: Min caliper
Nucleus: Eccentricity
Nucleus: Hematoxylin OD mean
Nucleus: Hematoxylin OD sum
Nucleus: Hematoxylin OD std dev
Nucleus: Hematoxylin OD max
Nucleus: Hematoxylin OD min
Nucleus: Hematoxylin OD range
Nucleus: Eosin OD mean
Nucleus: Eosin OD sum
Nucleus: Eosin OD std dev
Nucleus: Eosin OD max
Nucleus: Eosin OD min
Nucleus: Eosin OD range
Cell: Area
Cell: Perimeter
Cell: Circularity
Cell: Max caliper
Cell: Min caliper
Cell: Eccentricity
Cell: Eosin OD mean
Cell: Eosin OD std dev
Cell: Eosin OD max
Cell: Eosin OD min
Cytoplasm: Eosin OD mean
Cytoplasm: Eosin OD std dev
Cytoplasm: Eosin OD max
Cytoplasm: Eosin OD min
Nucleus/Cell area ratio

Smoothed: 25 µm: Nucleus: Area
Smoothed: 25 µm: Nucleus: Perimeter
Smoothed: 25 µm: Nucleus: Circularity
Smoothed: 25 µm: Nucleus: Max caliper
Smoothed: 25 µm: Nucleus: Min caliper
Smoothed: 25 µm: Nucleus: Eccentricity
Smoothed: 25 µm: Nucleus: Hematoxylin OD mean
Smoothed: 25 µm: Nucleus: Hematoxylin OD sum
Smoothed: 25 µm: Nucleus: Hematoxylin OD std dev
Smoothed: 25 µm: Nucleus: Hematoxylin OD max
Smoothed: 25 µm: Nucleus: Hematoxylin OD min
Smoothed: 25 µm: Nucleus: Hematoxylin OD range
Smoothed: 25 µm: Nucleus: Eosin OD mean
Smoothed: 25 µm: Nucleus: Eosin OD sum
Smoothed: 25 µm: Nucleus: Eosin OD std dev
Smoothed: 25 µm: Nucleus: Eosin OD max
Smoothed: 25 µm: Nucleus: Eosin OD min
Smoothed: 25 µm: Nucleus: Eosin OD range
Smoothed: 25 µm: Cell: Area
Smoothed: 25 µm: Cell: Perimeter
Smoothed: 25 µm: Cell: Circularity
Smoothed: 25 µm: Cell: Max caliper
Smoothed: 25 µm: Cell: Min caliper
Smoothed: 25 µm: Cell: Eccentricity
Smoothed: 25 µm: Cell: Eosin OD mean
Smoothed: 25 µm: Cell: Eosin OD std dev
Smoothed: 25 µm: Cell: Eosin OD max
Smoothed: 25 µm: Cell: Eosin OD min
Smoothed: 25 µm: Cytoplasm: Eosin OD mean
Smoothed: 25 µm: Cytoplasm: Eosin OD std dev
Smoothed: 25 µm: Cytoplasm: Eosin OD max
Smoothed: 25 µm: Cytoplasm: Eosin OD min
Smoothed: 25 µm: Nucleus/Cell area ratio
Smoothed: 25 µm: Nearby detection counts

Smoothed: 50 µm: Nucleus: Area
Smoothed: 50 µm: Nucleus: Perimeter
Smoothed: 50 µm: Nucleus: Circularity
Smoothed: 50 µm: Nucleus: Max caliper
Smoothed: 50 µm: Nucleus: Min caliper
Smoothed: 50 µm: Nucleus: Eccentricity
Smoothed: 50 µm: Nucleus: Hematoxylin OD mean
Smoothed: 50 µm: Nucleus: Hematoxylin OD sum
Smoothed: 50 µm: Nucleus: Hematoxylin OD std dev
Smoothed: 50 µm: Nucleus: Hematoxylin OD max
Smoothed: 50 µm: Nucleus: Hematoxylin OD min
Smoothed: 50 µm: Nucleus: Hematoxylin OD range
Smoothed: 50 µm: Nucleus: Eosin OD mean
Smoothed: 50 µm: Nucleus: Eosin OD sum
Smoothed: 50 µm: Nucleus: Eosin OD std dev
Smoothed: 50 µm: Nucleus: Eosin OD max
Smoothed: 50 µm: Nucleus: Eosin OD min
Smoothed: 50 µm: Nucleus: Eosin OD range
Smoothed: 50 µm: Cell: Area
Smoothed: 50 µm: Cell: Perimeter
Smoothed: 50 µm: Cell: Circularity
Smoothed: 50 µm: Cell: Max caliper
Smoothed: 50 µm: Cell: Min caliper
Smoothed: 50 µm: Cell: Eccentricity
Smoothed: 50 µm: Cell: Eosin OD mean
Smoothed: 50 µm: Cell: Eosin OD std dev
Smoothed: 50 µm: Cell: Eosin OD max
Smoothed: 50 µm: Cell: Eosin OD min
Smoothed: 50 µm: Cytoplasm: Eosin OD mean
Smoothed: 50 µm: Cytoplasm: Eosin OD std dev
Smoothed: 50 µm: Cytoplasm: Eosin OD max
Smoothed: 50 µm: Cytoplasm: Eosin OD min
Smoothed: 50 µm: Nucleus/Cell area ratio
Smoothed: 50 µm: Nearby detection counts

Table S2 Cox regression hazard ratios for recurrence-free survival or overall survival and low versus high Ki67 sugroups using DIA hot spot apps, DIA global scoring and manual hot spot scoring

	Recurrence-free survival				**Overall survival**			
Scoring method	**Hazard ratio**	**95% confidence interval**		***p***	**Hazard ratio**	**95% confidence interval**		***p***
		lower	**upper**			**lower**	**upper**	
APP05	3.66	1.39	9.66	0.009*	6.75	1.56	29.12	0.010*
APP06	11.06	1.50	81.50	0.018*	7.57	1.01	56.64	0.049*
APP08	9.26	1.26	68.16	0.029*	6.31	0.84	47.19	0.073
APP09	5.13	1.94	13.53	0.001*	5.97	1.75	20.41	0.004*
APP10	6.83	2.36	19.74	<0.001*	6.07	1.78	20.74	0.004*
APP11	5.79	2.00	16.74	0.001*	5.07	1.48	17.33	0.010*
APP12	5.25	1.58	17.41	0.007*	3.39	0.99	11.57	0.052
APP13	4.15	1.44	12.00	0.008*	2.66	0.89	7.96	0.081
APP14	4.54	1.37	15.08	0.014*	4.67	1.08	20.15	0.039*
APP15	5.80	1.74	19.25	0.004*	5.93	1.37	25.60	0.017*
APP16	7.68	1.04	56.57	0.045*	5.24	0.70	39.19	0.107
APP20	3.71	1.12	12.34	0.032*	8.10	1.08	60.59	0.042*
APP21	4.56	1.37	15.12	0.013*	9.92	1.33	74.16	0.025*
APP22	5.19	1.56	17.22	0.007*	5.31	1.23	22.90	0.025*
APP23	6.06	1.83	20.14	0.003*	6.17	1.43	26.66	0.015*
APP24	**6.88**	**2.07**	**22.87**	**0.002***	**6.93**	**1.61**	**29.91**	**0.009***
APP25	5.19	1.80	15.02	0.002*	4.53	1.33	15.48	0.016*
APP26	5.79	2.00	16.74	0.001*	5.07	1.48	17.33	0.010*
APP27	4.51	1.71	11.91	0.002*	8.42	1.95	36.35	0.004*
DIA global	**3.13**	**1.41**	**6.95**	**0.005***	**7.46**	**2.46**	**22.58**	**<0.001***
Manual hot spot	2.76	1.16	6.53	0.021*	2.27	0.87	5.92	0.093

**P* significant at a <0.05 level. DIA, digital image analysis.

Table S3 Cox regression hazard ratios for recurrence-free survival or overall survival and Ki67 low versus high, luminal A versus B-like surrogate subtypes and PAM50 luminal A versus B subtypes, among pN0 and pN1 cases

Scoring method	Recurrence-free survival			Overall survival		
	Hazard ratio	**95% confidence interval**	***p***	**Hazard ratio**	**95% confidence interval**	***p***
pN0						
Ki67 DIA hot spot APP24 scoring (<20% vs ≥20%)	48.80	0.20 - 12119.6	0.167	3.30	0.39 - 28.28	0.276
Ki67 DIA global scoring (<20% vs >20%)	**4.12**	1.01 - 16.74	**0.048***	3.57	0.42 - 30.62	0.245
Surrogate subtype DIA hot spot (lum A vs lum B-like)	31.79	0.05 - 18973.5	0.289	31.96	0.01 - 78122.8	0.384
Surrogate subtype DIA global (lum A vs lum B-like)	1.96	0.49 - 7.87	0.342	70.60	0.10 - 50983.9	0.205
PAM50 subtype (lum A vs lum B)	3.34	0.83 - 13.37	0.089	4.92	0.82 - 29.46	0.081
pN1						
Ki67 DIA hot spot APP24 scoring (<20% vs ≥20%)	6.90	0.86 - 55.30	0.069	6.23	0.76 - 50.98	0.088
Ki67 DIA global scoring (<20% vs >20%)	2.74	0.68 - 11.06	0.158	2.54	0.51 - 12.62	0.254
Surrogate subtype DIA hot spot (lum A vs lum B-like)	4.97	0.62 - 39.82	0.131	4.31	0.53 - 35.05	0.172
Surrogate subtype DIA global (lum A vs lum B-like)	1.81	0.45 - 7.23	0.402	2.81	0.57 - 13.94	0.207
PAM50 subtype (lum A vs lum B)	**6.57**	1.24 - 34.87	**0.027***	3.83	0.70 - 20.93	0.121

**P* significant at a <0.05 level. DIA, digital image analysis; RFS, recurrence-free survival; OS, overall survival.

Table S4 Univariate Cox proportional hazard models for recurrence-free survival

Variable	Hazard ratio	95% confidence interval		*p*
		lower	upper	
pT stage^				0.161
pT1 (ref)	-	-	-	0.165
pT2	1.91	0.80	4.57	0.147
pT3	3.37	0.87	13.05	0.079
Grade				0.003*
Grade (1 ref)	-	-	-	0.727
Grade 2	27272.91	<0.001	2.43E+65	0.887
Grade 3	36822.46	<0.001	3.28E+65	0.883
Mitotic score				0.214
Mitotic score (1 ref)	-	-	-	0.229
Mitotic score 2	1.86	0.77	4.50	0.169
Mitotic score 3	2.21	0.82	5.95	0.116
pN stage^				0.208
pN (pN0 ref)	-	-	-	-
pN1	1.82	0.72	4.58	0.205
pN stage^				0.005*
pN (pN0 ref)	-	-	-	0.002*
pN1	1.84	0.73	4.63	0.198
pN2	4.90	1.82	13.16	0.002*
pN3	7.05	1.91	26.10	0.003*

**P* significant at a <0.05 level. ^Pathologic T stage (pT) for invasive tumor and pathologic N stage (pN) for regional lymph nodes according to AJCC Breast Cancer Staging 7th Edition (TNM 7).

Table S5 Multivariate Cox proportional hazard models for recurrence-free survival

Variables in model	Hazard ratio	95% confidence interval	*p*
pN stage^ (pN0 ref)	-	-	-
pN1	1.98	0.79 - 4.99	0.148
pN2	5.22	1.94 - 14.06	0.001*
pN3	12.39	3.27 - 46.92	<0.001*
Ki67 DIA hot spot APP24 scoring (≥20% vs <20%)	8.42	2.49 - 28.45	0.001*
pN stage (pN0 ref)	-	-	-
pN1	1.54	0.61 - 3.89	0.365
pN2	4.27	1.52 - 12.02	0.006*
pN3	5.02	1.08 - 23.33	0.040*
Ki67 DIA global scoring (≥20% vs <20%)	3.23	1.45 - 7.20	0.004*
pN stage (pN0 ref)	-	-	-
pN1	1.44	0.57 - 3.65	0.436
pN2	4.15	1.47 - 11.73	0.007*
pN3	6.95	1.87 - 25.87	0.004*
Ki67 manual hot spot scoring (≥20% vs <20%)	2.78	1.16 - 6.65	0.022*

**P* significant at a <0.05 level. ^Pathologic N stage for regional lymph nodes according to AJCC Breast Cancer Staging 7th Edition (TNM 7). DIA, digital image analysis.

Table S6 Univariate Cox proportional hazard models for overall survival

Variable	**Hazard ratio**	**95% confidence interval**		***p***
		lower	**upper**	
pT stage^				0.161
pT1 (ref)	-	-	-	0.199
pT2	2.45	0.81	7.43	0.115
pT3	3.81	0.70	20.79	0.123
Grade				<0.001*
Grade (1 ref)	-	-	-	0.001
Grade 2	1.59	0.19	13.62	0.672
Grade 3	8.98	1.18	68.45	0.034
Mitotic score				0.009*
Mitotic score (1 ref)	-	-	-	0.011
Mitotic score 2	2.56	0.75	8.75	0.134
Mitotic score 3	6.11	1.83	20.38	0.003
pN stage^				0.112
pN (pN0 ref)	-	-	-	-
pN1	2.35	0.81	6.77	0.114
pN stage^				0.066
pN (pN0 ref)	-	-	-	0.061
pN1	2.37	0.82	6.82	0.111
pN2	4.04	1.14	14.33	0.030
pN3	6.19	1.25	30.76	0.026

**P* significant at a <0.05 level. ^Pathologic T stage (pT) for invasive tumor and pathologic N stage (pN) for regional lymph nodes according to AJCC Breast Cancer Staging 7th Edition (TNM 7).

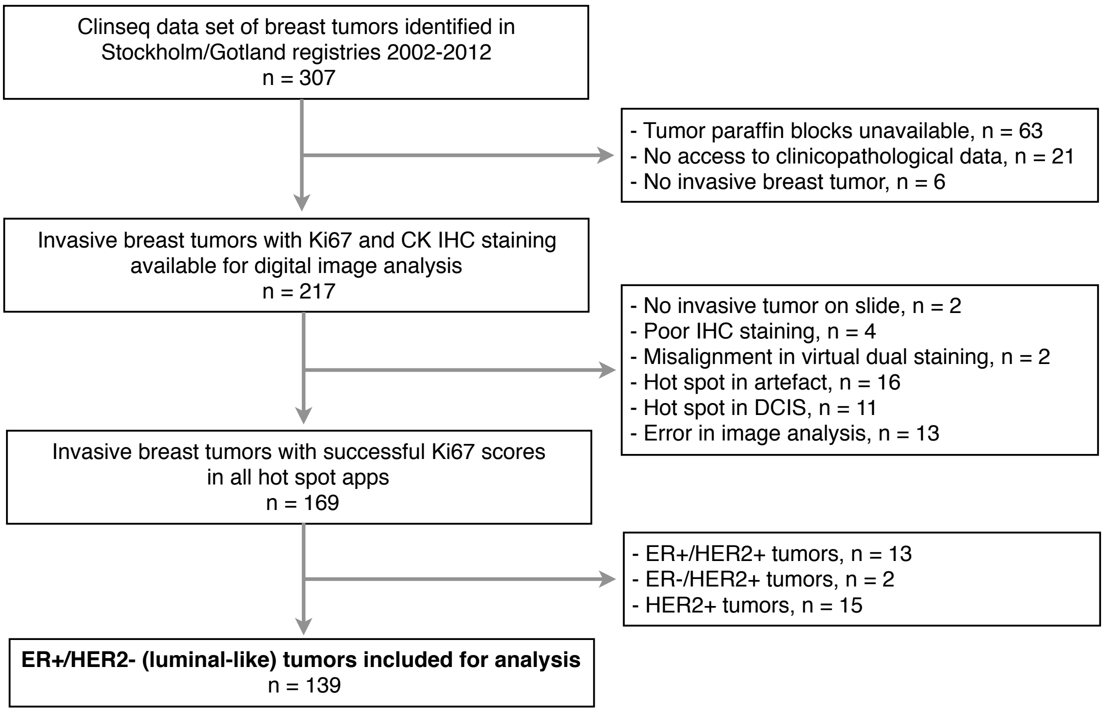

Fig. S1 CONSORT diagram of the study cohort. CK, cytokeratin; IHC, immunohistochemistry; DCIS, ductal carcinoma *in situ*; ER, estrogen receptor; HER2, human epidermal growth factor receptor 2.

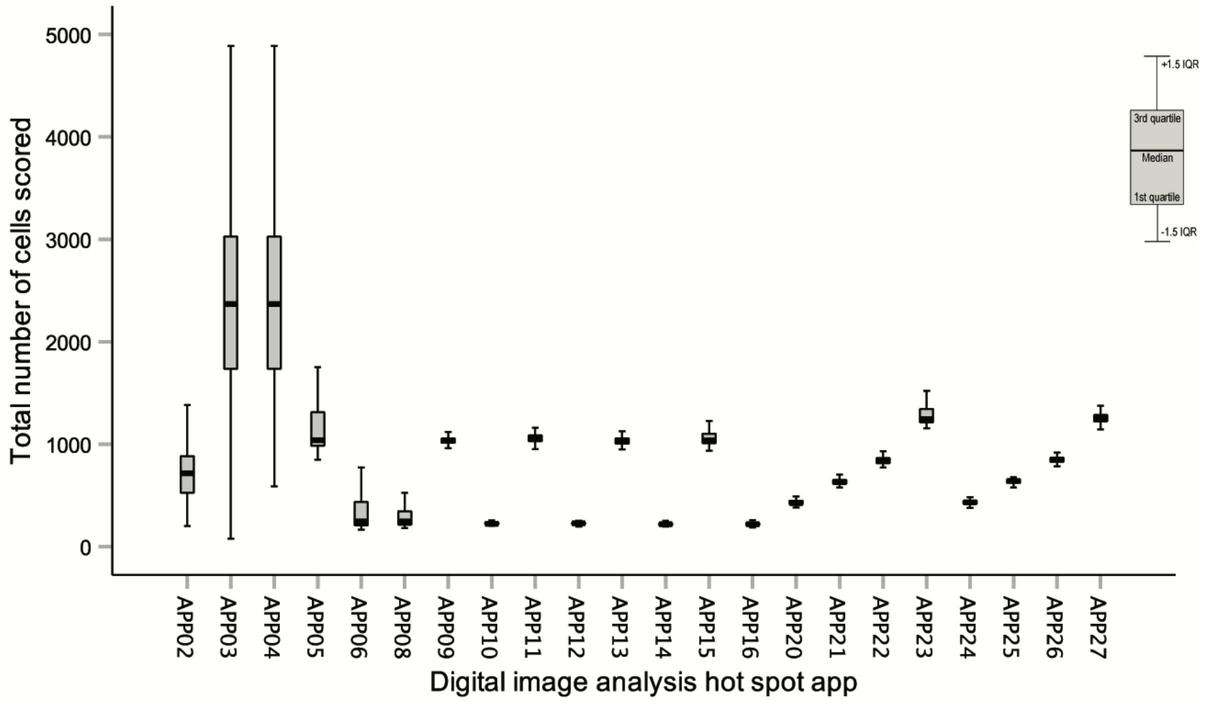

Fig. S2 Distribution of the total number of cells scored for each automated hot spot app. IQR, interquartile range.

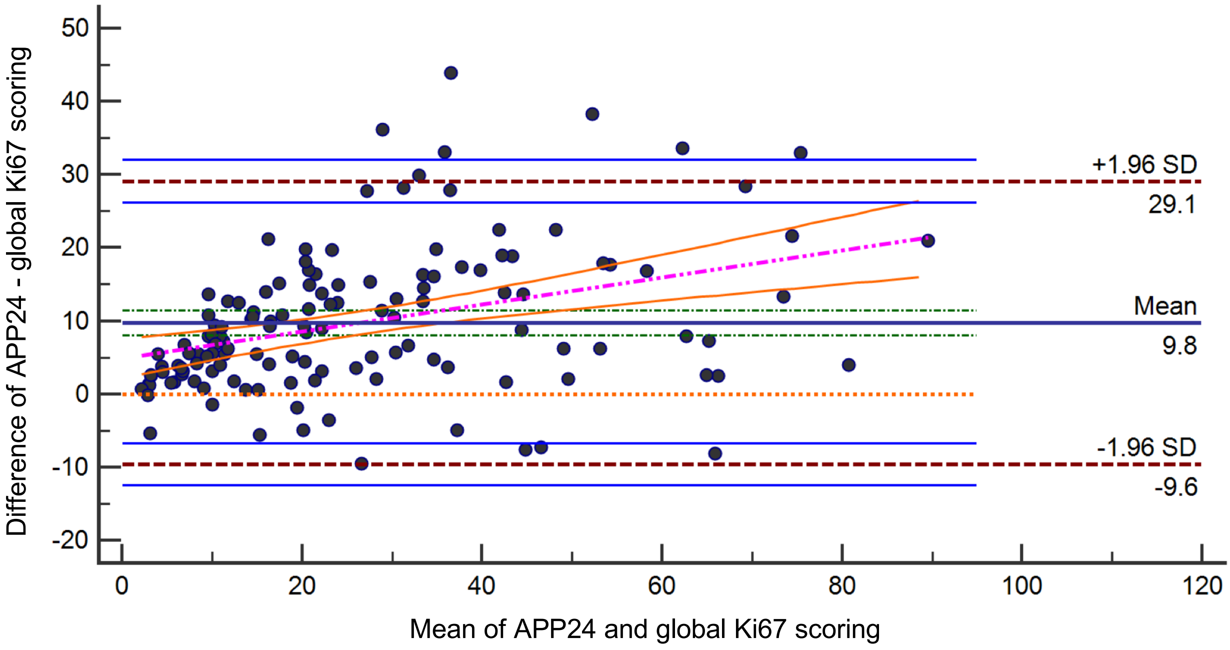

Fig. S3 Bland-Altman plot illustrating the difference between DIA hot spot APP24 and DIA global Ki67 values plotted against the mean Ki67 values of the paired DIA methods (mean Ki67 of DIA hot spot APP24 and DIA global Ki67 calculated for each case). The expected mean difference is 0, shown by dashed orange line. The observed mean difference is shown as a horizontal dark blue line and the limits of agreement as dashed brown lines. The regression line is shown in pink. DIA, digital image analysis. The 95% confidence intervals are shown for each metric.

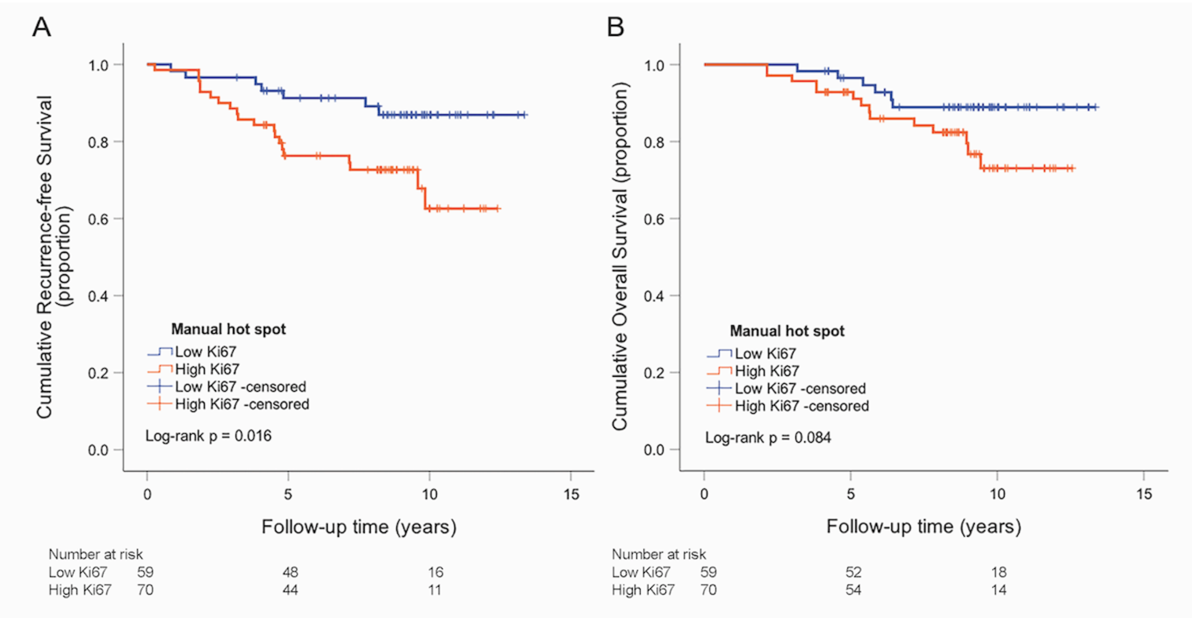

Fig. S4 Kaplan-Meier curves demonstrating associations between low versus high Ki67 subgroups and recurrence-free survival (a) or overall survival (b) using manual hot spot scoring.

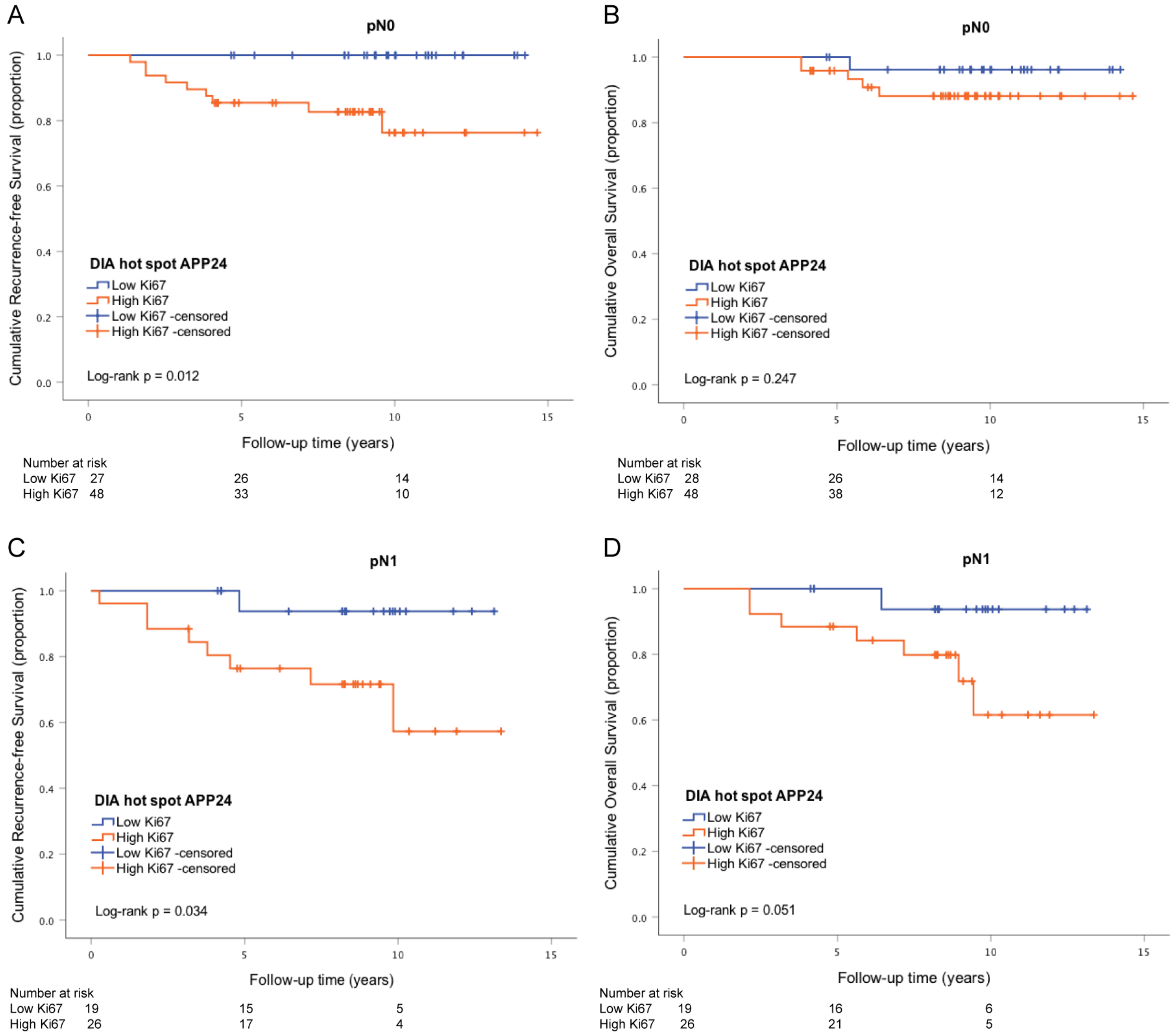

Fig. S5 Kaplan-Meier curves demonstrating associations between low versus high Ki67 subgroups and recurrence-free survival or overall survival in pN0 cases (a-b) and pN1 cases (c-d) using DIA hot spot APP24 scoring.

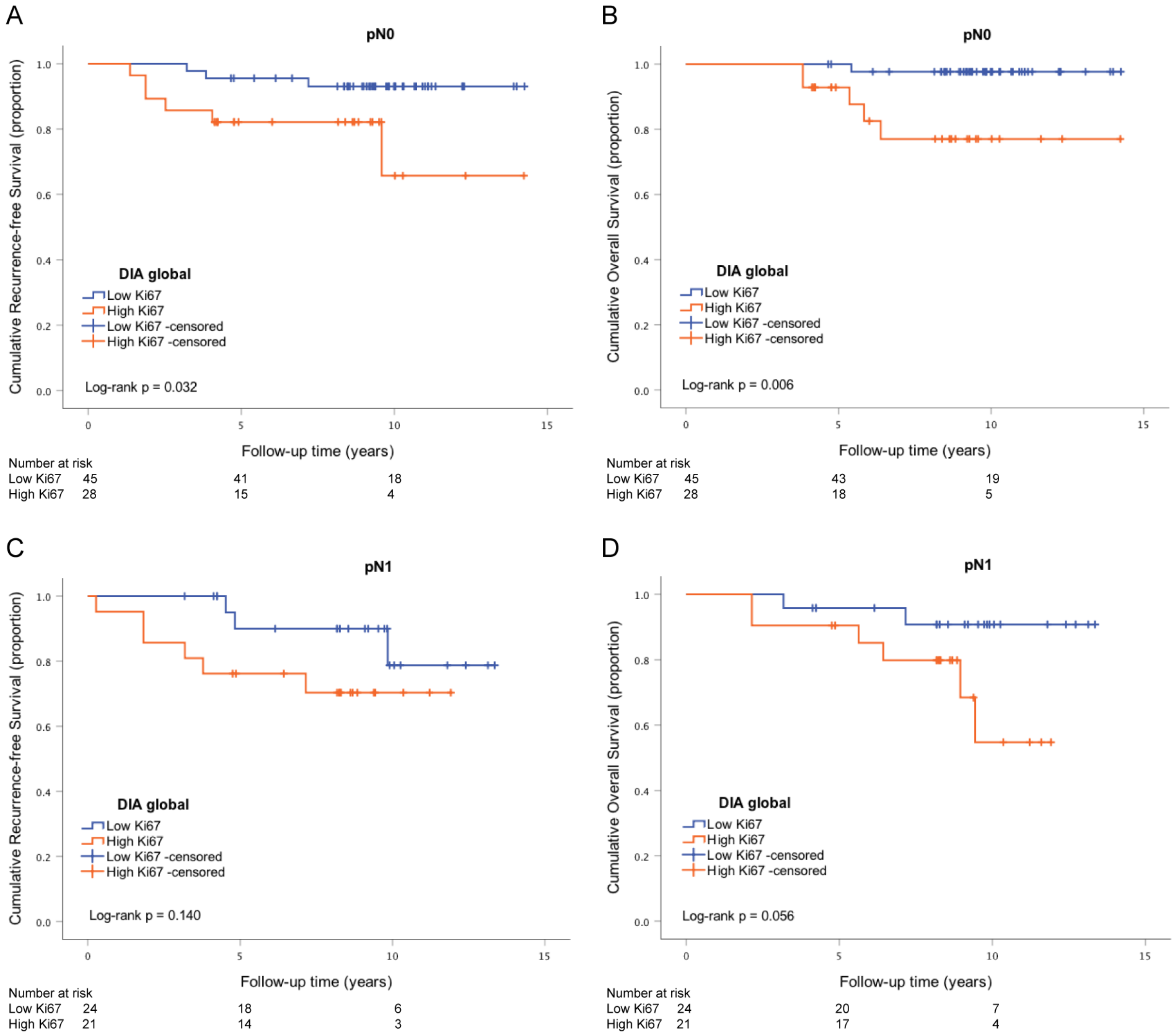

Fig. S6 Kaplan-Meier curves demonstrating associations between low versus high Ki67 subgroups and recurrence-free survival or overall survival in pN0 cases (a-b) and pN1 cases (c-d) using DIA global scoring.

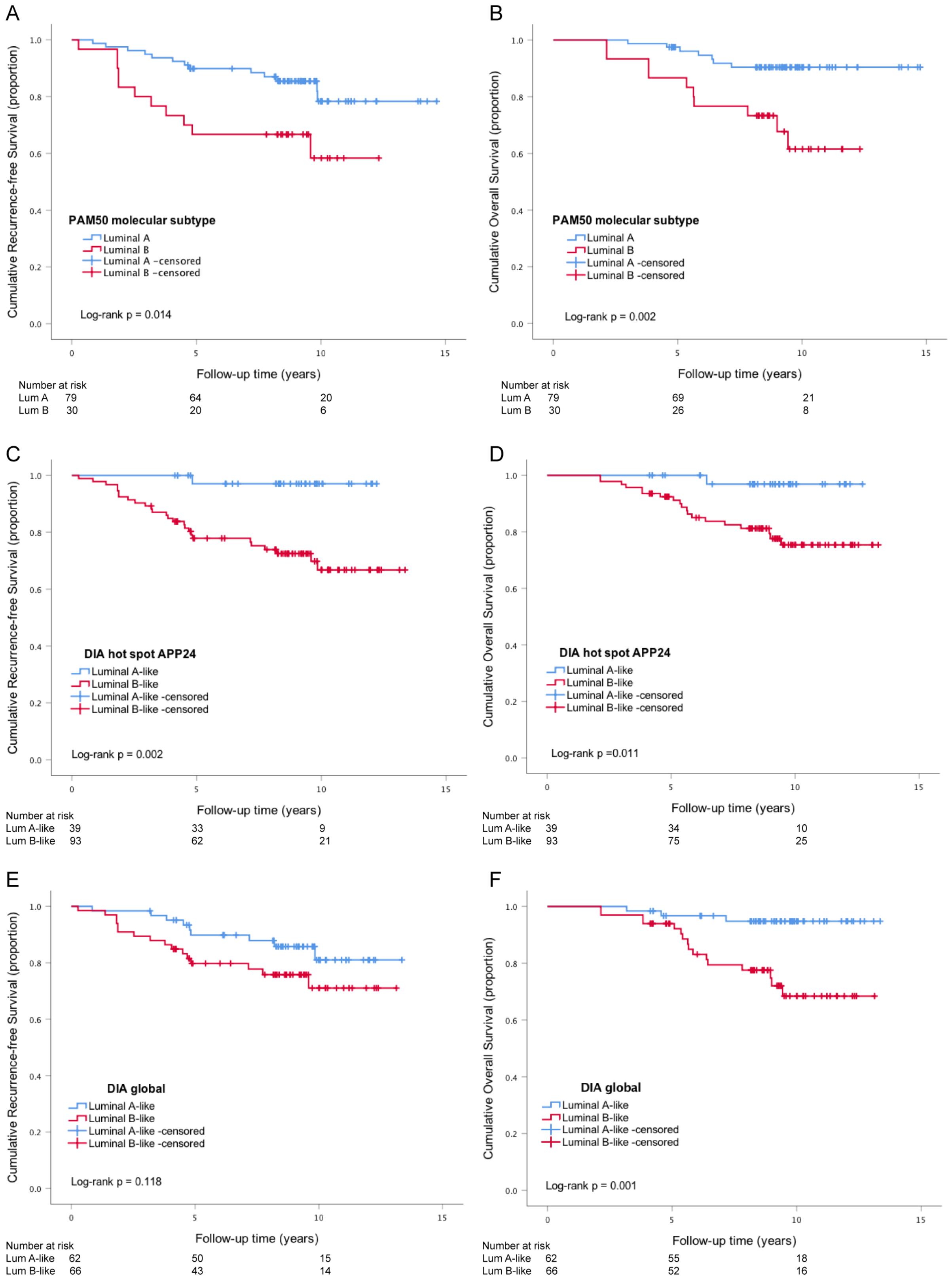

Fig. S7 Kaplan-Meier curves demonstrating associations between luminal A versus luminal B tumors and recurrence-free (a) or overall survival (b) determined by PAM50 molecular intrinsic subtypes. Associations between luminal A-like versus luminal B-like tumors and recurrence-free (c) or overall survival (d) using DIA hot spot APP24 scoring. Associations between luminal A-like versus luminal B-like tumors and recurrence-free (e) or overall survival (f) using DIA global scoring.