A stroma-related gene signature predicts resistance to epirubicin-containing neoadjuvant chemotherapy in breast cancer.


Supplementary information
SUPPLEMENTARY METHOD DESCRIPTION

PATIENT SELECTION (EORTC trial samples)

This study was performed in the context of a prospective phase III intergroup trial of neoadjuvant chemotherapy (EORTC 10994/BIG 00-01). Eligible patients had no evidence of metastatic disease, and had histologically confirmed large operable invasive tumor or locally advanced breast cancer. This sub-study was restricted to all cases evaluated at the EORTC data center on April 1st, 2005 meeting the following criteria: (1) estrogen receptor negative tumors defined as <10% of tumor cells stained positive for ER by immunohistochemistry of the pretreatment formalin-fixed biopsy; (2) patients who had completed the planned chemotherapy regimen with no major protocol violation; (3) non-T4 tumors; (4) good quality and >200 ng yield of RNA available from a pretreatment frozen biopsy. Ethical approval for the clinical trial and associated translational projects was obtained in all participating institutions. Patients gave signed informed consent for both the clinical and translational studies. Patients randomized to FEC received either six cycles of FEC 100, a European non-taxane regimen consisting of 500 mg m\(^{-2}\) 5-fluorouracil, 100 mg m\(^{-2}\) epirubicin, 500 mg m\(^{-2}\) cyclophosphamide, or six cycles of a modified (maximal cyclophosphamide dose/course 1200 mg/ m\(^{2}\), G-CSF given od 5-12) tailored and dose escalated FEC (Swedish patients\(^1\)). Clinical and pathological data are given in supplementary Tables 1 and 2. There were 28 pCR among 63 cases included in this study.

GENE EXPRESSION DATA GENERATION

Sample processing (EORTC trial samples)

Following completion of chemotherapy all patients underwent either tumorectomy or mastectomy. Pathological complete response (pCR) was defined as the disappearance of the invasive component of the primary tumor after treatment, with at most scattered tumor cells detected by the pathologist in the resection specimen. Analysis of pCR was performed locally in each center. All patients had one incisional or two trucut biopsies frozen before starting chemotherapy. Frozen sections of these biopsies were examined centrally by one pathologist (Dr V. Becette) and excluded if the tumor cell content was below 20%.

RNA was extracted from frozen sections as previously described\(^2\) and hybridized to Affymetrix X3P chips.

MIAME-compliant data of the EORTC 10994/BIG 00-01 trial were deposited in the Gene Expression Omnibus database (www.ncbi.nlm.nih.gov/geo) under accession number GSE4779.

Laser dissection microscopy and cell culture (colon cancer samples)

Fresh colon tumor tissue samples were collected from three patients, embedded in OCT, and frozen by immersion in dry ice/ethanol. Frozen sections (12 µm) were cut and mounted on membrane slides and stained with hematoxilin and eosin solution. RNA was prepared from three different sources. Epithelial and reactive stroma compartments were isolated using a laser dissecting microscope (PALM-Zeiss laser dissection microscope, Germany), coupled to a CCD camera. In addition, cell cultures of cancer-associated fibroblasts (CAF) were prepared, as described elsewhere\(^3\). Total
RNA of microdissected samples and cell cultures was extracted and analyzed using HU133 Plus 2.0 chips (Affymetrix, USA).

Microarray data processing
Raw data were processed with the statistical programming language R (cran.r-project.org), and Bioconductor packages (www.bioconductor.org). Gene expression was normalized with the robust multi-array (RMA) method implemented in the library affy. In the EORTC data only probesets that passed filtering criteria were used. Four selection criteria were applied to probesets: (1) A target sequence region of at least 56 nucleotides, (2) Annotation to a defined Entrez-gene, (3) A standard deviation of normalized expression superior to 0.5 and (4) For each Entrez-gene only the most variable probeset.

METAGENE CONSTRUCTION
The aim of the metagenes was to summarize the information contained in a cluster of functionally related genes (e.g. proliferation genes) into a single value that we refer to as metagene. For each of the nine functionally relevant gene clusters, a “representative gene” was chosen by human expertise. The representative gene is a gene regularly found grouped with functionally related genes when clustering breast cancer gene expression data. To identify groups of genes associated with each representative gene, a multiple linear regression model was used, with the gene expression levels of the representative genes as explanatory variables.

The data used were publicly available data from two studies, by van de Vijver (obtained from author’s web-site, Agilent platform, N=295, called NKI data) and by Wang (GEO database accession number GSE2034), Affymetrix platform, N=286, called EMC data). We used the 10317 genes that could be matched and aligned between these two platforms. Cross-platform mapping was performed by matching the Entrez-gene IDs. The aggregate of these two datasets is referred to as NKI-EMC dataset (N=581).

The multilinear regression model was fitted separately for each study and the associated t-statistics were combined using the fixed-effect (meta-analytical) method in order to correct for multiple testing. Supplementary Table 3 lists the t statistics and the corresponding p values for all genes and model coefficients. For all expression modules, the number of genes significantly associated (p < 0.05) with the representative gene was always greater than 50 genes. Each metagene was always computed as the average of the 50 genes with the highest positive meta-analytical t-statistics for the representative gene available in the study.

Stratified linear regression models provide the framework to allow easy adjustment for potential confounding effects and integration of data coming from different technological platforms. In all cases, the selection of the genes belonging to a module was performed using the external NKI-EMC data only; no pCR-npCR labels were used for the definition of the metagenes. Cross-platform mapping was performed by matching the Entrez-gene IDs. In case of redundancy, the most variable probe was chosen.
Gene Set Enrichment Assay (GSEA), validation of metagenes and interpretation of the stromal metagene

The ability of the linear model to identify groups of functionally related genes was verified by Gene Set Enrichment Analysis (GSEA; supplementary Fig. 1). GSEA is a non-parametric test that asks whether a predefined group of test genes lies near the top or bottom of a ranked gene list. Typically, the predefined list contains 10-100 genes and the ranked list contains all the genes on an array. A running total is kept, which increases every time a gene in the ranked list belongs to the test set, and decreases when it does not. To assess the significance of a GSEA score, it is normally compared with the empirical result obtained by randomly selecting genes in the test set or by resampling the order of the ranked list.

For each of the nine metagenes, a gene set known to be linked to the biological process we wished to test was selected from the MSigDB database (http://www.broad.mit.edu/gsea/, the genes are shown in supplementary table 5). GSEA was performed by ranking and weighting genes according to the t statistic from the linear model. Empirical p-values were obtained by randomly selecting the genes for inclusion in the predefined geneset 100,000 times.

In Fig. 4 and supplementary Fig. 7, GSEA was used to show that when we rank genes by similarity to decorin expression, the highest ranks are enriched for genes induced by TGF-b or Wnt pathway activation or by epithelial-mesenchymal transition (EMT). The test gene sets were selected from the MSigDB database and are shown in supplementary table 5.

PREDICTIVE MODELS FOR PATHOLOGICAL COMPLETE RESPONSE

For statistical analyses we used the statistical programming language R and its libraries (cran.r-project.org).

Estimation of prediction accuracy

The ability of the metagenes to classify the samples by their pCR status, was assessed with the area under the receiver operating characteristic curve (AUC) (Table 1). The 95% confidence intervals for the AUC were estimated by bootstrapping samples (1000 iterations). P-values were adjusted for multiple testing using the false discovery rate (FDR) method. While we used AUC as the only criterion for statistical significance, additional performance metrics such as sensitivity and specificity that depend on a cutoff were computed to further illustrate the observed characteristics of the predictor. The main results for these measures (Table 1c) were calculated at the cutoff with maximum Youden index for the EORTC dataset. We fitted a logistic regression trained on the mean-centered metagene and applied the same model and cutoff to the mean-centered metagene in the MDA dataset for (Table 1c). Full ROC curves are in supplementary Figure 2 along with plots showing PPV, NPV, sensitivity and specificity in function of the cutoff.
Multivariate model
Univariate and multivariate logistic regression (Table 2) was performed with the base statistic library in the R software. Statistical significance was assessed by Wald tests on the regression coefficients. All explanatory variables are binary and the coefficient is the log odds ratio between the two groups, when keeping other variables constant. Only the complete cases were used in the multivariate models.

Classifier based on variable selection by association with outcome
Initially we had applied classifiers with automatic feature selection and tested the prediction of pCR with cross-validation on the EORTC data. We used a few variants, for example classifiers built by selecting the 50 genes most strongly associated with pCR by a two-sample student t-test. A logistic regression for pCR was fitted on the training set using the average of the 50 genes as the predictive variable. Alternatively, the rank sum statistics was used for gene selection. The performance of the classifier was tested on the respective test set in three-fold cross-validation by pooling the predictions from the three test sets, so that each sample was classified once. The full cross-validation procedure was repeated 1000 times with different data partitions. The feature selection and predictive models were very unstable and no consistent predictive model was found.

External neoadjuvant dataset
The raw gene expression and tumour data from the study by Hess et al.\textsuperscript{10} (MDA data, Affymetrix platform) were obtained from the authors’ web page (http://www.bioinformatics.mdanderson.org/pubdata.html, MDA 133 project) and took the 51 tumors annotated as IHC-ER negative tumors (27 with pCR) in the clinical data file to test the response prediction of two selected classifiers under a very similar chemotherapy regimen. Data were normalized using RMA and only probesets with a standard variation greater than 0.5 were used.

Survival Analysis
Survival analysis was performed using the R library “survival”, significance was assessed with a log-rank test.

External survival analysis datasets
For the patient survival analysis, we used the cases without systemic treatment in the same two public datasets as for the metagene construction (NKI, EMC), and a third one (DUKE). The Duke University dataset is from the study by Bild et al.\textsuperscript{11}. It was obtained from the authors’ web page (http://data.cgt.duke.edu). A total of N=120 patients was used. Number of cases and events are for NKI (N=189, 81), for EMC NKI (N=286, 128), for Duke (N=120, 44). In each of these external datasets only probesets with a standard variation greater than 0.5 were used. When multiple probesets were mapped to the same Entrez-gene ID, only the most variable one was kept.
VARIATION OF THE METAGENE
In the definition of the metagenes, we fixed the representative gene and the total number of genes to be 50. A sensitivity analysis to test for the impact of varying the genes was performed. To do this, we ranked the genes by the strength of association in decreasing order of the t statistics. We then took consecutive non-overlapping groups of 15 genes from the top and computed their average (supplementary Fig. 3). In a second approach we exchanged the representative gene by another one from the original pool of 50 (supplementary Table 4).

BIOLOGICAL UNDERSTANDING OF THE STROMAL METAGENE

Intrinsic Analysis
Developed by the laboratory of Charles Perou, this approach identifies genes that are intrinsic to breast tumors. An “intrinsic” gene expression score is given by low gene expression variance between different physical samples of the same tumor compared to the variance between independent tumors. We performed the analysis in the same dataset as in Hu et al., composed of 26 breast tumor sample pairs and 86 additional independent tumor samples where the “between-subject variance” to “within- same tumor pair” variance was computed for every genes.

We have also permuted the sample labels to generate 26 random pairs and 86 non-paired samples to generated an empirical distribution of the mean score of the stromal metagene and to compare it to the value observed when no randomization is performed.

Epithelial vs Stroma Specificity
The analysis that compares gene expression in epithelial and stroma compartments was performed with two public breast cancer datasets and with existing unpublished colon cancer data introduced above. We took as indicator of expression specificity to the epithelial or stromal component, the log ratio of mean expressions in the epithelial fraction and in stroma or CAF fraction (Fig. 4f and supplementary Fig. 5).

In Fig. 4f, we used the breast cancer data from Boersma, where epithelial and reactive stroma fractions of breast tumors were obtained by laser micro-dissection and separately hybridized on Affymetrix HG-U133A chips. For consistency with our study, only the 13 non-inflammatory breast cancer (non-IBC) ER negative tumors were included that did not receive chemotherapy before the specimen was collected. In supplementary Fig. 5c, we used the breast cancer data of Finak, where the specificity of genes for epithelial versus stromal tumor components or cancer associated fibroblasts is analyzed with an unpublished dataset from colon specimens, described above.

The same six gene sets were used for Fig. 4f and supplementary Fig. 6.

Gene Lists used to characterize the stromal metagene
The reference epithelial (E) and stromal (I) gene lists (Fig. 4f, supplementary Fig. 5) were taken from table 2 of the Finak et al. study. P (proliferation) and S (stroma) are the metagenes described in this study. For the desmoid-type fibromatosis tumors (D) and mammospheres (M), we assembled signatures by taking the top 50 genes with...
the strongest association with the respective phenotype, as reported by the original authors.

**GSEA test (supplementary Fig. 7)**
This has been described above.

**Correlation between signatures**
Correlation between signatures was performed as follows. For each signature, the average expression of all genes included in the same signature was calculated for each sample of the EORTC dataset. Pearson correlation was then computed between pairs of tested signatures.
DEFINITION OF TERMS AS USED IN THIS WORK

FEC: 5-Fluorouracil, Epirubicin, Cyclophosphamide; this is a widely prescribed pre-operative chemotherapy regimen.

T-FAC: Chemotherapy regimen with Paclitaxel and 5-Fluorouracil, Doxorubicin and Cyclophosphamide.

pCR: Pathological complete response. Disappearance of the tumour after treatment, with at most scattered tumor cells detected by the pathologist in the resection specimen.

npCR: Non-pathological complete response.

MIAME: Minimum Information About a Microarray Experiment. An international standard for the annotation of microarray data.

Multiple Regression: Linear regression with two or more independent variables.

Gene Signature: A general term for an expression pattern associated with a particular state or outcome.

Prototype: A gene identified as a typical representative of a large, highly correlated cluster of genes. These cluster genes were observed to be made of functionally related genes that consistently cluster together regardless of the dataset being analyzed.

Expression Module: A group of genes significantly associated, in terms of similarity of expression, with that of the prototype gene.

Metagene: Average expression value of a subset of genes all belonging to the same expression module. Therefore a metagene is a "virtual" gene that summarizes the information contained in many real genes into a single value per sample.

HUGO name: The unique name given by the Human Genome Organisation to a gene. Use of HUGO names is encouraged in order to prevent confusion when, as is often the case, multiple different names have been used in the literature.

Sensitivity: In this study, the test’s sensitivity measures the ability of a classification function to predict pCR when it is truly present. Sensitivity is the proportion of all pCR for which there is a positive prediction, determined as the number of true positives divided by the sum of true positives and false negatives.

Specificity: In this study, the test’s specificity measures the ability of a classification function to predict the absence of pCR when a patient is truly npCR. Specificity is the proportion of npCR patients for which there is a correct prediction, expressed as the number of true negatives divided by the sum of true negatives and false positives.

ROC curve: Receiver Operating Characteristic (curve). A plot of sensitivity vs. (1-specificity) for a classification function. The AUC (area under the ROC curve) is a useful global measure for how well the two classes are separated, independent from a particular threshold.

PPV: Positive Predictive Value. The proportion of the correct decisions among the cases declared positive by a particular classification function and a particular threshold, that is the ratio of true positives to the number of positive calls.

NPV: Negative Predictive Value. The proportion of predicted npCR patients (negative tests) that are truly npCR.

Bootstrapping: Sampling with replacement from a set of data to produce simulated data sets and determine approximately the variability of a parameter estimate. The term "95% confidence interval of the AUC" is used here for the range of values bounded by the 2.5th and 97.5th percentiles of the bootstrap distribution of the AUC.

DTF: Desmoid-type fibromatosis. A form of fibroblastic tumor occurring in patients with germline mutations in the adenomatous polyposis coli gene (APC).

Multiple regression for the selection of expression modules:

\[ GENE_i = \beta_0 + \beta_{1i}ESR1 + \beta_{2i}CLCA2 + \beta_{3i}FABP4 + \beta_{4i}GZMA + \beta_{5i}CD83 + \beta_{6i}MX1 + \beta_{7i}DCN + \beta_{8i}ADM + \beta_{9i}TPX2 + \epsilon_i \]  

(\textit{sample's index are removed for clarity})

\( GENE_i \): the expression of gene \( i \) is an independent variable of the linear model. Its variation over the set of profiled tumors is decomposed into linear terms given by the prototype genes as explanatory variables.  
\( ESR1, CLCA2, FABP4, GZMA, CD83, MX1, DCN, ADM, TPX2 \): the expression vectors of the nine prototype genes.  
\( \beta_{0i} \): the intercept term for gene \( i \)  
\( \beta_{ji} \): the regression coefficient for prototype \( j \) and gene \( i \). It is a measure of the correlation between the expression vector of the genes \( j \) and \( i \), adjusted by the presence of the other explanatory variables in the model.  
\( \epsilon_i \): the unexplained variation (residual) term for gene \( i \).

**HUGO names of the prototypes** (the independent variables):  
**ESR1**: estrogen receptor \( \alpha \), classic marker that distinguishes ER+ and ER- breast cancer subtype  
**CLCA2**: chloride-activated calcium channel 2. CLCA2 is a marker that, conjointly with ESR1, distinguishes the molecular apocrine subtype postulated by Farmer et al.\(^2\) from the luminal and basal subtypes  
**FABP4**: fatty acid binding protein 4, a marker for adipocytes  
**GZMA**: granzyme A, a marker for T lymphocytes  
**CD83**: cluster of differentiation 83, a marker for B lymphocytes  
**MX1**: myxovirus resistance gene 1, a marker for interferon signaling  
**DCN**: decorin, a marker for stroma  
**ADM**: adrenomedullin, a marker for hypoxia  
**TPX2**: aurora kinase targeting subunit, a marker for proliferation.
References

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Supplementary Figures

P Farmer, H Bonnefoi, P Anderle, D Cameron, P Wirapati, V Becette, S André, M Piccart, M Campone, E Brain, G MacGrogan, T Petit, J Jassem, F Bibeau, E Blot, J Bogaerts, M Aguet, J Bergh, R Iggo and M Delorenzi
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Supplementary Figure 1. GSEA testing the expression modules derived from the linear model. The x-axis represents genes used for the linear model ranked according to the t-statistic for the coefficient of the indicated representative gene in the regression fit. The y-axis shows the normalized GSEA statistic (maximum possible score is 1) for the indicated gene sets, which correspond to the biological processes targeted by the representative genes in the linear model. The gene sets were selected from the MSigDB database (http://www.broad.mit.edu/gsea/). The p-values were obtained by replacing the genes in the selected geneset with randomly chosen genes 100,000 times.
Supplementary Figure 2. Prediction of pCR using the stromal metagene. (a,b) EORTC dataset. (c,d) MDA dataset. (a,c) ROC curves. (b,d) Performance metrics. The x-axis shows the cutoff used for calculation of the accuracy (ACC), sensitivity (SEN), specificity (SPE), positive and negative predictive values (PPV, NPV) and Youden index (YOU). The results in Table 1c were calculated at the maximum Youden index of the EORTC dataset.
Supplementary Figure 3. Metagene walk. The y-axis shows the AUC for prediction of response in the EORTC dataset. The x-axis shows a sequence of stromal metagenes, each dot corresponding to a different set of 15 genes. To create these metagenes, the genes used for the linear model were ranked by the t-statistic for the stromal representative gene (decorin), then grouped into sequential windows of 15 genes. Red dots are metagenes where all the genes in the metagene were significantly associated with the stromal representative gene in the linear model (p 0.05). The error bars show the 95% confidence intervals of an empirical distribution calculated by bootstrapping. When the lower boundary is over 0.5, the metagene predicts response better than chance at a p 0.05.
Supplementary Figure 4. Normal tissue content of the biopsies. (a) H.E staining of a breast tumor biopsy showing normal, non-malignant breast tissue (NT) and tumor tissue (T). Scale bar: 0.2 mm. (b) Correlation of normal tissue content with metagene expression. Tumors were divided into three groups by a pathologist, based on the amount of normal, non-malignant breast tissue. There was almost no difference in the means of the stromal metagene scores for the three groups and only non statistically significant difference (Wilcoxon test, α=0.05).
**Supplementary Figure 5.** Gene expression in microdissected tissue and cancer-associated fibroblasts. The analysis was performed as in Fig. 4f but using E/S ratios derived from different reference tissue. (a) Colon cancer epithelial versus reactive stromal tissue. (b) Colon cancer epithelial tissue versus colon cancer-associated fibroblasts. (c) Non-tumor epithelial versus stromal tissues Finak, 2006. Asterisks indicate significant differences between expression levels in E and S (sign test, p < 0.001).

**Supplementary Figure 6.** Stromal metagene and other signatures. Correlation of the stromal metagene with (a) mammosphere, (b) EMT and (c) SFT signatures. Each dot is a single tumour (pCR, red; non-pCR, blue). r = Pearson correlation.
Supplementary Figure 7. GSEA testing of Wnt and TGF–β functions. GSEA testing the rank of (a) Wnt and (b) TGF–β signature genes in the stromal gene list. The x-axis shows genes expressed by breast tumors ranked according to the t-statistic for the stromal representative gene (decorin) in the linear regression model. See also Supplementary Figure 1.
### Supplementary Table 1. Clinical characteristics

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**Abbreviations:**

NA: not assessable;

pCR: pathologic complete response.
LEGENDS FOR THE SUPPLEMENTARY TABLES

Supplementary Table 1. Clinical data. Characterisation of the 63 patients of the EORTC study included in this project by standard clinical variables; all are ER negative.

Supplementary Table 2. Pathological data. The PF numbers (column 9) correspond to the patient identifiers used in the study by Farmer et al.; The HB numbers (column 1) correspond to the patient identifiers used in the study by Bonnefoi et al.

Supplementary Table 3. Output of the linear regression model. The table lists the t-statistics and p-values generated by multiple regression in the NKI-EMC dataset using the “representative genes” shown in row 1 as independent variables and the genes in column 1 as dependent variables. Ranking by t-statistic identifies genes with similar expression to the representative gene, where positive t-statistics selects positively correlated representative and dependent genes only.

Supplementary Table 4. Varying the stromal representative gene. The top 50 genes selected by the linear model when decorin (DCN) was used as the stromal representative gene are shown in the first signature column. To check for overfitting, DCN was iteratively replaced in the model with each gene in the original stromal metagene. This generated 49 new stromal metagenes, which are listed in signature columns 2-50. The top row shows the stromal representative gene used in each new model. All 49 new signatures were tested for prediction of response to chemotherapy. The AUC and 95% confidence intervals for each metagene in the EORTC and MDA datasets are shown in green below each gene list. The bottom row shows the number of genes in the original metagene present in the new metagene.

Supplementary Table 5. Published gene signatures used in this study. Each column is a list of genes used in the comparison of gene expression in stromal and epithelial compartments (columns 1-4), characterization of our stromal metagene with the GSEA approach and/or correlation (columns 3-8), or for the validation of the nine metagenes (columns 9-17), as described in the main or supplementary text.

References