Supplementary Materials

Intra-tumor heterogeneity of the estrogen receptor and the long-term risk of fatal breast cancer

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SUPPLEMENTARY METHODS

The Stockholm Tamoxifen (STO-3) trial

From the original randomized trial cohort, 808 patients had formalin-fixed paraffin-embedded (FFPE) tissue blocks of primary breast cancer tumor available for molecular analyses, and of these, 81 patients were excluded because there was insufficient invasive tumor present for analysis, **Supplementary Figure 1**.

ER, PR, HER2, and Ki-67

Immunohistochemistry (IHC) was done for ER, progesterone receptor [PR], human epidermal growth factor receptor 2 [HER2], and Ki-67 using DAKO Link48 Autostainer. The antibodies used were: ER (SP1; Spring Bioscience M301), PR (PgR 636; DAKO IR068), HER2 (HercepTest; DAKO SK001), and Ki67 (MIB-1; DAKO M7240), with EnVision+ detection, following standard recommended procedures and with per-run positive controls assessed by quantitative image analysis to ensure consistent run-to-run staining intensity.

Prior to scoring, the pathologists were trained to recognize the boundary thresholds for staining intensity (0 versus +1; +1 versus +2; +2 versus +3) using a validated training set built as a computer based training and testing tool (1). The inter-rater reliability between the ATHENA pathologists that scored the slides in our study was assessed in a separate publication (Kappa value 0.8 for ER) (1). We computed the total percentage of cells stained positive for ER (at intensity levels +1, +2 and +3) and the ER H-score defined as the sum of the percent of ER-positive tumor cells at each intensity level multiplied by an ordinal value corresponding to the intensity level (0=none, 1=weak, 2=moderate, and 3=strong) (2, 3).

Intra-tumor heterogeneity of ER

For each patient, the intra-tumor heterogeneity of ER was calculated using Rao's quadratic entropy (QE, continuous score) (4, 5). Rao's quadratic entropy uses the

Simpson index (6) together with a distance matrix as weights to better quantify intra-tumor heterogeneity, which in our study is the staining intensity of ER within the tumor (see Equation 1A and 1B). The multiplied proportions as denoted “pi*pj” in Equation 1A are defined by the proportion of tumor cells positively stained for ER at intensity “i“ (0+, 1+, 2+, or 3+) multiplied by the proportion of tumor cells at intensity “j” (0+, 1+, 2+, or 3+). Furthermore, the distance matrix (d_{ij}) defines the weight for the difference in intensity “i” and “j” according to Equation 1B. For each tumor, the proportion of tumor cells positively stained for ER at each intensity are multiplied in pairs together with the weight from the distance matrix in Equation 1B according to Equation 1A. Noteworthy is that products from pairs with equal intensity, i.e. i=j, are by definition set to 0 due to a weight of 0. For instance, since the product when i=0+ and j=0+ is set to 0, the first product from Equation 1A would give us the proportion of tumor cells at intensity 1+ and at intensity 0+ multiplied together, then multiplied by the weight according to the distance matrix for i=1+ and j=0+, which is ‘1’. The weighted product estimates for each intensity i,j pairs were then summarized to obtain the QE continuous score for each patient.

Equation 1A

$$QE = \sum_{i=0}^{3}\sum_{j=0}^{3} d_{ij}\, p_i p_j$$

Equation 1B

$$\bar{D} = \begin{array}{c|cccc} & 3+ & 2+ & 1+ & 0+ \\ \hline 3+ & 0 & 1 & 2 & 3 \\ 2+ & 1 & 0 & 1 & 2 \\ 1+ & 2 & 1 & 0 & 1 \\ 0+ & 3 & 2 & 1 & 0 \end{array}$$

Four representative patient tumors according to low/ high intra-tumor heterogeneity of ER (predefined cut-off at the 2nd tertile (67%) for high intra-tumor heterogeneity) and ER H-score, respectively, are shown in **Supplementary Figure 2**. The images are captured at the same magnification.

Intrinsic subtypes (PAM50)

Tumors were assigned to one of five molecular subtypes (Luminal A, Luminal B, HER2-enriched, Basal-like, Normal-like) using the PAM50 classifier as described in

Parker et al (7). Specifically, we used log2-scaled upper quartile normalized expression data. We generated a subsample of our cohort balanced for ER status comprising all 113 ER-negative patients and a randomly selected 113 ER-positive patients to mirror the ER distribution in the PAM50 classifier training set. We computed the median of each gene across this subsample and adjusted the expression levels within each sample to this median. Data was mapped by gene symbol to the genes within the PAM50 classifier. Genes represented by multiple probes were collapsed by averaging, as per recommended for long oligo platforms.

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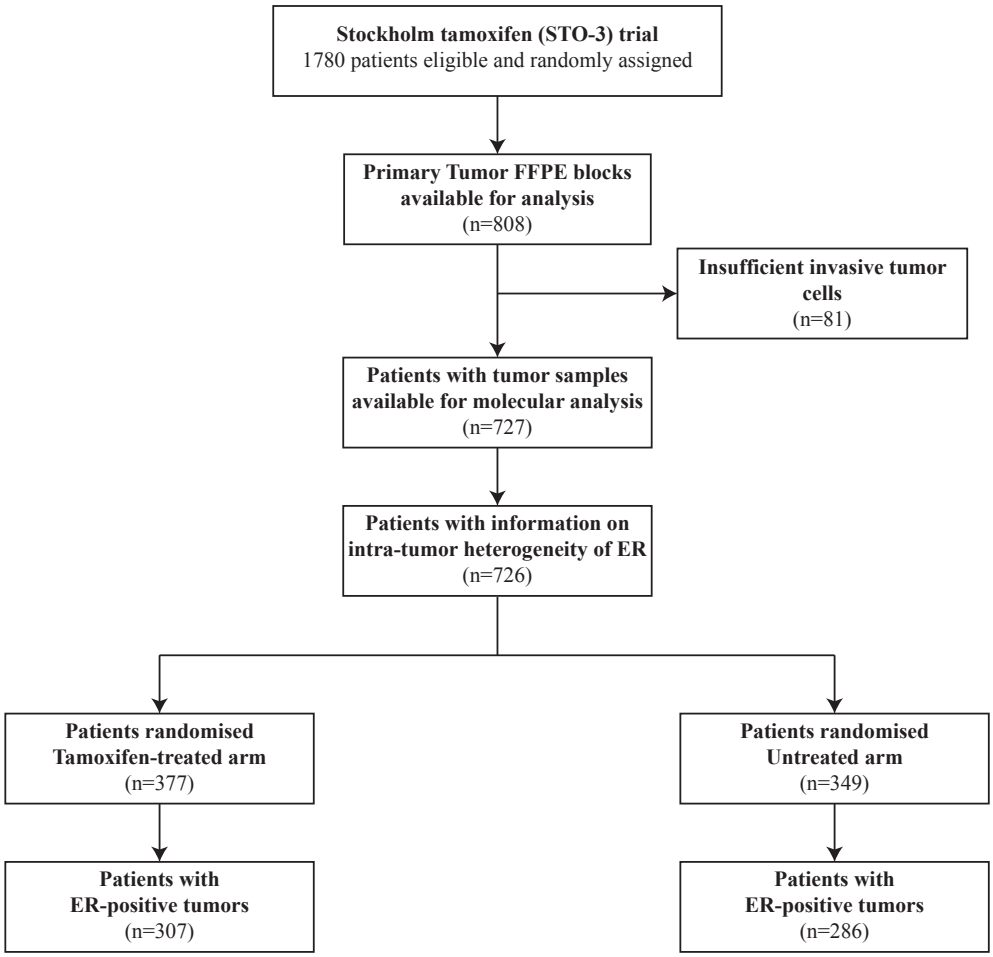
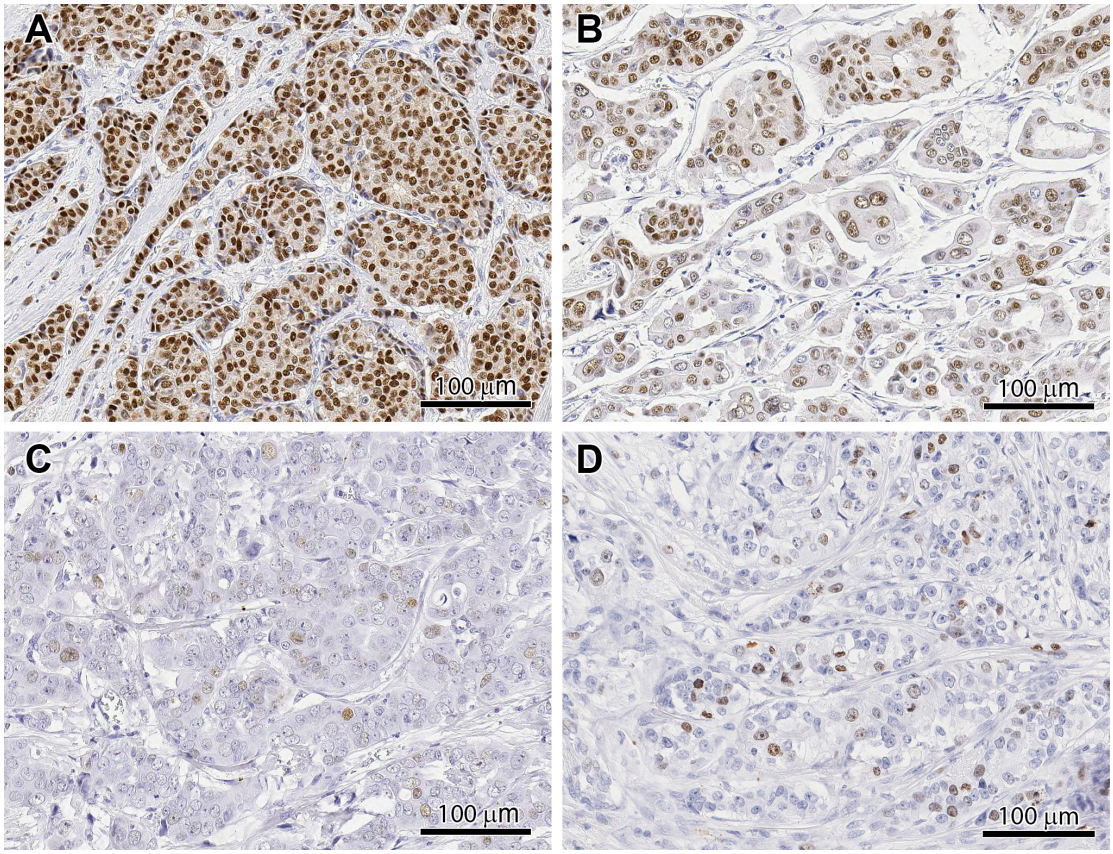
SUPPLEMENTARY FIGURES AND TABLES

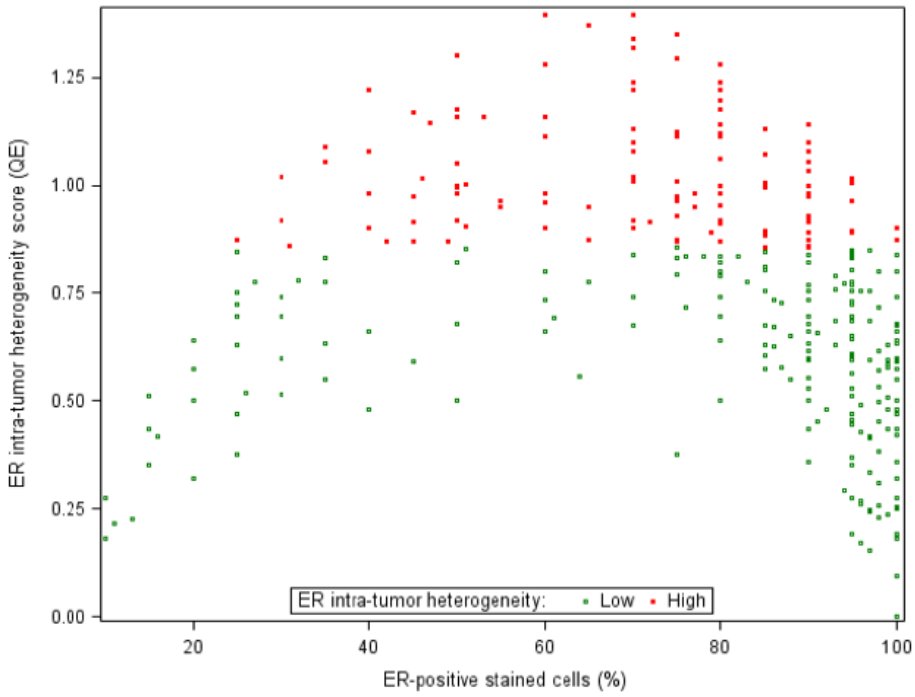


Supplementary Figure 1. Consort diagram for the Stockholm tamoxifen (STO-3) trial.

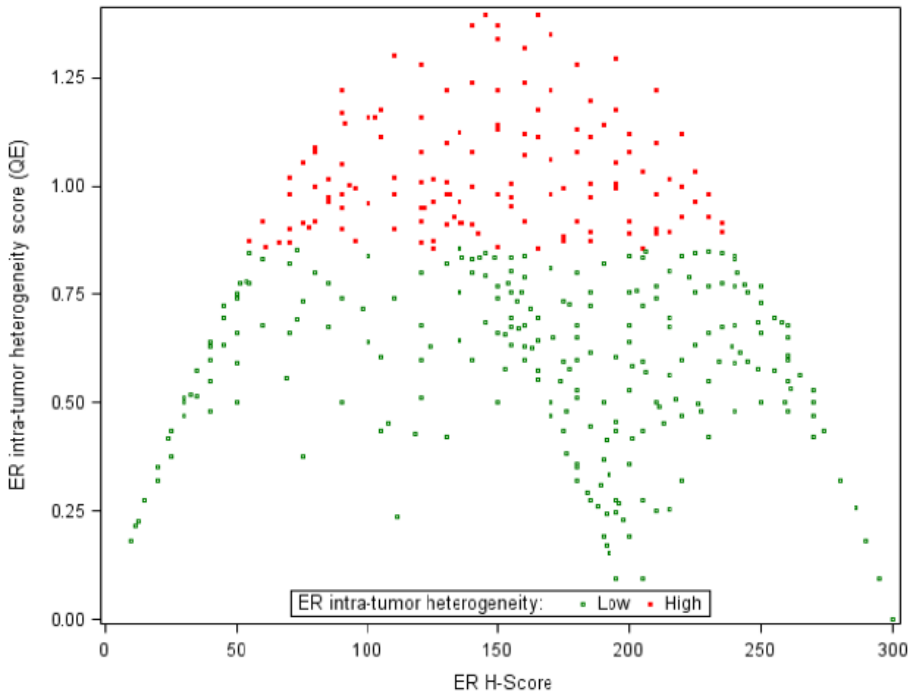


Supplementary Figure 2. Estrogen receptor (ER) immunohistochemistry in four representative patients (according to low/ high intra-tumor heterogeneity of ER and ER H-score, respectively). **A**) Low intra-tumor heterogeneity of ER and high ER H-score. **B**) High intra-tumor heterogeneity of ER and high ER H-score. **C**) Low intra-tumor heterogeneity of ER and low ER H-score. **D**) High intra-tumor heterogeneity of ER and low ER H-score. Scale bar=100 µm.

A.



B.



Supplementary Figure 3. Intra-tumor heterogeneity of ER (QE, continuous score) by percentage of ER-positive tumor cells, and the ER H-score. **A**) Intra-tumor heterogeneity of ER by percentage of ER-positive tumor cells. **B**) Intra-tumor heterogeneity of ER by the ER H-score defined as the sum of the percent of ER-positive tumor cells at each intensity levels multiplied by an ordinal value corresponding to the intensity level (0=none, 1=weak, 2=moderate, and 3=strong)

Supplementary Table 1. Risk of long-term breast cancer-specific death by intra-tumor heterogeneity of the estrogen receptor (ER) in ER-positive breast cancer – All covariate effects presented

STO-3 trial Patients included	COVARIATE		Breast cancer-specific survival*	
			Crude estimates adjusted for age and period of diagnosis	**Adjusted estimates** for patient and tumor characteristics
	Main	Reference	HR (95% CI)	HR (95% CI)
All patients	Intra-tumor heterogeneity High	Intra-tumor heterogeneity Low	**1.64 (1.11-2.44)**	**1.98 (1.31-3.00)** §
	Age at diagnosis 45-54	Age at diagnosis 65-73	1.27 (0.71-2.27)	1.24 (0.67-2.28) §
	Age at diagnosis 55-64	Age at diagnosis 65-73	0.69 (0.45-1.06)	0.81 (0.52-1.27) §
	Period at diagnosis 1976-79	Period at diagnosis 1985-90	1.58 (0.91-2.74)	1.48 (0.84-2.61) §
	Period at diagnosis 1980-84	Period at diagnosis 1985-90	1.49 (0.96-2.30)	1.34 (0.86-2.09) §
	Tamoxifen untreated arm	Tamoxifen treated arm		**2.36 (1.54-3.59)** §
	Tumor grade 1	Tumor grade 2		0.67 (0.36-1.26) §
	Tumor grade 3	Tumor grade 2		**1.98 (1.19-3.31)** §
	Tumor size < 20 mm	Tumor size ≥ 20 mm		**0.48 (0.31-0.76)** §
	ER positive stained cells	Continuous covariate		1.00 (0.98-1.02) §
	ER H-Score	Continuous covariate		1.00 (1.00-1.01) §
	PR status Negative	PR status Positive		1.47 (0.96-2.25) §
	HER2 status Positive	HER2 status Negative		1.10 (0.47-2.56) §
	Ki-67 status Positive	Ki-67 status Negative		1.36 (0.84-2.20) §
Tamoxifen treated arm	Intra-tumor heterogeneity High	Intra-tumor heterogeneity Low	1.94 (0.99-3.80)	**2.15 (1.07-4.34)** ¶
	Age at diagnosis 45-54	Age at diagnosis 65-73	1.97 (0.78-4.99)	2.09 (0.77-5.63) ¶
	Age at diagnosis 55-64	Age at diagnosis 65-73	0.71 (0.33-1.51)	0.77 (0.35-1.71) ¶
	Period at diagnosis 1976-79	Period at diagnosis 1985-90	**2.48 (1.04-5.93)**	2.52 (0.99-6.37) ¶
	Period at diagnosis 1980-84	Period at diagnosis 1985-90	1.71 (0.78-3.75)	1.32 (0.58-3.00) ¶
	Tumor grade 1	Tumor grade 2		0.51 (0.15-1.75) ¶
	Tumor grade 3	Tumor grade 2		1.99 (0.81-4.87) ¶
	Tumor size < 20 mm	Tumor size ≥ 20 mm		0.57 (0.25-1.29) ¶
	ER positive stained cells	Continuous covariate		1.03 (0.99-1.07) ¶
	ER H-Score	Continuous covariate		0.99 (0.98-1.01) ¶
	PR status Negative	PR status Positive		1.55 (0.78-3.09) ¶
	Ki-67 status Positive	Ki-67 status Negative		0.78 (0.30-2.02) ¶
Untreated arm	Intra-tumor heterogeneity High	Intra-tumor heterogeneity Low	1.52 (0.93-2.50)	**1.91 (1.12-3.27)** ¶
	Age at diagnosis 45-54	Age at diagnosis 65-73	0.85 (0.39-1.82)	0.85 (0.38-1.90) ¶
	Age at diagnosis 55-64	Age at diagnosis 65-73	0.68 (0.40-1.16)	0.79 (0.45-1.41) ¶
	Period at diagnosis 1976-79	Period at diagnosis 1985-90	1.10 (0.53-2.28)	1.07 (0.50-2.27) ¶
	Period at diagnosis 1980-84	Period at diagnosis 1985-90	1.31 (0.77-2.23)	1.17 (0.68-2.04) ¶
	Tumor grade 1	Tumor grade 2		0.68 (0.32-1.43) ¶
	Tumor grade 3	Tumor grade 2		**1.96 (1.05-3.64)** ¶
	Tumor size < 20 mm	Tumor size ≥ 20 mm		**0.47 (0.26-0.85)** ¶
	ER positive stained cells	Continuous covariate		0.98 (0.96-1.01) ¶
	ER H-Score	Continuous covariate		1.01 (1.00-1.02) ¶
	PR status Negative	PR status Positive		1.33 (0.77-2.32) ¶
	Ki-67 status Positive	Ki-67 status Negative		**1.82 (1.02-3.24)** ¶
Luminal A tumor subtype	Intra-tumor heterogeneity High	Intra-tumor heterogeneity Low	1.83 (0.99-3.39)	**2.43 (1.18-4.99)** ¶
	Age at diagnosis 45-54	Age at diagnosis 65-73	1.18 (0.46-3.04)	1.05 (0.39-2.84) ¶
	Age at diagnosis 55-64	Age at diagnosis 65-73	0.65 (0.33-1.28)	0.86 (0.42-1.79) ¶
	Period at diagnosis 1976-79	Period at diagnosis 1985-90	1.61 (0.69-3.75)	1.75 (0.71-4.32) ¶
	Period at diagnosis 1980-84	Period at diagnosis 1985-90	1.37 (0.68-2.76)	1.09 (0.52-2.30) ¶
	Tamoxifen untreated arm	Tamoxifen treated arm		**2.39 (1.23-4.65)** ¶
	Tumor grade 1	Tumor grade 2		0.71 (0.30-1.66) ¶
	Tumor grade 3	Tumor grade 2		1.34 (0.35-5.16) ¶
	Tumor size < 20 mm	Tumor size ≥ 20 mm		**0.29 (0.14-0.61)** ¶
	ER positive stained cells	Continuous covariate		1.01 (0.98-1.05) ¶
	ER H-Score	Continuous covariate		1.00 (0.99-1.01) ¶
	PR status Negative	PR status Positive		1.72 (0.87-3.39) ¶
	Ki-67 status Positive	Ki-67 status Negative		1.46 (0.53-4.04) ¶

* 25-year breast cancer-specific survival

§ Modeled by multivariable proportional hazard (Cox) analyses adjusting for treatment arm, age and calendar period of diagnosis, ER-positive stained cells, ER H-Score, progesterone receptor (PR) status, HER2 status, Ki-67 status, tumor grade, tumor size

¶ Modeled by multivariable proportional hazard (Cox) analyses adjusting for age and calendar period of diagnosis, ER-positive stained cells, ER H-Score, progesterone receptor (PR) status, Ki-67 status, tumor grade, tumor size. The Luminal A analysis was additionally adjusted for treatment arm