SUPPLEMENTAL MATERIAL

Synchronous detection of circulating tumor cells in blood and disseminated tumor cells in bone marrow predicts adverse outcome in early breast cancer

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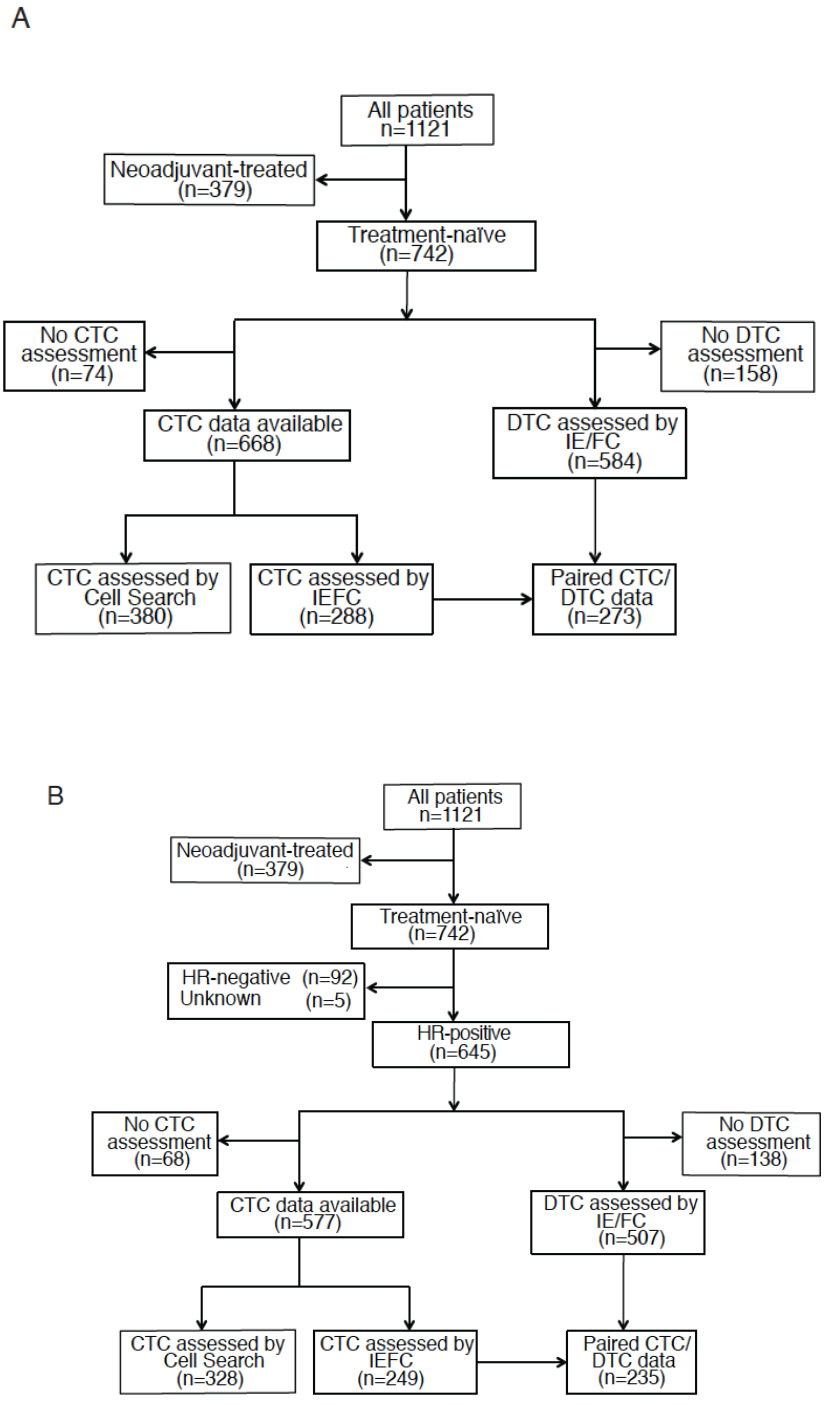
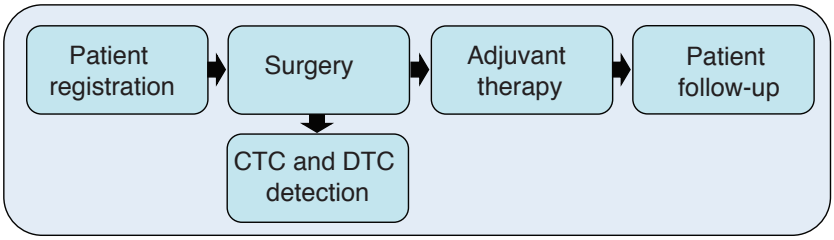


Supplementary Figure 1. Flow diagram of patients and samples. (A) All patients, (B) Hormone-receptor positive subset. CTCs in blood were enumerated either by CellSearch or by IE/FC. DTCs in bone marrow were enumerated by IE/FC.

A



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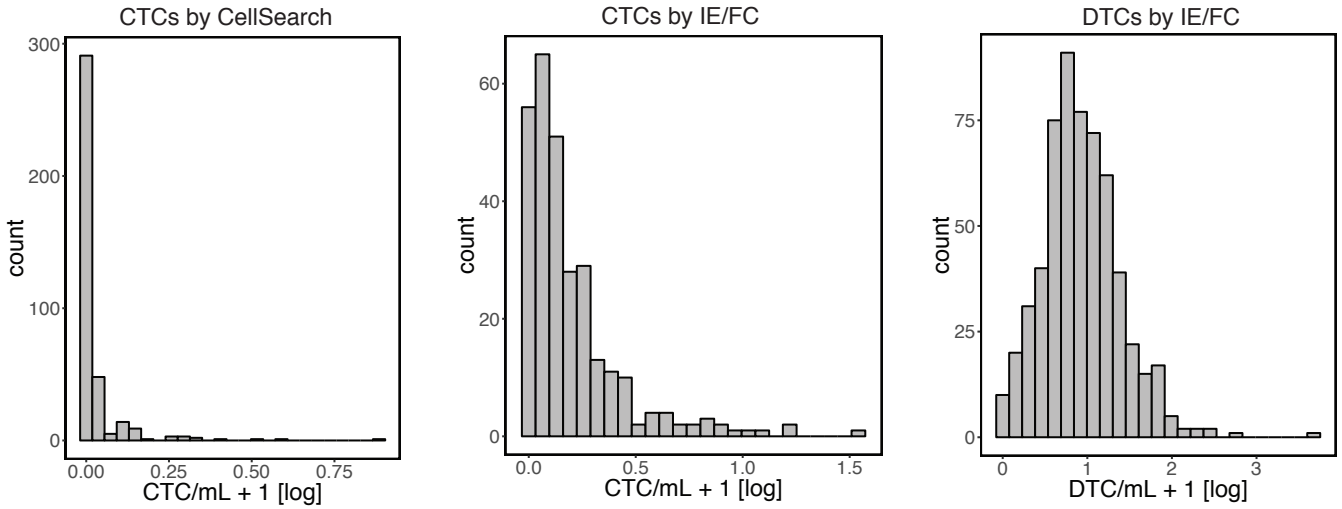
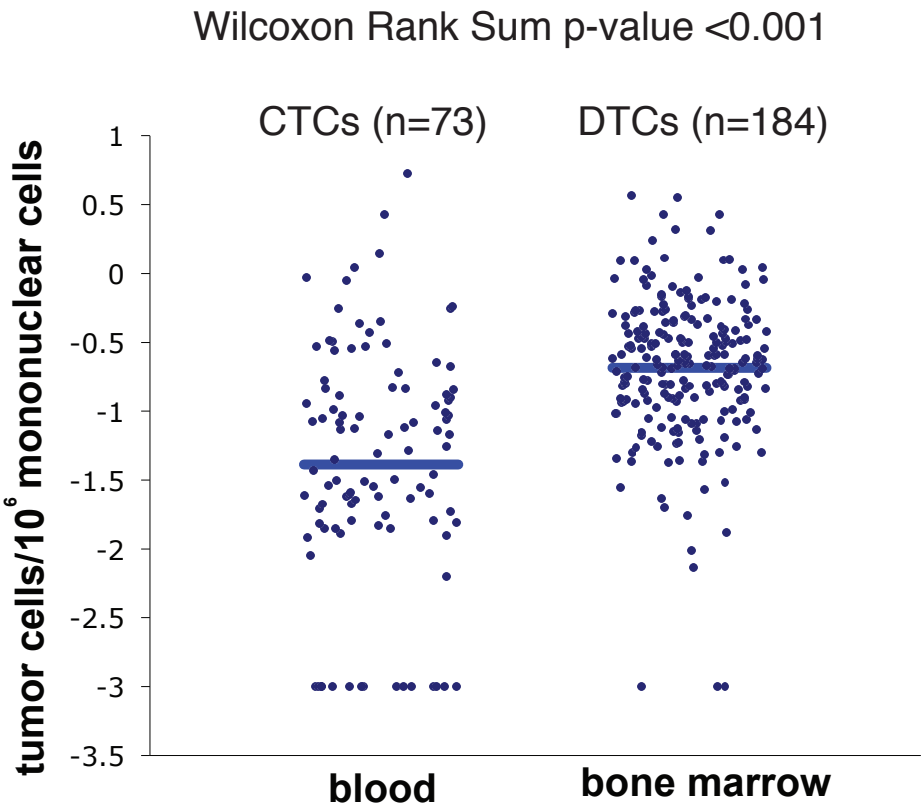
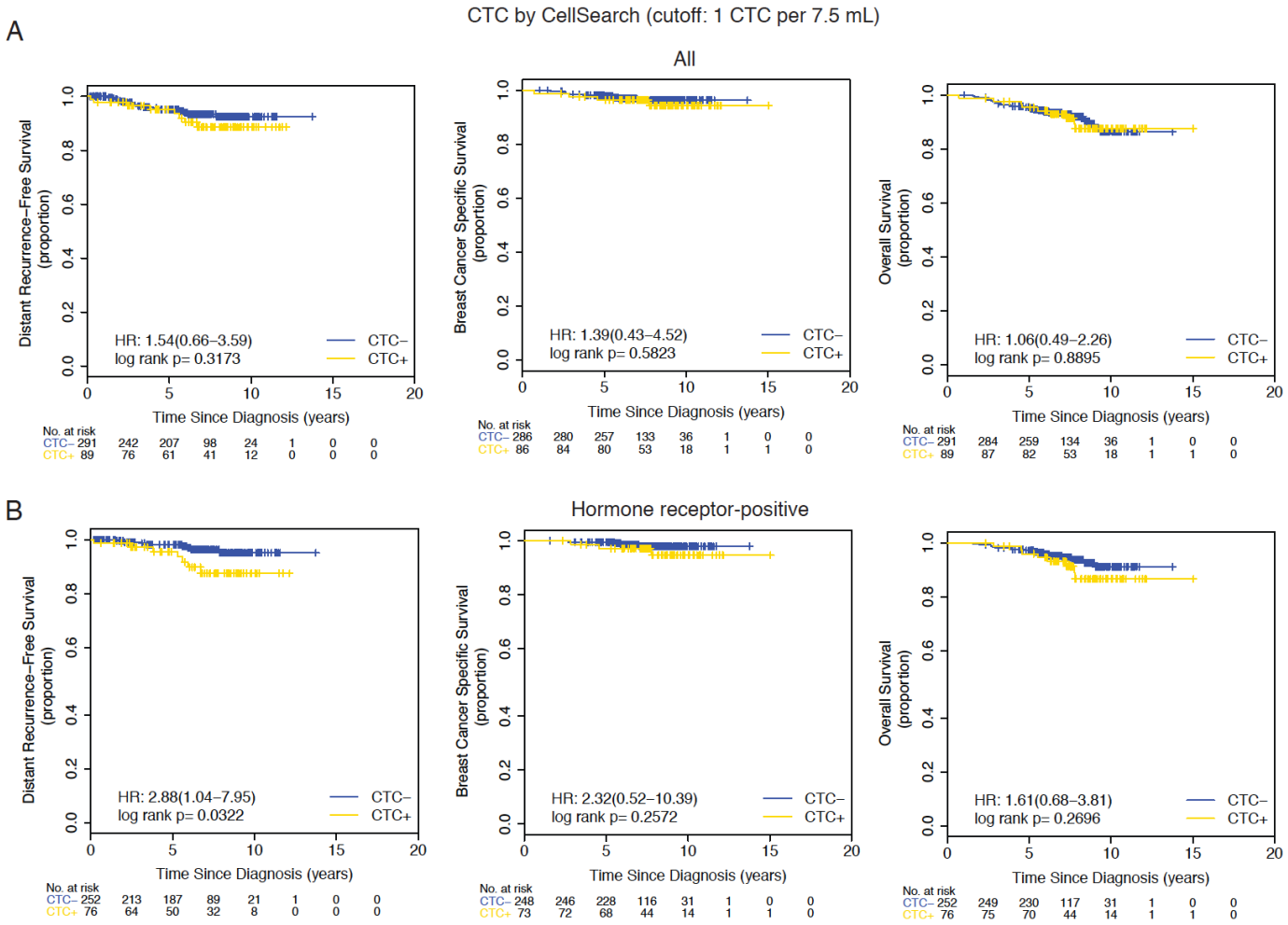


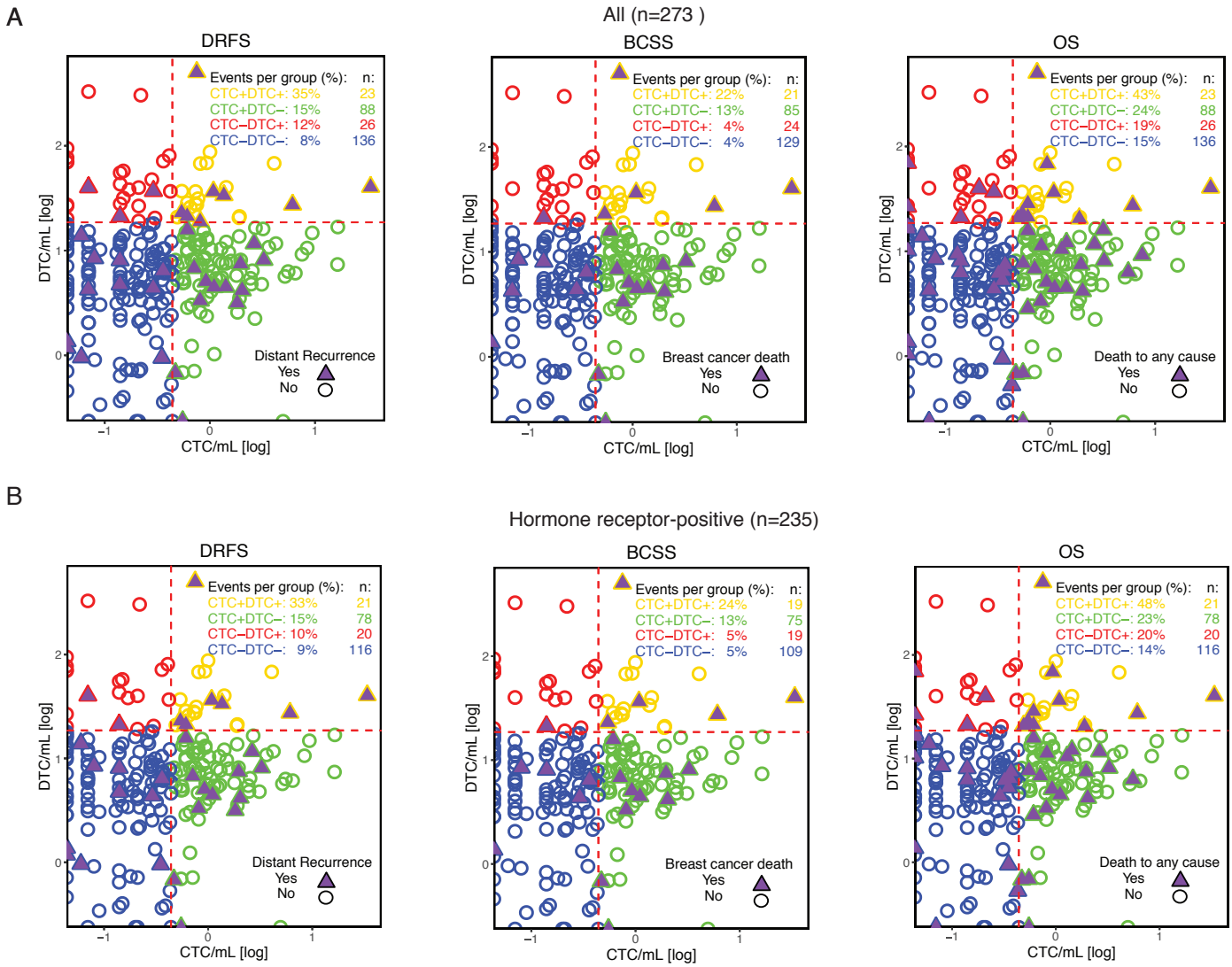
Supplementary Figure 2. Study schema and frequency distribution of CTCs and DTCs. (A) Blood and bone marrow samples were collected immediately prior to surgery and were analyzed for the presence of CTCs and DTCs, respectively. (B) Frequency distribution of CTC/mL and DTC/mL. For plotting purposes, "1" was added to each value and $\log_{10}$-transformation was performed.



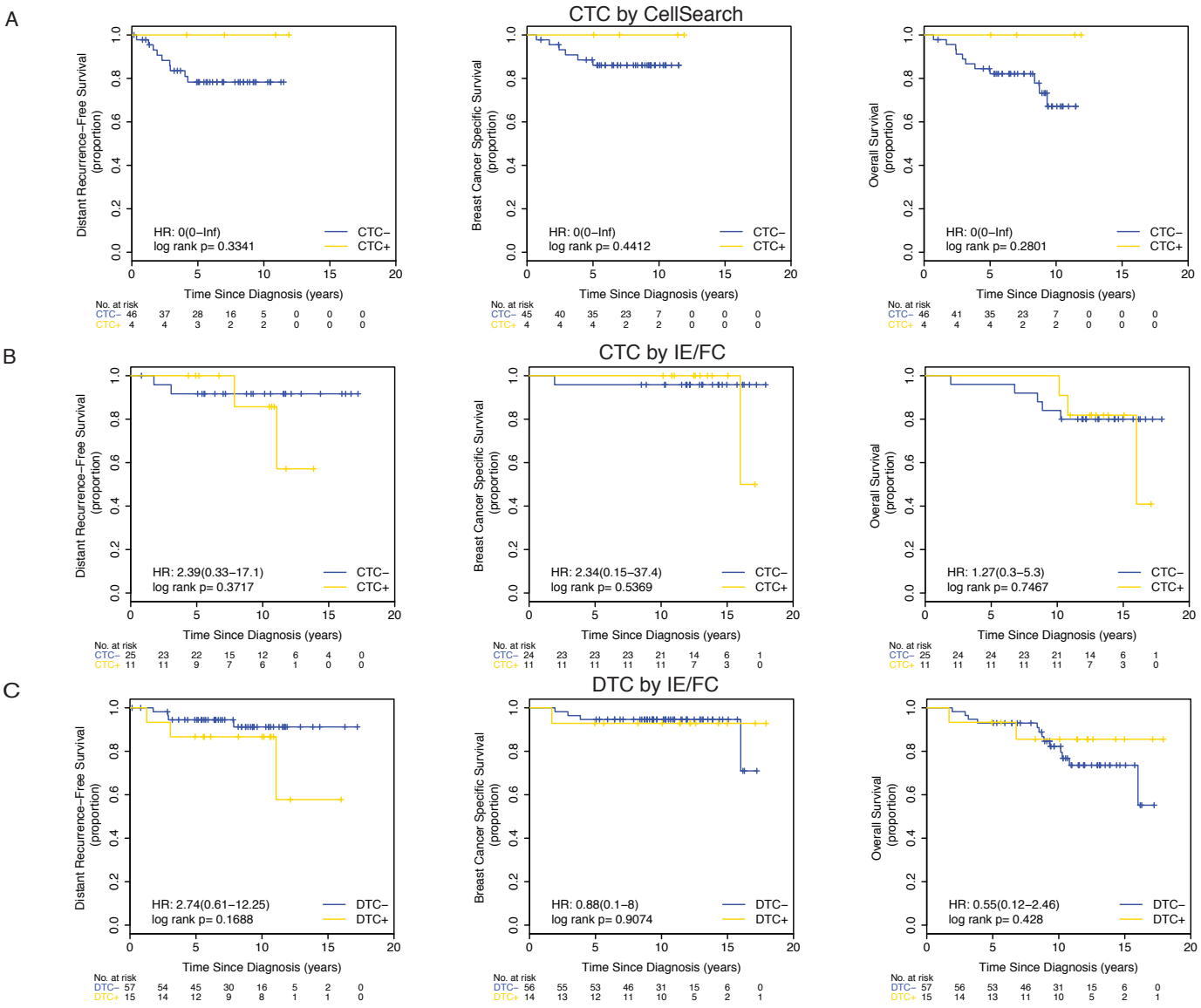
Supplementary Figure 3. Tumor cells per 10^6 mononuclear cells in blood (circulating tumor cells or CTCs) and bone marrow (disseminated tumor cells or DTCs). Plot showing log transformed values and median (horizontal line) for CTCs per 10^6 mononuclear cells in blood (n=73) and DTCs per 10^6 mononuclear cells in bone marrow (n=184). Values below the limit of detection were replaced with 0.001.



Supplementary Figure 4. Circulating tumor cells (CTCs) detected by CellSearch and patient outcome. (A). All patients , B) Hormone receptor-positive subset. Kaplan-Meier plots for distant recurrence-free survival (DRFS), breast cancer-specific survival (BCSS), and overall survival (OS) are shown. Dichotomization into CTC-positive and CTC-negative was based on the cutoff of ≥1 CTCs per 7.5 mLs of blood. Detection of CTCs by CellSearch in hormone receptor-positive, treatment-naïve patients with early breast cancer was significantly correlated with reduced DRFS (p=0.0322).



Supplementary Figure 5. CTC/DTC status and patient outcome. (A) All patients (n=273), (B) Hormone receptor-positive subset (n=235). Scatter plots showing patients' DTC per mL vs. CTC per mL and the percentage of events (distant recurrence or death) in each group. For plotting purposes, "1" was added to each cell/mL value and then $\log_{10}$-transformation was performed. The vertical red dashed line represents the cutoff >0.44 CTC per mL), while horizontal red dashed line represents the cutoff >18.61 DTC per mL. The survival endpoints include distant recurrence-free survival (DRFS), breast cancer-specific survival (BCSS), and overall survival (OS).



Supplementary Figure 6. Survival curves according to circulating tumor cell (CTC) and disseminated tumor cells (DTC) status using optimized cutoffs in hormone receptor-negative subset. Kaplan-Meier plots for shown for (A) CTCs by IE/FC (cutoff >0.44 CTC per mL), (B) CTCs detected by CellSearch (cutoff ≥2 CTC per 7.5 mLs), and (C) DTCs by IE/FC (cutoff >18.61 DTC per mL).

SUPPLEMENTARY TABLES

Supplementary Table 1. Detection of circulating tumor cells (CTC) in blood and disseminated tumor cells (DTCs) in bone marrow from early breast cancer patients.

	CTC		DTC
	CellSearch n=380	IE/FC n=288	IE/FC n=584
Cutoff (mean background levels + 2xSD) cells/mL	≥1*	>0.54	>4.16
% Positivity	23	38	68
Cutoff (optimized) cells/mL	≥2*	>0.44	>18.61
% Positivity	9	41	19
Range	0-6.67	0-33.74	0-4743.20
Standard deviation	0.42	2.74	198.55
Mean (cells/mL)	0.09	1.01	23.31
Wilcoxon rank-sumTest (cells/mL)			
p<0.001			
p<0.001			
p<0.001			

* based on literature; CTCs per 7.5 mL blood

Supplementary Table 2. Association between circulating tumor cell (CTC) and disseminated tumor cell (DTC) status vs. clinicopathologic variables. Numbers in bold are considered statistically significant.

Cell type	**CTC**					**CTC**					**DTC**				
Method	CellSearch					IE/FC					IE/FC				
Cutoff		≥1 CTC per 7.5 mLs		≥2 CTC per 7.5 mLs			>0.54 CTC per mL		>0.44 CTC per mL			>4.16 DTC per mL		>18.61 DTC per mL	
	Total	CTC+	p value*	CTC+	p value*	Total	CTC+	p value*	CTC+	p value*	Total	DTC+	p value*	DTC+	p value*
Sample size: n (%)	380 (100%)	89 (23%)		36 (9%)		288 (100%)	109 (38%)		116(40%)		584 (100%)	399 (68%)		109 (19%)	
Age			0.658		0.072			0.248		0.253			0.197		0.175
median (range)	53.5 (25-82)	54 (33-78)		56 (35-78)		53 (25-78)	54 (31-77)		53(31-77)		53 (25-82)	52 (25-81)		52 (31-78)	
Tumor size			0.735		0.238			0.070		**0.050**			0.491		0.808
median (range)	1.6 (0-15.2)	1.6 (0-15.2)		1.6 (0-15.2)		1.3 (0-24)	1.3 (0-24)		1.9 (0-24)		1.5 (0-24)	1.5 (0-24)		1.7 (0-24)	
Nodal status			0.780		1.000			0.699		1.000			0.692		0.348
Negative	268 (74%)	63 (75%)		24 (75%)		192 (67%)	75 (69%)		78(67%)		398 (70%)	272 (70%)		71 (66%)	
Positive	96 (26%)	21 (25%)		8 (25%)		93 (33%)	34 (31%)		38(33%)		168 (30%)	118 (30%)		36 (34%)	
HR status**			0.721		1.000			0.364		0.275			0.177		0.628
Negative	50 (13%)	13 (15%)		4 (11%)		36 (13%)	11 (10%)		11 (10%)		72 (12%)	44 (11%)		15 (14%)	
Positive	328 (87%)	76 (85%)		32 (89%)		249 (87%)	97 (90%)		104 (90%)		507 (88%)	351 (89%)		93 (86%)	
HER2 status			0.057		**0.011**			0.614		0.404			0.788		0.630
Negative	330 (90%)	72 (85%)		27 (77%)		230 (84%)	89 (86%)		96 (86%)		491 (87%)	331 (87%)		91 (86%)	
Positive	35 (10%)	13 (15%)		8 (23%)		44 (16%)	15 (14%)		15 (14%)		72 (13%)	50 (13%)		15 (14%)	
Grade			0.415		0.601			0.120		0.358			0.957		0.466
1	105 (29%)	21 (25%)		10 (29%)		105 (39%)	46 (46%)		46 (44%)		187 (34%)	127 (34%)		29 (28%)	
2	172 (48%)	40 (47%)		14 (41%)		106 (39%)	32 (32%)		36 (34%)		256 (46%)	175 (46%)		51 (50%)	
3	85 (23%)	24 (28%)		10 (29%)		59 (22%)	21 (21%)		23 (22%)		115 (21%)	77 (20%)		22 (22%)	
Pathological stage			0.273		0.176			0.306		0.254			0.205		0.422
0	12 (3%)	3 (3%)		0 (0%)		2 (1%)	0 (0%)		0 (0%)		5 (1%)	4 (1%)		1 (1%)	
1	201 (53%)	52 (59%)		24 (69%)		171 (60%)	63 (58%)		66 (57%)		335 (58%)	221 (56%)		59 (55%)	
2	138 (37%)	25 (28%)		8 (23%)		85 (30%)	30 (28%)		33 (29%)		189 (32%)	139 (35%)		34 (31%)	
3	26 (7%)	8 (9%)		3 (9%)		29 (10%)	15 (14%)		16 (14%)		53 (9%)	33 (8%)		14 (13%)	

* For continuous clinical variables (age and tumor size), association with DTC/CTC positivity was assessed using a t-test.
For categorical variables, association with DTC/CTC positivity was assessed using a Fisher's Exact test.
**HR- hormone receptor status (ER-postive or PR-positive)

Supplementary Table 3. Prognostic significance of standard clinicopathologic variables. Univariate Cox regression analysis was performed to estimate hazard ratio (HR) and 95% confidence interval (CI). Numbers in bold (Wald p <0.05) are considered statistically significant.

	DRFS		BCSS		OS	
Variable	HR [95% CI]	p-value	HR [95% CI]	p-value	HR [95% CI]	p-value
Age at Diagnosis	1.00 [0.98-1.03]	0.8438	1.00 [0.97-1.03]	0.7533	1.04 [1.02-1.06]	**0.0002**
Invasive Tumor Size	1.21 [1.12-1.31]	**>0.0001**	1.20 [1.12-1.30]	**>0.0001**	1.16 [1.08-1.24]	**>0.0001**
Node-pos vs Node-neg	3.40 [2.07-5.58]	**>0.0001**	4.03 [2.11-7.68]	**>0.0001**	2.19 [1.46-3.28]	**0.0002**
HR-pos vs. HR-neg	0.56 [0.30-1.02]	0.0592	0.57 [0.26-1.24]	0.1574	0.59 [0.36-0.97]	**0.0382**
HER2-pos vs. HER2-neg	1.93 [1.07-3.50]	**0.029**	1.98 [0.94-4.17]	0.0707	1.74 [1.05-2.88]	**0.0314**
Grade						
Grade 2 vs. Grade 1	1.98 [0.95-4.15]	0.0689	3.10 [1.03-9.35]	**0.0446**	1.22 [0.72-2.07]	0.4511
Grade 3 vs. Grade 1	4.71 [2.29-9.70]	**>0.0001**	8.78 [3.00-25.71]	**>0.0001**	2.64 [1.57 -4.45]	**0.0003**
Pathological stage						
Stage 2 vs. Stage 0/1	3.93 [2.30-6.72]	**>0.0001**	4.24 [2.11-8.49]	**>0.0001**	2.60 [1.72-3.91]	**>0.0001**

Supplementary Table 4. CTC and DTC detection and clinical outcomes. Univariate Cox regression analyses was performed to determine associations between CTC/DTC levels (as continuous and binary variables) and clinical outcomes: distant recurrence-free survival (DRFS), breast cancer-specific survival (BCSS), and overall survival (OS). Numbers in bold (Wald $p < 0.05$) were considered statistically significant.

					DRFS		BCCS		OS	
Cell type	**Method**	**Sample size**	**Cutoff Rationale**	**Cutoff**	**HR [95% CI]**	**p-value**	**HR [95% CI]**	**p-value**	**HR [95% CI]**	**p-value**
CTC	IE/FC	n=288		Continuous	1.20[1.00-1.44]	0.0534	1.25[1.05-1.48]	**0.0119**	1.17[0.99-1.39]	0.0651
			Mean levels in controls + 2 x SD	>0.54 cells/mL	1.96[1.03-3.74]	**0.0420**	2.73[1.20-6.24]	**0.0172**	1.63[0.98-2.72]	0.0616
			Optimized cutoff	>0.44 cells/mL	2.16[1.12-4.16]	**0.0220**	3.63[1.51-8.76]	**0.0041**	1.79[1.07-3.00]	**0.0255**
CTC	CellSearch	n=380		Continuous	1.11[0.92-1.34]	0.2763	1.15[0.94-1.41]	0.1606	1.08[0.88-1.32]	0.4647
			Literature	≥1 cells per 7.5 mLs	1.54[0.66-3.59]	0.3210	1.39[0.43-4.52]	0.5840	1.06[0.49-2.26]	0.8895
				≥2 cells per 7.5 mLs	3.12[1.24-7.87]	**0.0157**	2.74[0.75-9.95]	0.1265	1.48[0.57-3.82]	0.4196
DTC	IE/FC	n=584		Continuous	1.56[0.89-2.75]	0.1218	1.20[1.11-1.31]	**<0.0001**	1.19[1.09-1.29]	**<0.0001**
			Mean levels in controls + 2 x SD	>4.16 cells/mL	0.97[0.54-1.75]	0.9213	1.42[0.64-3.15]	0.3893	1.21[0.74-1.97]	0.4417
			Optimized cutoff	>18.61 cells/mL	1.77[0.97-3.22]	0.0634	1.48[0.67-3.29]	0.3350	1.32[0.79-2.20]	0.2890

Supplementary Table 5. CTC and DTC detection and clinical outcomes. Multivariate Cox regression analysis was performed to adjust for age at diagnosis, tumor size, nodal status, hormone receptor (HRS) and HER2 status, grade and pathological stage. The survival endpoints were recurrence-free survival (DRFS), breast cancer-specific survival (BCSS), and overall survival (OS). Numbers in bold (Wald $p < 0.05$) were considered statistically significant. The summary is presented in Table 2 in the main text. HR-hazard ratio, CI-confidence interval.

All patients	CTCs by IE/FC (n=288)						CTCs by CellSearch (n=380)						DTCs by IE/FC (n=584)					
	DRFS		**BCSS**		**OS**		**DRFS**		**BCSS**		**OS**		**DRFS**		**BCSS**		**OS**	
	HR [95% CI]	**p-value**	**HR [95% CI]**	**p-value**	**HR [95% CI]**	**p-value**	**HR [95% CI]**	**p-value**	**HR [95% CI]**	**p-value**	**HR [95% CI]**	**p-value**	**HR [95% CI]**	**p-value**	**HR [95% CI]**	**p-value**	**HR [95% CI]**	**p-value**
Positive vs. Negative	1.92 [0.93-3.95]	0.0759	3.55 [1.29-9.72]	**0.0138**	1.9 [1.06-3.39]	**0.0301**	4.93 [1.56-15.64]	**0.0067**	4.51 [0.76-26.56]	0.0962	1.63 [0.53-4.98]	0.3924	1.46 [0.75-2.81]	0.2631	1.48 [0.64-3.42]	0.3542	1.24 [0.71-2.15]	0.4491
Age at Diagnosis (continuous)	1.02 [0.98-1.06]	0.2751	1.02 [0.98-1.07]	0.3766	1.05 [1.02-1.08]	**0.0003**	1 [0.96-1.05]	0.8453	0.99 [0.93-1.06]	0.8114	1.04 [1-1.08]	**0.0385**	1.01 [0.98-1.04]	0.3632	1.02 [0.99-1.06]	0.2450	1.05 [1.03-1.08]	**>0.0001**
Tumor size (cm) at surgery (continuous)	1.11 [0.91-1.35]	0.3172	1.11 [0.87-1.41]	0.4166	0.97 [0.77-1.22]	0.7985	0.93 [0.73-1.2]	0.5942	1.17 [0.74-1.86]	0.5017	1 [0.78-1.27]	0.9765	1 [0.87-1.16]	0.9527	1.04 [0.88-1.23]	0.6599	0.98 [0.83-1.15]	0.7902
Stage II/III vs. Stage 0/I	2.85 [0.92-8.84]	0.0695	2.1 [0.47-9.42]	0.3305	2.32 [0.97-5.53]	0.0577	3.98 [1.2-13.17]	**0.0239**	2.75 [0.5-15.3]	0.2470	2.38 [0.9-6.29]	0.0802	2.96 [1.2-7.31]	0.0185	3.85 [1.15-12.89]	**0.0289**	2.53 [1.26-5.08]	**0.0088**
HRS+ vs. HRS-	2.45 [0.52-11.56]	0.2569	2.94 [0.34-25.48]	0.3273	1.14 [0.43-2.99]	0.7966	0.48 [0.17-1.39]	0.1755	0.42 [0.09-1.87]	0.2531	0.69 [0.25-1.93]	0.4855	2.31 [0.77-6.91]	0.1355	2.3 [0.64-8.28]	0.2020	1.29 [0.62-2.67]	0.4980
HER2+ vs. HER2-	1.73 [0.74-4.06]	0.2052	1.36 [0.49-3.79]	0.5522	1.74 [0.83-3.65]	0.1427	0.48 [0.12-1.88]	0.2887		0.9983	0.81 [0.26-2.54]	0.7168	1.36 [0.63-2.92]	0.4379	1.11 [0.43-2.88]	0.8298	1.44 [0.78-2.68]	0.2444
Node+ vs. Node-	1.8 [0.66-4.88]	0.2480	3.9 [1.01-15.09]	**0.0490**	1.82 [0.83-4]	0.1357	0.98 [0.37-2.61]	0.9751	0.36 [0.08-1.66]	0.1925	0.67 [0.27-1.65]	0.3867	1.74 [0.79-3.85]	0.1721	2.01 [0.75-5.36]	0.1623	1.44 [0.77-2.69]	0.2487
Grade 2 vs. Grade 1	1.75 [0.71-4.3]	0.2242	3.12 [0.9-10.89]	0.0741	1.28 [0.64-2.55]	0.4894	4.19 [0.5-34.87]	0.1849		0.9989	1.32 [0.41-4.26]	0.6432	1.32 [0.61-2.88]	0.4845	2.46 [0.79-7.72]	0.1223	1.32 [0.73-2.37]	0.3593
Grade 3 vs. Grade 1	2.08 [0.82-5.28]	0.1250	3.99 [1.12-14.16]	**0.0323**	1.65 [0.77-3.54]	0.1989	10.76 [1.24-93.34]	**0.0312**		0.9988	3.23 [0.86-12.11]	0.0827	2.2 [0.93-5.2]	0.0727	4.58 [1.35-15.56]	**0.0147**	1.99 [1-3.93]	**0.0489**

Hormone-receptor positive subset	CTCs by IE/FC (n=249)						CTCs by CellSearch (n=328)						DTCs by IE/FC (n=507)					
	DRFS		**BCSS**		**OS**		**DRFS**		**BCSS**		**OS**		**DRFS**		**BCSS**		**OS**	
	HR [95% CI]	**p-value**	**HR [95% CI]**	**p-value**	**HR [95% CI]**	**p-value**	**HR [95% CI]**	**p-value**	**HR [95% CI]**	**p-value**	**HR [95% CI]**	**p-value**	**HR [95% CI]**	**p-value**	**HR [95% CI]**	**p-value**	**HR [95% CI]**	**p-value**
Positive vs. Negative	1.75 [0.84-3.64]	0.1317	2.80 [1.02-7.69]	**0.0454**	1.72 [0.93-3.17]	0.0830	21.2 [4.25-105.3]	**0.0002**	9.94 [1.43-69.18]	**0.0204**	2.04 [0.62-6.73]	0.2398	1.40 [0.69-2.83]	0.3563	1.56 [0.64-3.83]	0.3263	1.46 [0.81-2.63]	0.2103
Age at Diagnosis (continuous)	1.04 [0.99-1.08]	0.0792	1.04 [0.99-1.10]	0.0989	1.06 [1.03-1.1]	**0.0006**	0.98 [0.92-1.04]	0.4619	1.01 [0.91-1.13]	0.8328	1.05 [1.01-1.10]	**0.0115**	1.02 [0.99-1.05]	0.1912	1.03 [0.99-1.08]	0.1003	1.05 [1.03-1.08]	**>0.0001**
Tumor size (cm) at surgery (continuous)	1.11 [0.91-1.36]	0.2843	1.14 [0.89-1.45]	0.3037	0.98 [0.78-1.23]	0.8516	0.74 [0.54-0.99]	**0.0494**	0.94 [0.5-1.77]	0.8438	0.97 [0.74-1.28]	0.8515	0.99 [0.86-1.15]	0.9298	1.02 [0.86-1.22]	0.8007	0.97 [0.83-1.15]	0.7523
Stage II/III vs. Stage 0/I	2.21 [0.63-7.81]	0.2166	1.06 [0.21-5.43]	0.9472	1.74 [0.61-4.99]	0.3027	63.3 [4.72-849.0]	**0.0017**		0.9988	4.21 [1.16-15.27]	**0.0289**	2.43 [0.9-6.58]	0.0795	2.34 [0.63-8.72]	0.2056	2.34 [1.05-5.22]	**0.0378**
HER2+ vs. HER2-	2.3 [0.98-5.45]	0.0571	1.64 [0.57-4.68]	0.3551	2.45 [1.13-5.3]	**0.0230**	1.31 [0.32-5.45]	0.7084		0.9995	1.19 [0.33-4.31]	0.7964	1.82 [0.84-3.97]	0.1313	1.51 [0.56-4.04]	0.4108	2.13 [1.1-4.13]	**0.0257**
Node+ vs. Node-	2.76 [0.88-8.67]	0.0818	8.17 [1.60-41.7]	**0.0115**	2.77 [1.06-7.25]	**0.0385**	0.47 [0.12-1.83]	0.2765	0.25 [0.04-1.72]	0.1599	0.93 [0.32-2.73]	0.8973	2.43 [0.99-5.99]	0.0535	3.51 [1.09-11.3]	**0.0354**	1.94 [0.95-3.97]	0.0708
Grade 2 vs. Grade 1	1.78 [0.72-4.43]	0.2148	3.13 [0.89-11.0]	0.0752	1.18 [0.57-2.43]	0.6613	6.09 [0.58-63.7]	0.1317		0.9993	1.16 [0.35-3.87]	0.8089	1.33 [0.61-2.91]	0.4728	2.48 [0.79-7.78]	0.1202	1.26 [0.69-2.29]	0.4539
Grade 3 vs. Grade 1	1.92 [0.74-4.96]	0.1783	3.92 [1.09-14.1]	**0.0369**	1.56 [0.71-3.44]	0.2734	28.9 [2.44-341.8]	**0.0076**		0.9992	1.93 [0.43-8.63]	0.3918	1.92 [0.8-4.63]	0.1471	4.07 [1.16-14.22]	**0.0280**	1.66 [0.8-3.44]	0.1755

Supplementary Table 6. Number of events according to hormone receptor status. The survival endpoints include distant recurrence-free survival (DRFS), breast cancer-specific survival (BCSS), and overall survival (OS).

		Hormone receptor-positive (n=645)	Hormone receptor-neg (n=92)
Events	**Endpoints**	n (%)	n (%)
Distant recurrence	*DRFS*	52 (8%)	13 (14%)
Breast cancer specific death	*BCSS*	32 (5%)	8 (9%)
Death (any cause)	*OS*	78 (12%)	19 (21%)

Supplementary Table 7. **Evaluating the clinical significance of circulating tumor cells (CTCs) and disseminated tumor cells (DTCs).** List of studies that contemporaneously assessed DTCs and CTCs and their corresponding results (see .xlsx file).