Prognostic stromal and intratumoral CD3, CD8 and FOXP3 in adjuvantly treated breast cancer: do they add information over stromal tumor-infiltrating lymphocyte density?

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SUPPLEMENTARY INFORMATION

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Supplementary Figure S2: Descriptive statistics of continuous measurements for CD3, CD8 and FOXP3 markers, as well as stromal TIL density.

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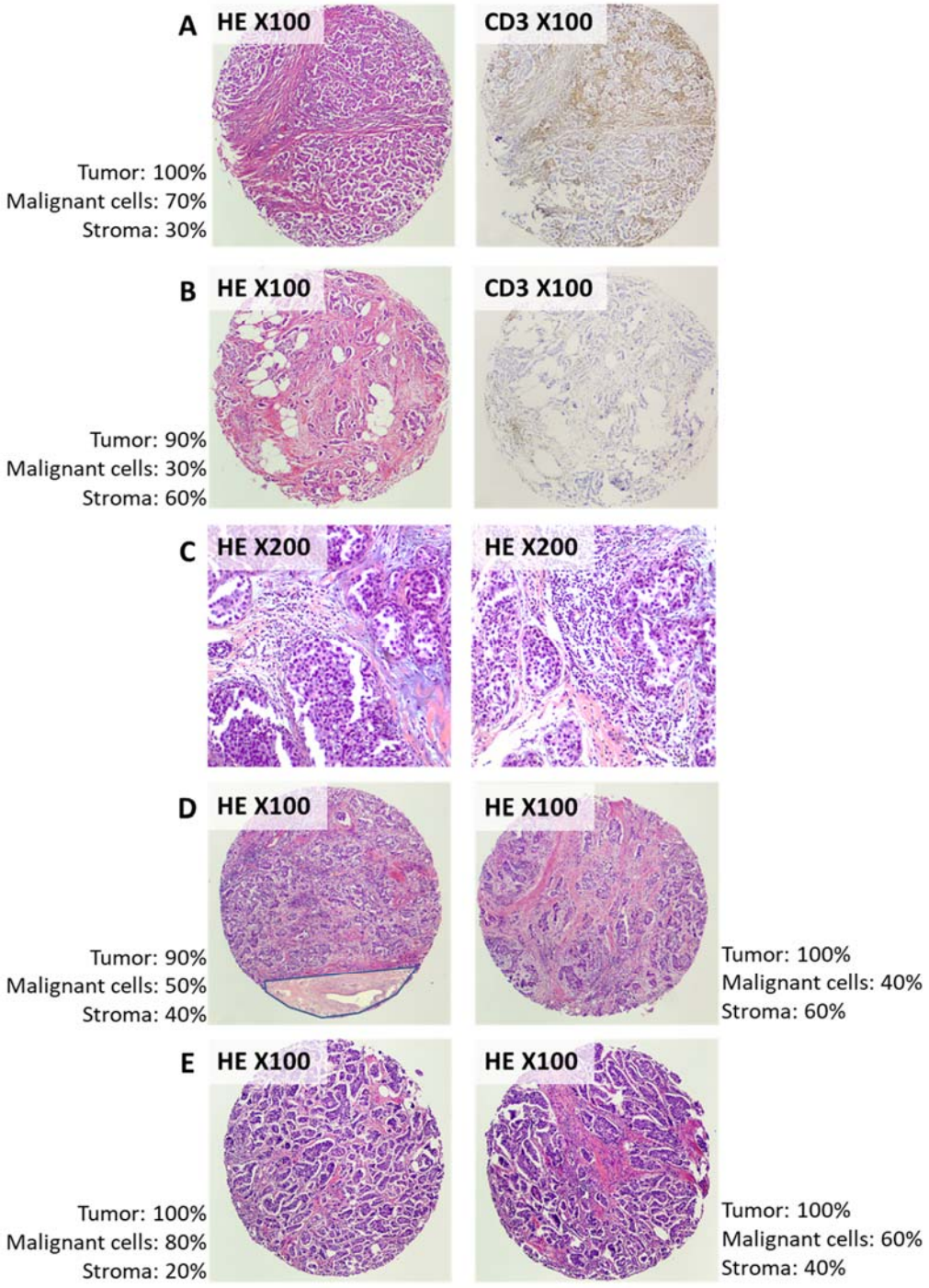
Supplementary Figure S1: Examples of area records for the assessment of the surface occupied by each tumor compartment on 1.5mm cores. A – E: Two pictures from the same tumor are shown. Areas on H&E slides matched to IHC were recorded (**A & B**). Tumor area: stroma + malignant cells. The area of each tumor compartment (stroma or malignant cells) and the lymphocytic density within the same compartment varied in different cores from the same tumor (**C – E**). Non-tumor elements were excluded, e.g., fat in **B**, remnants of breast tissue in **D (left).** Original magnifications are shown.



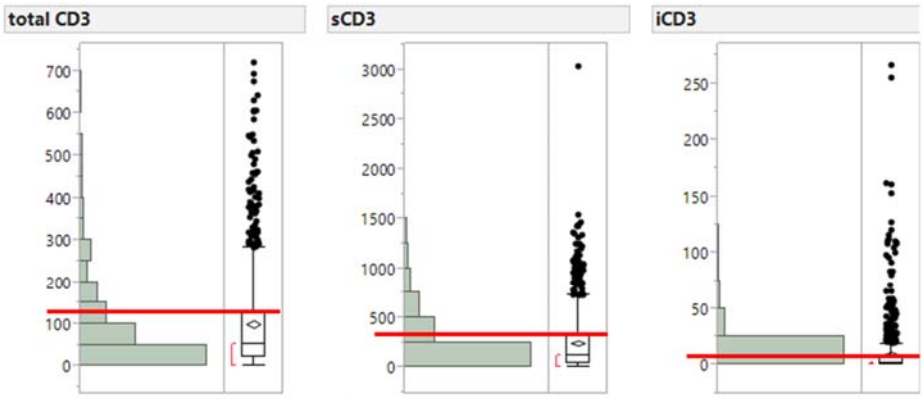
Supplementary Figure S2: Descriptive statistics of continuous measurements for CD3, CD8 and FOXP3 markers, as well as stromal TIL density.

Distributions and cut-offs are shown for each marker, whereby:

- sCD3, sCD8, sFOXP3: positive cell counts per mm^2 stromal surface;
- iCD3, iCD8, iFOXP3: positive cell counts per mm^2 malignant cell surface (intratumoral);
- total CD3, total CD8, total FOXP3: sum of stromal and intratumoral positive cell counts per mm^2 of total tumor surface (stroma + malignant cells).

Based on the many outliers (>10% of eligible tumors for each CD3, CD8, FOXP3), the cut-off for high/low per marker was set at the upper quartile (75% percentile, red lines).

Stromal TIL density was used as a continuous variable only.



Quantiles		
100.0%	maximum	719.604
99.5%		648.737
97.5%		465.301
90.0%		259.023
75.0%	quartile	127.247
50.0%	median	53.035
25.0%	quartile	23.2287
10.0%		10.276
2.5%		3.95558
0.5%		1.69851
0.0%	minimum	1.13234

Summary Statistics	
Mean	99.096978
Std Dev	120.8166
Std Err Mean	4.4057283
Upper 95% Mea	107.74599
Lower 95% Mean	90.447971
N	752

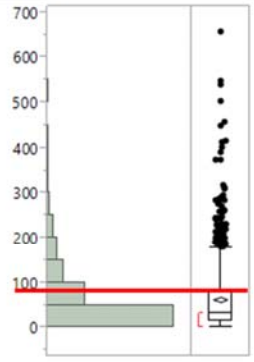
Quantiles		
100.0%	maximum	3038.45
99.5%		1472.93
97.5%		1129.49
90.0%		653.806
75.0%	quartile	318.754
50.0%	median	118.243
25.0%	quartile	47.3157
10.0%		21.3163
2.5%		8.1623
0.5%		3.10156
0.0%	minimum	1.88724

Summary Statistics	
Mean	244.74936
Std Dev	308.19867
Std Err Mean	11.238849
Upper 95% Mea	266.81266
Lower 95% Mean	222.68607
N	752

Quantiles		
100.0%	maximum	266.1
99.5%		160.688
97.5%		92.3567
90.0%		25.6617
75.0%	quartile	7.48155
50.0%	median	1.41543
25.0%	quartile	0
10.0%		0
2.5%		0
0.5%		0
0.0%	minimum	0

Summary Statistics	
Mean	9.6822458
Std Dev	24.522893
Std Err Mean	0.8942579
Upper 95% Mea	11.437788
Lower 95% Mean	7.9267033
N	752

total CD8



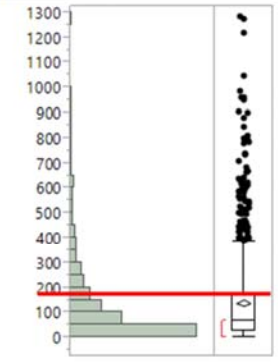
Quantiles

100.0%	maximum	656.759
99.5%		485.624
97.5%		276.232
90.0%		158.23
75.0%	quartile	79.8301
50.0%	median	32.3526
25.0%	quartile	13.7073
10.0%		5.66171
2.5%		1.41543
0.5%		0.56617
0.0%	minimum	0

Summary Statistics

Mean	61.998208
Std Dev	78.085771
Std Err Mean	2.6337677
Upper 95% Mea	67.167423
Lower 95% Mean	56.828992
N	879

sCD8



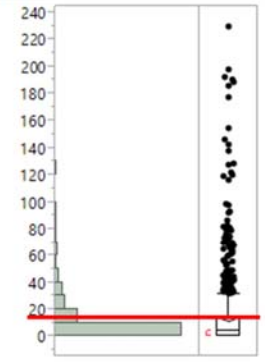
Quantiles

100.0%	maximum	1283.32
99.5%		1022.51
97.5%		669.214
90.0%		358.103
75.0%	quartile	171.739
50.0%	median	66.0533
25.0%	quartile	28.0989
10.0%		11.3234
2.5%		3.39703
0.5%		0.74815
0.0%	minimum	0

Summary Statistics

Mean	138.53289
Std Dev	184.35633
Std Err Mean	6.2181848
Upper 95% Mea	150.73713
Lower 95% Mean	126.32865
N	879

iCD8



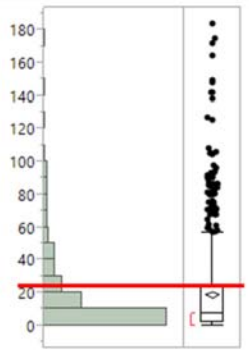
Quantiles

100.0%	maximum	230.007
99.5%		189.533
97.5%		86.8129
90.0%		33.9703
75.0%	quartile	13.2107
50.0%	median	4.11761
25.0%	quartile	0.80882
10.0%		0
2.5%		0
0.5%		0
0.0%	minimum	0

Summary Statistics

Mean	13.464118
Std Dev	26.594906
Std Err Mean	0.8970239
Upper 95% Mea	15.22468
Lower 95% Mean	11.703557
N	879

total FOXP3



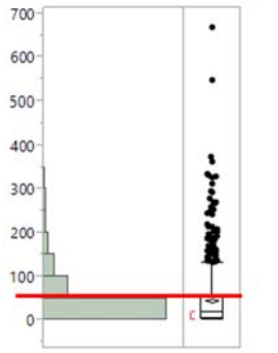
Quantiles

100.0%	maximum	184.006
99.5%		167.639
97.5%		96.8404
90.0%		52.5659
75.0%	quartile	24.0623
50.0%	median	7.64331
25.0%	quartile	1.99825
10.0%		0
2.5%		0
0.5%		0
0.0%	minimum	0

Summary Statistics

Mean	19.088608
Std Dev	27.921534
Std Err Mean	1.0471393
Upper 95% Mea	21.144468
Lower 95% Mean	17.032748
N	711

sFOXP3



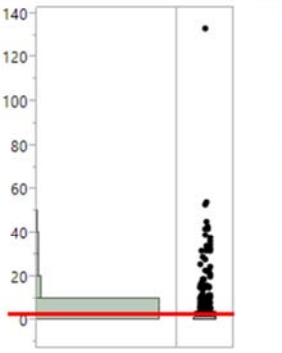
Quantiles

100.0%	maximum	668.082
99.5%		450.773
97.5%		255.768
90.0%		118.896
75.0%	quartile	54.8751
50.0%	median	17.4207
25.0%	quartile	4.24628
10.0%		0
2.5%		0
0.5%		0
0.0%	minimum	0

Summary Statistics

Mean	44.712114
Std Dev	71.835749
Std Err Mean	2.6940511
Upper 95% Mea	50.001374
Lower 95% Mean	39.422854
N	711

iFOXP3



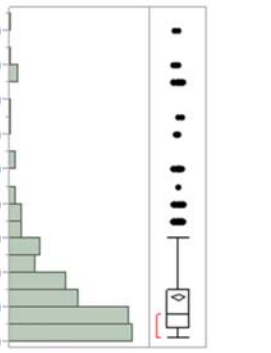
Quantiles

100.0%	maximum	133.365
99.5%		48.6152
97.5%		29.5218
90.0%		7.97358
75.0%	quartile	1.41543
50.0%	median	0
25.0%	quartile	0
10.0%		0
2.5%		0
0.5%		0
0.0%	minimum	0

Summary Statistics

Mean	2.8400989
Std Dev	8.5244165
Std Err Mean	0.3196906
Upper 95% Mea	3.4677509
Lower 95% Mean	2.2124469
N	711

stromal TIL density (%)



Quantiles

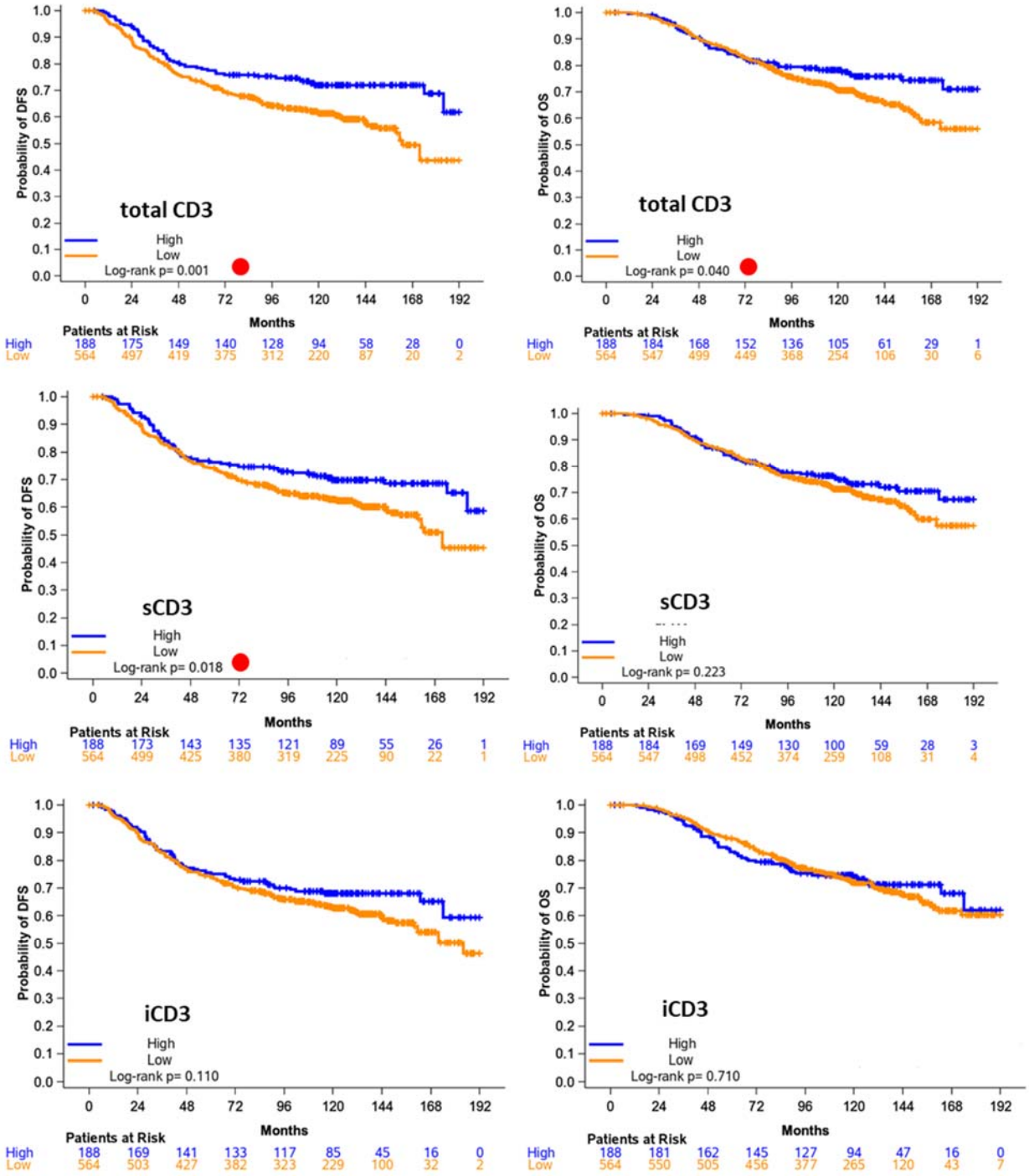
100.0%	maximum	90
99.5%		80
97.5%		71.5
90.0%		30
75.0%	quartile	15
50.0%	median	8
25.0%	quartile	4
10.0%		2
2.5%		1
0.5%		1
0.0%	minimum	1

Summary Statistics

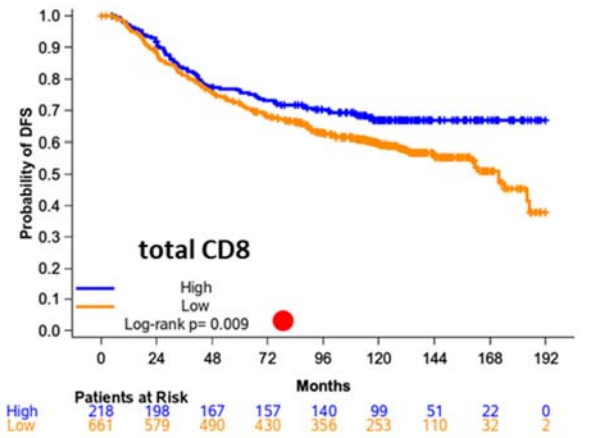
Mean	13.139335
Std Dev	14.877608
Std Err Mean	0.487071
Upper 95% Mea	14.095218
Lower 95% Mean	12.183452
N	933

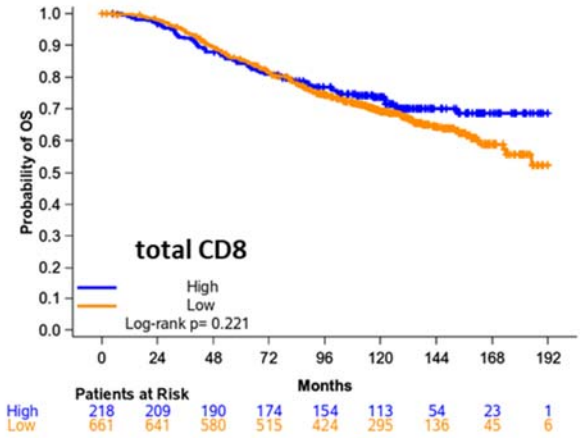
Supplementary Figure S3: Lymphocytic subsets (counts/mm^2) assessed as categorical variables (high/low) on patient disease-free survival (DFS) and overall survival (OS). Kaplan-Meier curves and log-lank test results are shown.

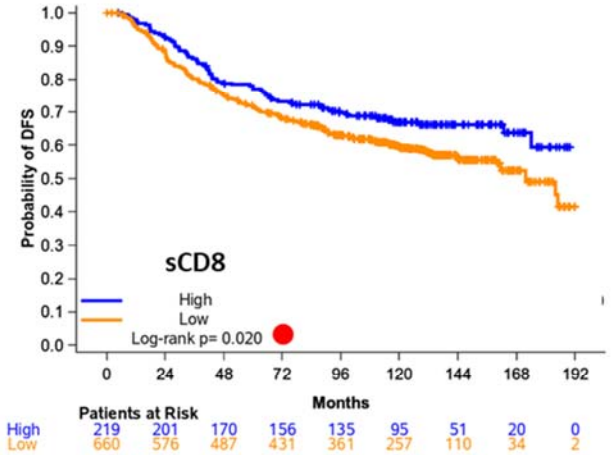
A. CD3 markers in the entire cohort (DFS on the left, OS on the right; red circle: significant results)

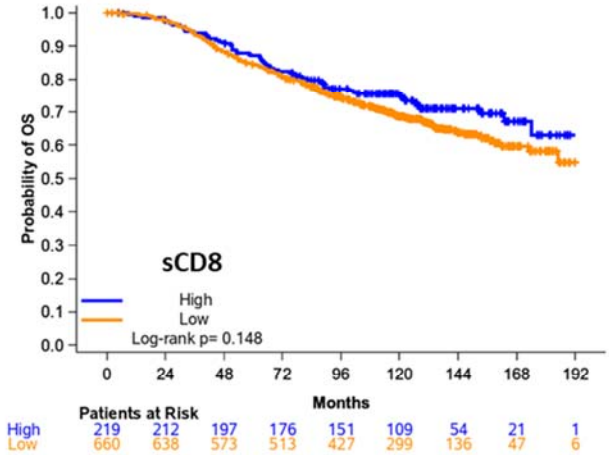


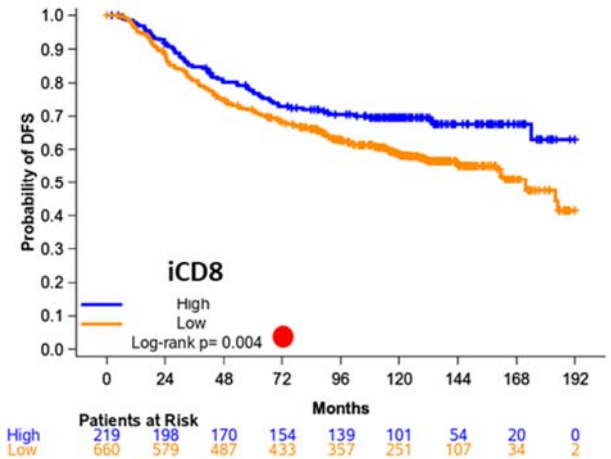
B. CD8 markers in the entire cohort (DFS on the left, OS on the right; red circle: significant results)

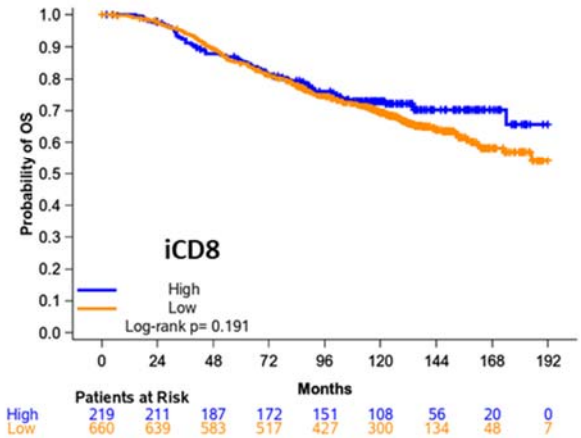




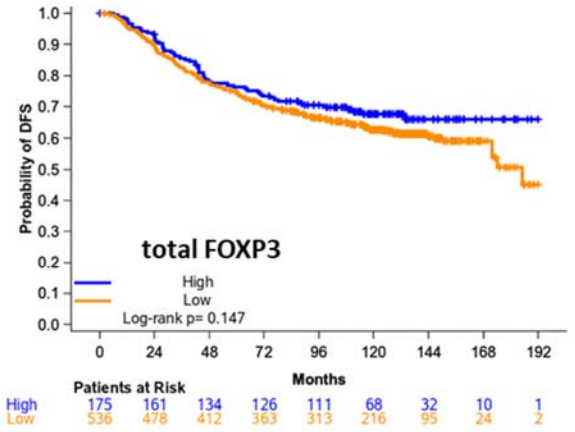


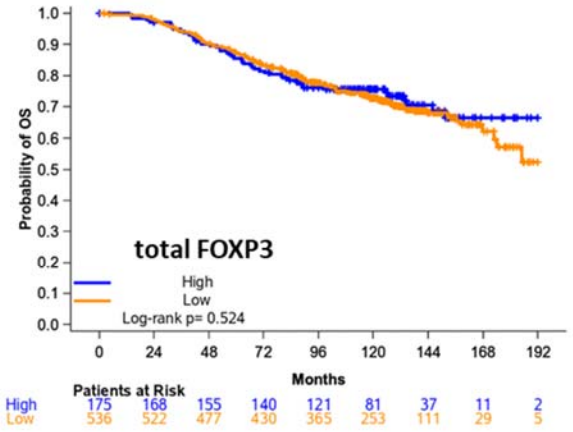


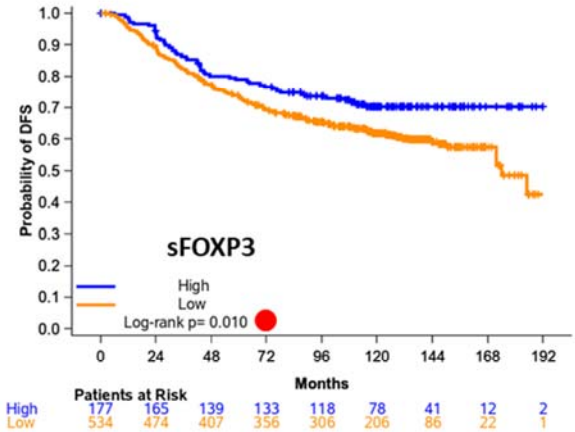


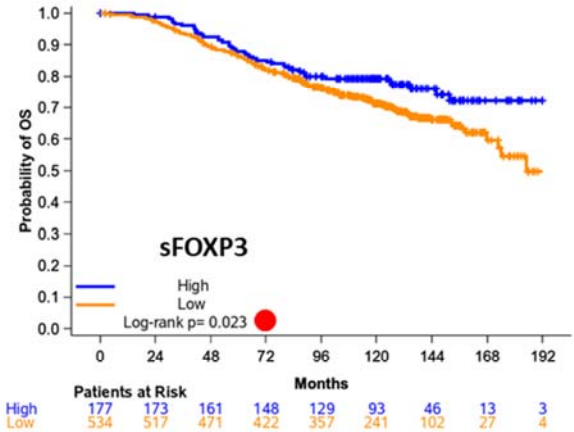


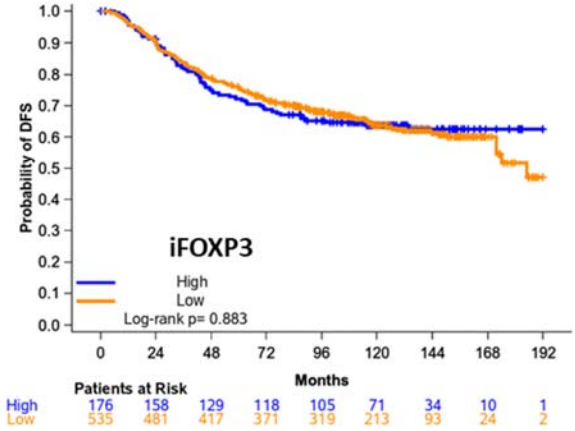
C. FOXP3 markers in the entire cohort (DFS on the left, OS on the right; red circle: significant results)

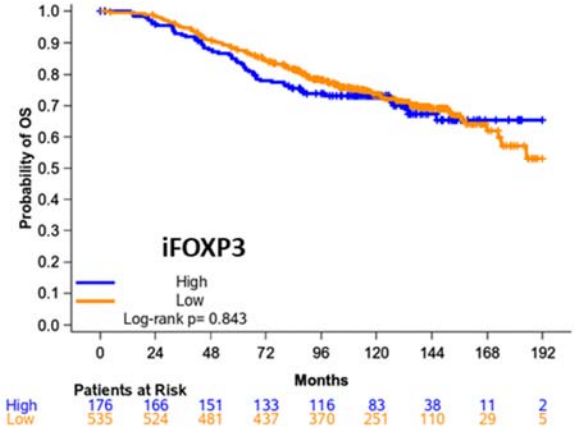




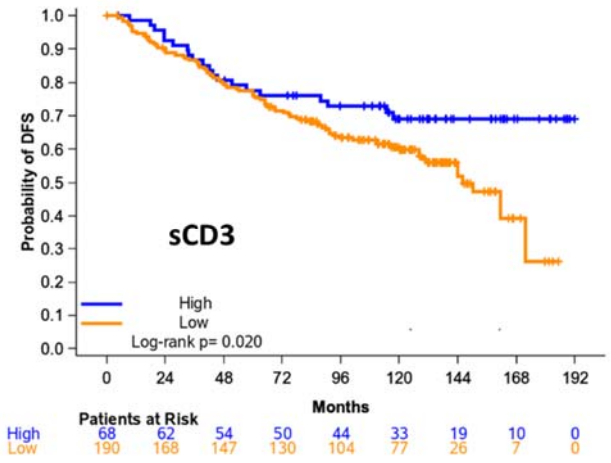


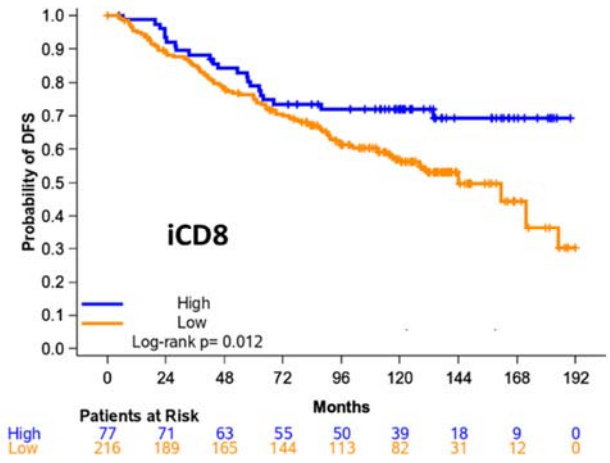




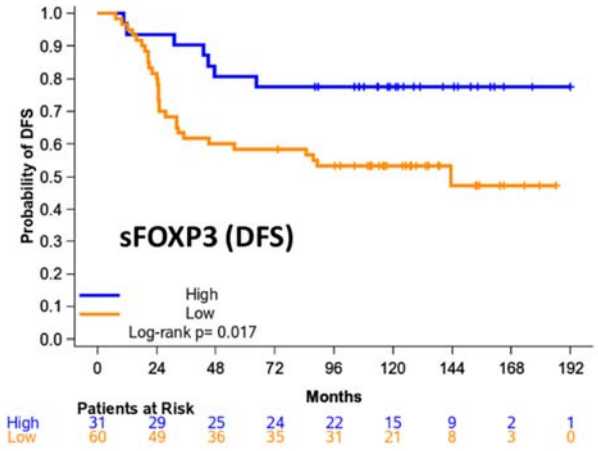


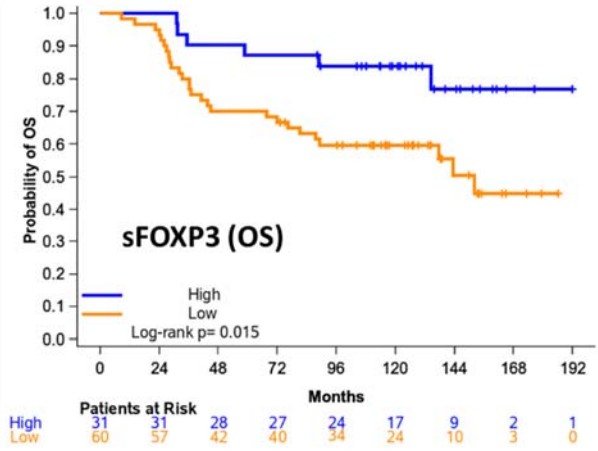
D. Significant marker impact on the outcome of patients with luminal-B tumors



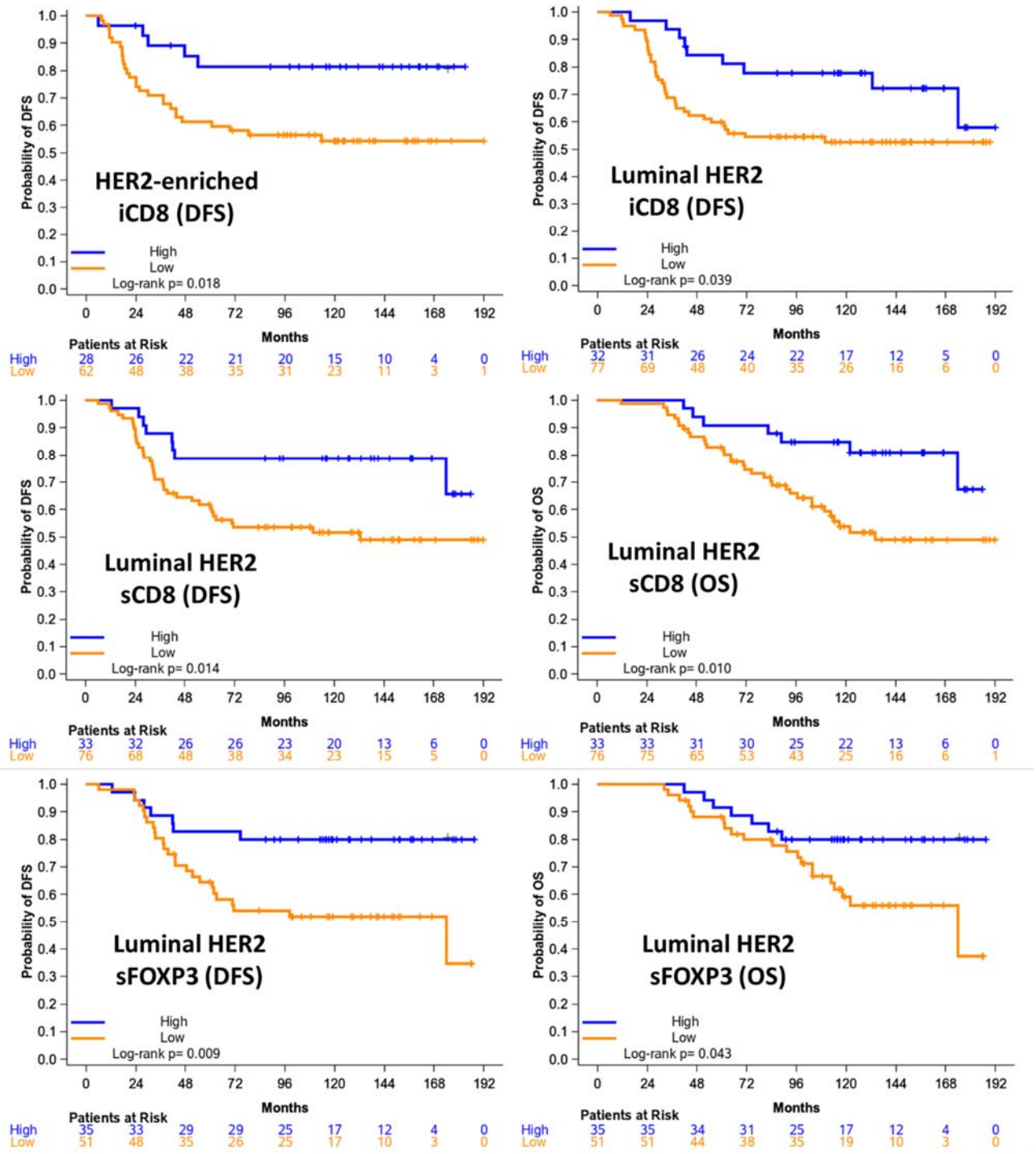


E. Significant marker impact on the outcome of patients with TNBC





F. **Significant marker impact on the outcome of patients with HER2-positive tumors, as indicated**



Supplementary Table S1: Clinical trial characteristics.

Trial	Accrual period	N[1]	n[2]	Treatment schedule	Eligibility criteria	References
HE10/97 Australian New Zealand Clinical Trials Registry ACTRN12611000506998	**1997 - 2000**	**595**	**265**	**E-T-CMF:** Epirubicin 110 mg/m^2 q 2 weeks x 3 followed by paclitaxel 250 mg/m^2 q 2 weeks x 3 followed by cyclophosphamide 840 mg/m^2, methotrexate 57 mg/m^2, fluorouracil 840 mg/m^2 (CMF) q 2 weeks x 3. GCSF support in all cycles. **E-CMF:** Epirubicin 110 mg/m^2 q 2 weeks x 4 followed by CMF q 2 weeks x 4. GCSF support in all cycles. Patients with ER/PgR-positive tumors received tamoxifen 20 mg daily for five years. Premenopausal patients received additional treatment with an LH-RH analog for two years. All patients who underwent partial mastectomy or with tumors >5 cm and/or with ≥4 infiltrated axillary nodes, irrespectively of the type of surgery, were irradiated. Radiation therapy and hormonal therapy were administered after the completion of chemotherapy.	Eligible were women with: histologically confirmed epithelial breast cancer; pathological stage T1-3 N1 M0 or T3 N0 M0; Eastern Cooperative Oncology Group performance status 0-1; normal cardiac function; and adequate bone marrow, hepatic and renal function.	Fountzilas G, Skarlos D, et al. Postoperative dose-dense sequential chemotherapy with epirubicin, followed by CMF with or without paclitaxel, in patients with high-risk operable breast cancer: a randomized phase III study conducted by the Hellenic Cooperative Oncology Group. Ann Oncol. 2005;16(11):1762-71.
HE10/00 Australian New Zealand Clinical Trials Registry ACTRN12609001036202	**2000 - 2005**	**1,086**	**746**	**E-T-CMF:** As in the HE10/97 trial. **ET-CMF:** Epirubicin 83 mg/m^2 + paclitaxel 187 mg/m^2 q 3 weeks x 4 followed by cyclophosphamide 840 mg/m^2, methotrexate 57 mg/m^2, fluorouracil 840 mg/m^2 (CMF) q 2 weeks x 3. GCSF support in all cycles. Premenopausal patients received hormonal therapy as in the HE10/97 trial. Postmenopausal patients received tamoxifen 20 mg daily for 2-3 years followed 2-3 years of daily examestane 25 mg. Criteria for irradiation were the same as in the HE10/97 trial.	Eligible were women with: histologically confirmed epithelial breast cancer; pathological stage T1-4 N1-2 M0; Eastern Cooperative Oncology Group performance status 0-1; normal cardiac function; and adequate bone marrow, hepatic and renal function.	Gogas H, Dafni U, et al. Postoperative dose-dense sequential versus concomitant administration of epirubicin and paclitaxel in patients with node-positive breast cancer: 5-year results of the Hellenic Cooperative Oncology Group HE 10/00 phase III Trial. Breast Cancer Res Treat. 2012;132(2):609-19.

[1]N, number of patients enrolled in the trials; [2]n, number of patients included in the current study with tumor tissue blocks available.

Supplementary Table S2: Antibodies and staining conditions for CD3, CD8 and FOXP3 immunohistochemistry.

Protein	CD3*	CD8**	FOXP3***
Antibody clone	PS1	C8/144B	SP97
Source	mouse	mouse	rabbit
Antibody dilution	1:180	1:60	1:80
Antigen retrieval / 98^{0}C	Citric acid 20'min	EDTA 20'min	EDTA 20'min
Antibody incubation	20'min	20'min	20'min
Staining system	Polymer HRP/DAB	Polymer HRP/DAB	Polymer HRP/DAB

*: Novocastra/Leica Biosystems, Newcastle, UK; **: Dako, Glostrup, Denmark; ***: Spring Bioscience, Fremont, CA.

Supplementary Table S3: Spearman correlations among continuous lymphocytic markers.

A. Spearman's rho values for all IHC CD3, CD8, FOXP3 and TIL density, stromal (s) and intraepithelial (i)

	sCD3	iCD3	sCD8	iCD8	sFOXP3	iFOXP3	sTIL density
sCD3	1	0.591	**0.883**	0.575	0.497	0.394	0.445
iCD3		1	0.557	**0.659**	0.422	0.402	0.34
sCD8			1	0.585	0.521	0.349	0.463
iCD8				1	0.428	0.448	0.378
sFOXP3					1	**0.633**	0.469
iFOXP3						1	0.358
sTIL density							1

B. Spearman's rho values for total IHC and mRNA expression values

	Total CD3	Total CD8	Total FOXP3	CD3 mRNA	CD8 mRNA	FOXP3 mRNA
Total CD3	1	**0.884**	0.463	0.369	0.33	0.323
Total CD8		1	0.496	0.429	0.382	0.368
Total FOXP3			1	0.377	0.275	0.384
CD3 mRNA				1	**0.694**	**0.647**
CD8 mRNA					1	**0.609**
FOXP3 mRNA						1

Note: rho values indicating strong correlations are shown in bold

Supplementary Table S4: Associations of CD3, CD8, FOXP3 and sTIL density with clinicopathological parameters.

	CD3	sCD3			iCD3			Total CD3		
	N(%)	Low	High	p-value	Low	High	p-value	Low	High	p-value
Age	752	51.9(22.3,79.3)	53.9(23.8,76.9)	0.33	52.3(22.3,79.3)	52.9(23.8,76.9)	0.97	52.4(22.3,79.3)	52.6(23.8,77.1)	0.46
Ki67	727	20.0(0.00,95.0)	35.0(1.00,98.0)	**<0.001**	25.0(0.00,95.0)	33.5(2.0,98.0)	**<0.001**	20.0(0.00,95.0)	35.0(1.00,98.0)	**<0.001**
Age (categorical)	752(100.0)			0.070			0.64			0.97
≤52.7	383(50.9)	298(52.8)	85(45.2)		290(51.4)	93(49.5)		287(50.9)	96(51.1)	
>52.7	369(49.1)	266(47.2)	103(54.8)		274(48.6)	95(50.5)		277(49.1)	92(48.9)	
Menopausal status	752(100.0)			0.13			0.87			0.87
Postmenopausal	400(53.2)	291(51.6)	109(58.0)		301(53.4)	99(52.7)		301(53.4)	99(52.7)	
Premenopausal	352(46.8)	273(48.4)	79(42.0)		263(46.6)	89(47.3)		263(46.6)	89(47.3)	
Tumor size	752(100.0)			0.26			0.60			**0.035**
≤2	224(29.8)	160(28.4)	64(34.0)		163(28.9)	61(32.4)		154(27.3)	70(37.2)	
2-5	431(57.3)	327(58.0)	104(55.3)		329(58.3)	102(54.3)		334(59.2)	97(51.6)	
>5	97(12.9)	77(13.7)	20(10.6)		72(12.8)	25(13.3)		76(13.5)	21(11.2)	
Nodal status	752(100.0)			0.80			0.14			0.93
0-3	302(40.2)	225(39.9)	77(41.0)		218(38.7)	84(44.7)		227(40.2)	75(39.9)	
≥4	450(59.8)	339(60.1)	111(59.0)		346(61.3)	104(55.3)		337(59.8)	113(60.1)	
Grade	750(100.0)			**<0.001**			**<0.001**			**0.001**
I-II	365(48.7)	294(52.3)	71(37.8)		299(53.2)	66(35.1)		293(52.1)	72(38.3)	
III-IV	385(51.3)	268(47.7)	117(62.2)		263(46.8)	122(64.9)		269(47.9)	116(61.7)	
ER/PgR status	735(100.0)			**0.009**			**0.001**			0.083
Negative	164(22.3)	110(20.0)	54(29.2)		107(19.5)	57(30.8)		114(20.8)	50(26.9)	
Positive	571(77.7)	440(80.0)	131(70.8)		443(80.5)	128(69.2)		435(79.2)	136(73.1)	
HER2 status	742(100.0)			**0.003**			**0.038**			**0.004**
Negative	569(76.7)	441(79.3)	128(68.8)		436(78.6)	133(71.1)		440(79.3)	129(69.0)	
Positive	173(23.3)	115(20.7)	58(31.2)		119(21.4)	54(28.9)		115(20.7)	58(31.0)	
Subtypes	728(100.0)			**<0.001**			**<0.001**			**<0.001**
Luminal A	209(28.7)	180(86.1)	29(13.9)		174(83.3)	35(16.7)		180(86.1)	29(13.9)	
Luminal B	258(35.4)	190(73.6)	68(26.4)		197(76.4)	61(23.6)		190(73.6)	68(26.4)	
Luminal HER2	97(13.3)	64(66.0)	33(34.0)		66(68.0)	31(32.0)		59(60.8)	38(39.2)	
HER2-enriched	73(10.0)	48(65.8)	25(34.2)		51(69.9)	22(30.1)		53(72.6)	20(27.4)	
TNBC	91(12.5)	62(68.1)	29(31.9)		56(61.5)	35(38.5)		61(67.0)	30(33.0)	
	CD8	**sCD8**			**iCD8**			**Total CD8**		
	N(%)	**Low**	**High**	**p-value**	**Low**	**High**	**p-value**	**Low**	**High**	**p-value**
Age	879	52.3(22.3,79.3)	53.5(23.8,76.9)	0.99	52.3(22.3,79.3)	53.4(23.8,76.9)	0.49	52.4(22.3,79.3)	53.1(23.8,77.1)	0.38
Ki67	831	25.0(0.00,95.0)	35.0(1.00,98.0)	**<0.001**	25.0(0.00,98.0)	35.0(1.00,98.0)	**<0.001**	23.8(0.00,95.0)	35.0(1.00,98.0)	**<0.001**
Age (categorical)	879 (100.0)			0.19			0.19			0.65
≤52.7	443(50.4)	341(51.7)	102(46.6)		341(51.7)	102(46.6)		336(50.8)	107(49.1)	
>52.7	436(49.6)	319(48.3)	117(53.4)		319(48.3)	117(53.4)		325(49.2)	111(50.9)	
Menopausal status	879 (100.0)			0.80			0.57			0.78
Postmenopausal	471(53.6)	352(53.3)	119(54.3)		350(53.0)	121(55.3)		356(53.9)	115(52.8)	
Premenopausal	408(46.4)	308(46.7)	100(45.7)		310(47.0)	98(44.7)		305(46.1)	103(47.2)	
Tumor size	879 (100.0)			**0.041**			0.99			0.21
≤2	255(29.0)	177(26.8)	78(35.6)		191(28.9)	64(29.2)		183(27.7)	72(33.0)	
2-5	514(58.5)	396(60.0)	118(53.9)		386(58.5)	128(58.4)		390(59.0)	124(56.9)	
>5	110(12.5)	87(13.2)	23(10.5)		83(12.6)	27(12.3)		88(13.3)	22(10.1)	
Nodal status	879 (100.0)			0.34			0.76			0.82
0-3	353(40.2)	259(39.2)	94(42.9)		267(40.5)	86(39.3)		264(39.9)	89(40.8)	
≥4	526(59.8)	401(60.8)	125(57.1)		393(59.5)	133(60.7)		397(60.1)	129(59.2)	
Grade	876 (100.0)			**<0.001**			**<0.001**			**0.001**
I-II	429(49.0)	345(52.5)	84(38.4)		351(53.4)	78(35.6)		343(52.1)	86(39.4)	
III-IV	447(51.0)	312(47.5)	135(61.6)		306(46.6)	141(64.4)		315(47.9)	132(60.6)	
ER/PgR status	844 (100.0)			**0.009**			**0.004**			**0.006**
Negative	199(23.6)	136(21.4)	63(30.3)		134(21.1)	65(31.0)		135(21.3)	64(30.6)	
Positive	645(76.4)	500(78.6)	145(69.7)		500(78.9)	145(69.0)		500(78.7)	145(69.4)	
HER2 status	854 (100.0)			0.067			0.061			**0.002**
Negative	650(76.1)	500(77.6)	150(71.4)		498(77.7)	152(71.4)		506(78.7)	144(68.2)	
Positive	204(23.9)	144(22.4)	60(28.6)		143(22.3)	61(28.6)		137(21.3)	67(31.8)	
Subtypes	833(100.0)			**<0.001**			**<0.001**			**<0.001**
Luminal A	232(27.9)	203(87.5)	29(12.5)		198(85.3)	34(14.7)		201(86.6)	31(13.4)	
Luminal B	293(35.2)	213(72.7)	80(27.3)		216(73.7)	77(26.3)		218(74.4)	75(25.6)	
Luminal HER2	109(13.1)	76(69.7)	33(30.3)		77(70.6)	32(29.4)		73(67.0)	36(33.0)	
HER2-enriched	90(10.8)	64(71.1)	26(28.9)		62(68.9)	28(31.1)		60(66.7)	30(33.3)	
TNBC	109(13.1)	72(66.1)	37(33.9)		72(66.1)	37(33.9)		75(68.8)	34(31.2)	
	FOXP3	**sFOXP3**			**iFOXP3**			**Total FOXP3**		
	N(%)	**Low**	**High**	**p-value**	**Low**	**High**	**p-value**	**Low**	**High**	**p-value**
Age	711	52.5(24.5,78.9)	52.9(22.3,76.9)	0.36	52.8(24.5,78.9)	51.3(22.3,76.9)	0.04	52.4(27.5,78.9)	53.1(22.3,76.9)	0.21
Ki67	681	20.0(0.00,95.0)	35.0(2.0,98.0)	**<0.001**	20.0(0.00,95.0)	37.0(5.0,98.0)	**<0.001**	20.0(0.00,95.0)	37.0(5.0,98.0)	**<0.001**
Age (categorical)	711 (100.0)			0.78			0.50			0.53
≤52.7	360(50.6)	272(50.9)	88(49.7)		267(49.9)	93(52.8)		275(51.3)	85(48.6)	
>52.7	351(49.4)	262(49.1)	89(50.3)		268(50.1)	83(47.2)		261(48.7)	90(51.4)	
Menopausal status	711 (100.0)			0.92			0.48			0.54
Postmenopausal	388(54.6)	292(54.7)	96(54.2)		296(55.3)	92(52.3)		296(55.2)	92(52.6)	
Premenopausal	323(45.4)	242(45.3)	81(45.8)		239(44.7)	84(47.7)		240(44.8)	83(47.4)	
Tumor size	711 (100.0)			0.66			0.49			0.84

≤2	205(28.8)	152(28.5)	53(29.9)		149(27.9)	56(31.8)		153(28.5)	52(29.7)	
2-5	416(58.5)	311(58.2)	105(59.3)		315(58.9)	101(57.4)		313(58.4)	103(58.9)	
>5	90(12.7)	71(13.3)	19(10.7)		71(13.3)	19(10.8)		70(13.1)	20(11.4)	
Nodal status	711 (100.0)			**0.007**			0.17			**0.049**
0-3	292(41.1)	204(38.2)	88(49.7)		212(39.6)	80(45.5)		209(39.0)	83(47.4)	
≥4	419(58.9)	330(61.8)	89(50.3)		323(60.4)	96(54.5)		327(61.0)	92(52.6)	
Grade	709 (100.0)			**<0.001**			**<0.001**			**<0.001**
I-II	364(51.3)	304(57.1)	60(33.9)		298(55.9)	66(37.5)		299(56.0)	65(37.1)	
III-IV	345(48.7)	228(42.9)	117(66.1)		235(44.1)	110(62.5)		235(44.0)	110(62.9)	
ER/PgR status	691 (100.0)			**<0.001**			**0.009**			**0.003**
Negative	166(24.0)	107(20.6)	59(34.5)		112(21.6)	54(31.4)		111(21.3)	55(32.4)	
Positive	525(76.0)	413(79.4)	112(65.5)		407(78.4)	118(68.6)		410(78.7)	115(67.6)	
HER2 status	701 (100.0)			**<0.001**			**0.020**			**<0.001**
Negative	536(76.5)	427(81.0)	109(62.6)		415(78.6)	121(69.9)		424(80.2)	112(65.1)	
Positive	165(23.5)	100(19.0)	65(37.4)		113(21.4)	52(30.1)		105(19.8)	60(34.9)	
Subtypes	683 (100.0)			**<0.001**			**<0.001**			**<0.001**
Luminal A	209(30.6)	191(91.4)	18(8.6)		185(88.5)	24(11.5)		187(89.5)	22(10.5)	
Luminal B	222(32.5)	164(73.9)	58(26.1)		158(71.2)	64(28.8)		161(72.5)	61(27.5)	
Luminal HER2	86(12.6)	51(59.3)	35(40.7)		57(66.3)	29(33.7)		55(64.0)	31(36.0)	
HER2-enriched	75(11.0)	47(62.7)	28(37.3)		53(70.7)	22(29.3)		48(64.0)	27(36.0)	
TNBC	91(13.3)	60(65.9)	31(34.1)		59(64.8)	32(35.2)		63(69.2)	28(30.8)	

	stromal TIL density					CLUSTERS			
	N	**Mean±std**	**Median**	**min - max**	**p-value**	**N**	**Low (clstr 1)**	**High (clstr 2)**	**p-value**
Age						511	52.1(24.5,78.9)	54.2(22.3,76.9)	0.77
Ki67						499	20.0(0.00,95.0)	40.0(1.00,98.0)	**<0.001**
Age (categorical)	933				0,43	511 (100.0)			0.37
≤52.7	468	13.05±14.65	10	1 to 90		261(51.1)	212(52.1)	49(47.1)	
>52.7	465	13.23±15.12	8	1 to 90		250(48.9)	195(47.9)	55(52.9)	
Menopausal status	933				0,52	511 (100.0)			0.86
Postmenopausal	505	12.82±14.13	8	1 to 90		276(54.0)	219(53.8)	57(54.8)	
Premenopausal	428	13.51±15.73	9	1 to 90		235(46.0)	188(46.2)	47(45.2)	
Tumor size	933				**0.005**	511 (100.0)			0.27
≤2	275	15.16±17.34	10	1 to 90		298(58.3)	241(59.2)	57(54.8)	
2-5	541	12.66±13.6	10	1 to 90		146(28.6)	110(27.0)	36(34.6)	
>5	117	10.62±13.8	5	1 to 90		67(13.1)	56(13.8)	11(10.6)	
Nodal status	933				0,34	511 (100.0)			0.34
0-3	385	12.95±13.69	8	1 to 90		210(41.1)	163(40.0)	47(45.2)	
≥4	548	13.27±15.67	8	1 to 90		301(58.9)	244(60.0)	57(54.8)	
Grade	929				**<0.001**	510 (100.0)			**<0.001**
I-II	463	11.04±14.03	5	1 to 90		257(50.4)	221(54.4)	36(34.6)	
III-IV	466	15.27±15.44	10	1 to 90		253(49.6)	185(45.6)	68(65.4)	
ER/PgR status	887				**<0.001**	502 (100.0)			0.40
Negative	208	17.73±18.96	10	1 to 90		117(23.3)	90(22.5)	27(26.5)	
Positive	679	11.87±13.43	5	1 to 90		385(76.7)	310(77.5)	75(73.5)	
HER2 status	903				**<0.001**	507 (100.0)			0.05
Negative	685	11.3±12.73	5	1 to 90		390(76.9)	319(78.8)	71(69.6)	
Positive	216	18.82±19.24	10	1 to 90		117(23.1)	86(21.2)	31(30.4)	
Subtypes	885				**<0.001**	460(100)			**0.006**
Luminal A	269	8.37±8.78	5	1 to 75		137(29.7)	125(91.2)	12(8.8)	
Luminal B	293	11.88±11.67	8	1 to 80		154(33.5)	126(81.8)	28(18.2)	
Luminal HER2	115	19.24±20.57	10	1 to 90		61(13.3)	44(72.1)	17(27.9)	
HER2-enriched	97	18.52±17.94	12	1 to 75		47(10.2)	36(76.6)	11(23.4)	
TNBC	111	17.05±19.86	10	1 to 90		61(13.3)	46(82.0)	15(18.0)	

Note: Significant associations are shown in bold

Supplementary Table S5: Hazard ratios (95% CI) estimated from univariate Cox regression for each of the clinicopathological parameters with respect to the total follow-up period.

Parameter	Categories	N of patients	N of events	HR	95% CI	Wald's p
DFS						
Age (median cut-off)	>52.7 vs. ≤52.7	505 vs. 506	211 vs. 191	1.12	0.92-1.36	0.25
Menopausal status	Premenopausal vs. Postmenopausal	463 vs. 548	164 vs. 238	0.77	0.63-0.94	**0.011**
Breast surgery	Breast-conserving vs. MRM	315 vs. 694	98 vs. 302	0.64	0.55-0.76	**<0.001**
Tumor size						**<0.001**
	2-5 vs. ≤2	580 vs. 302	237 vs. 98	1.36	1.07-1.72	**0.011**
	>5 vs. ≤2	129 vs. 302	67 vs. 98	1.96	1.43-2.67	**<0,001**
Nodal status	≥4 vs. 0-3	603 vs. 408	296 vs. 106	2.22	1.78-2.77	**<0,001**
Hormonal therapy	Yes vs. No	804 vs. 205	315 vs. 87	0.8	0.63-1.01	**0.062**
Radiation therapy	Yes vs. No	770 vs. 214	322 vs. 72	1.28	0.99-1.65	**0.062**
Paclitaxel	Yes vs. No	866 vs.145	331 vs. 71	0.82	0.63-1.06	0.13
Histological grade	III-IV vs. I-II	511 vs. 496	211 vs. 189	1.17	0.96-1.43	0.11
ER/PgR status	Positive vs. Negative	737 vs. 225	279 vs. 93	0.81	0.64-1.03	**0.081**
HER2 status	Positive vs. Negative	232 vs. 744	94 vs. 290	1.11	0.88-1.40	0.38
Subtypes						**0.011**
	HER2-enriched vs. Luminal B	103 vs. 327	38 vs. 138	0.95	0.66-1.36	0.77
	Luminal A vs. Luminal B	273 vs. 327	84 vs. 138	0.68	0.52-0.89	**0.005**
	Luminal-HER2 vs. Luminal B	123 vs. 327	51 vs. 138	1.01	0.73-1.39	0.96
	TNBC vs. Luminal B	122 vs. 327	55 vs. 138	1.2	0.88-1.65	0.24
Ki67				1.003	0.999-1.007	0.11
OS						
Age (median cut-off)	>52.7 vs. ≤52.7	505 vs. 506	173 vs. 152	1.2	0.96-1.49	0.10
Menopausal status	Premenopausal vs. Postmenopausal	463 vs. 548	131 vs. 194	0.74	0.59-0.92	**0.008**
Breast surgery	Breast-conserving vs. MRM	315 vs. 694	79 vs. 244	0.67	0.52-0.86	**0.002**
Tumor size						**<0,001**
	2-5 vs. ≤2	580 vs. 302	189 vs. 76	1.39	1.06-1.81	**0.016**
	>5 vs. ≤2	129 vs. 302	60 vs. 76	2.24	1.60-3.14	**<0,001**
Nodal status	≥4 vs. 0-3	603 vs. 408	250 vs. 75	2.52	1.95-3.27	**<0,001**
Hormonal therapy	Yes vs. No	804 vs. 205	250 vs. 75	0.73	0.57-0.95	**0.018**
Radiation therapy	Yes vs. No	770 vs. 214	263 vs. 55	1.28	0.96-1.72	**0.092**
Paclitaxel	Yes vs. No	866 vs. 145	266 vs. 59	0.84	0.63-1.12	0.24
Histological grade	III-IV vs. I-II	511 vs. 496	176 vs. 148	1.22	0.98-1.52	**0.076**
ER/PgR status	Positive vs. Negative	737 vs. 225	222 vs. 79	0.8	0.62-1.03	**0.087**
HER2 status	Positive vs. Negative	232 vs. 744	79 vs. 230	1.09	0.85-1.41	0.49
Subtypes						**<0.001**
	HER2-enriched vs. Luminal B	103 vs. 327	29 vs. 113	0.81	0.54-1.22	0.32
	Luminal A vs. Luminal B	273 vs. 327	57 vs. 113	0.59	0.43-0.81	**0.001**
	Luminal-HER2 vs. Luminal B	123 vs. 327	46 vs. 113	1.09	0.77-1.53	0.64
	TNBC vs. Luminal B	122 vs. 327	50 vs. 113	1.35	0.97-1.89	0.076
Ki67				1.007	1.002-1.011	**0.003**

MRM, modified radical mastectomy.

Significant p-values are shown in bold.

Supplementary Table S6: Cox multivariate regression analysis for DFS and OS in the entire cohort. sTIL density and lymphocytic subsets examined as single markers.

Parameter	Events/Total	HR (95% CI)	P-value
DFS			
Model 1 *			
sCD3			
High	55/180	0.69 (0.51-0.94)	**0.017**
Low	205/528	Reference	--
Model 2 **			
sCD8			
High	67/203	0.74 (0.56-0.98)	**0.032**
Low	247/606	Reference	--
Model 3 ***			
iCD8			
High	62/203	0.64 (0.48-0.85)	**0.002**
Low	252/606	Reference	--
Model 4 ******			
sFOXP3			
High	48/167	0.69 (0.50-0.97)	**0.03**
Low	192/497	Reference	--
Model 5 +			
total CD3			
High	51/181	0.60 (0.44-0.82)	**0.002**
Low	209/527	Reference	--
Model 6 ++			
total CD8			
High	64/204	0.68 (0.52-0.91)	**0.008**
Low	250/605	Reference	--
sTIL density (10% increment) #		0.86 (0.79-0.94)	**0.001**
OS			
Model 1^			
sFOXP3			
High	39/167	0.71 (0.49-1.03)	0.071
Low	155/497	Reference	--
Model 2^^			
total CD3			
High	44/181	0.68 (0.49-0.96)	**0.029**
Low	163/527	Reference	--
sTIL density (10% increment) ##		0.86 (0.78-0.95)	**0.003**

Notes:

1. Significant results in bold
2. In all models, markers were adjusted for menopausal status, tumor size, nodal status, histological grade, radiation therapy and subtypes.
3. The following clinicopathological parameters retained their statistical significance ($p<0.050$) in the respective multivariate analyses: *tumor size ($p=0.011$), nodal status ($p<0.001$)** tumor size ($p=0.007$), nodal status ($p<0.001$), *** tumor size ($p=0.007$), nodal status ($p<0.001$), **** nodal status ($p<0.001$), + nodal status ($p<0,001$) and tumor size ($p=0,016$), ++ nodal stauts ($p<0,001$), ^ nodal status ($p<0.001$), ^^ nodal status ($p<0,001$), tumor size ($p=0,009$) and subtypes ($p=0,018$), # nodal status ($p<0.001$) and subtypes ($p=0.009$); ## nodal status ($p<0.001$), tumor size ($p=0.016$) and subtypes ($p<0.001$);