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Decreased expression of *ABAT* and *STC2* hallmarks ER-positive inflammatory breast cancer and endocrine therapy resistance in advanced disease

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ABSTRACT

Background: Patients with Estrogen Receptor α-positive (ER+) Inflammatory Breast Cancer (IBC) are less responsive to endocrine therapy compared with ER+ non-IBC (nIBC) patients. The study of ER+ IBC samples might reveal biomarkers for endocrine resistant breast cancer.
Materials & methods: Gene expression profiles of ER+ samples from 201 patients were explored for genes that discriminated between IBC and nIBC. Classifier genes were applied onto clinically annotated expression data from 947 patients with ER+ breast cancer and validated with RT-qPCR for 231 patients treated with first-line tamoxifen. Relationships with metastasis-free survival (MFS) and progression-free survival (PFS) following adjuvant and first-line endocrine treatment, respectively, were investigated using Cox regression analysis.
Results: A metagene of six genes including the genes encoding for 4-aminobutyrate aminotransferase (*ABAT*) and Stanniocalcin-2 (*STC2*) were identified to distinguish 22

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ABAT
STC2

ER+ IBC from 43 ER+ nIBC patients and remained discriminatory in an independent series of 136 patients. The metagene and two genes were not prognostic in 517 (neo)adjuvant untreated lymph node-negative ER+ nIBC breast cancer patients. Only *ABAT* was related to outcome in 250 patients treated with adjuvant tamoxifen. Three independent series of in total 411 patients with advanced disease showed increased metagene scores and decreased expression of *ABAT* and *STC2* to be correlated with poor first-line endocrine therapy outcome. The biomarkers remained predictive for first-line tamoxifen treatment outcome in multivariate analysis including traditional factors or published signatures. In an exploratory analysis, ABAT and STC2 protein expression levels had no relation with PFS after first-line tamoxifen.

Conclusions: This study utilized ER+ IBC to identify a metagene including *ABAT* and *STC2* as predictive biomarkers for endocrine therapy resistance.

1. Introduction

Breast cancer is the most prevalent form of cancer in women in the United States and Europe (Torres-Arzayus et al., 2010). The majority of patients with breast cancer bear tumors expressing detectable levels of the Estrogen Receptor (ER). For these patients, targeted therapies are available including strategies directed at the receptor itself, such as tamoxifen and fulvestrant. In addition, estrogen deprivation offers another therapeutic strategy that can be achieved by ovarian ablation, or LHRH analogs, in the premenopausal patient, or with aromatase inhibitors (AIs) in the postmenopausal setting. These therapies are highly effective; adjuvant endocrine therapy has been shown to reduce mortality from ER+ breast cancer to the same degree as adjuvant chemotherapy (Early Breast Cancer Trialists' Collaborative, G, 2005). Unfortunately, part of the patients with ER+ breast cancer show *de novo* resistance to endocrine therapy, whereas others initially benefit but ultimately relapse due to acquired endocrine resistance (Leary et al., 2010). Predicting, modulating and/or restoring endocrine responsiveness remain important clinical priorities for which molecular targets are urgently needed.

Inflammatory breast cancer (IBC) is a rare (~5%) but aggressive form of locally advanced breast cancer. At time of diagnosis, virtually all patients with IBC have lymph node metastases and 1/3 of the patients have metastases in distant organs. As a consequence, the prognosis for patients with IBC is dismal (Dawood et al., 2011; Dirix et al., 2006). Analysis of the Surveillance, Epidemiology and End Results (SEER)-database revealed that IBC is characterized by atypical clinicopathological features (Dawood et al., 2011), including frequent absence of ER protein expression (Hance et al., 2005). Our research group and others have shown that this IBC-specific clinicopathological profile is corroborated at the molecular level by a distinct gene expression profile (Bertucci et al., 2004; Van Laere et al., 2007a; Van Laere et al., 2005). Exploration of this gene expression profile led to the discovery of pronounced activation of the transcription factor NFkB in IBC (Lerebours et al., 2008; Van Laere et al., 2006) and more recently to the observation that TGFβ-signaling is repressed (Van Laere et al., 2008). Furthermore, we demonstrated that the IBC-specific expression profile harbors the molecular traits of aggressive tumor cell behavior in general (Van Laere et al., 2008), including stem cell biology (Van Laere et al., 2010). As such, we consider IBC, although occurring rarely, as a suitable example to elucidate mechanisms responsible for tumor cell dissemination, metastasis and drug resistance in breast cancer in general.

The majority (depending on the reference up to 66%) of patients with IBC lack ER protein expression, but ER+ tumor samples from patients with IBC exist. Clinically, patients with ER+ IBC are less responsive to endocrine treatment as compared to patients with other forms of ER+ breast cancer. In light of molecular heterogeneity and our previous results, we reasoned that studying ER+ IBC focusing on endocrine treatment response might provide new insights into molecular resistance mechanisms of endocrine therapy. In the current study, we evaluated expression profiles from patients with ER+ IBC and nIBC. The purpose of this study was 1) to identify differentially expressed genes between IBC and nIBC, 2) assess their accuracy to predict ER+ IBC, and 3) to define their relationship with endocrine therapy response in clinical samples. Discriminatory genes were identified by gene expression arrays, of which two genes remained deregulated in an independent series of ER+ samples between patients with and without IBC. When applied onto clinically annotated expression series from patients with ER+ breast cancer treated with endocrine therapy either in the adjuvant or advanced setting, decreased expression of these two genes were linked with poor responsiveness to endocrine therapy. These two genes when validated with quantitative real-time PCR for mRNA expression and with immunohistochemistry for protein expression, demonstrated predictive value only at the mRNA level.

2. Materials and methods

2.1. Study design and patient samples

The present study describes a retrospective analysis performed in accordance with the Code of Conduct of the

Federation of Medical Scientific Societies in the Netherlands, Belgium and France, and is reported following the REMARK recommendations (McShane et al., 2006). The local medical ethics committees have approved the study. Follow-up, tumor staging, and response to therapy was defined by standard International Union Against Cancer (Geneva, Switzerland) classification criteria (Hayward et al., 1978). Samples were recruited from the Translational Cancer Research Unit (TCRU, Antwerp, Belgium), the Institut Poali-Calmettes (IPC, Marseille France), the Erasmus Medical Center (EMC, Rotterdam, the Netherlands) and the Netherlands Cancer Institute (NKI, Amsterdam, the Netherlands). The ER-status of the tumors was established by immunohistochemistry (≥10% positive tumor cells) or EIA (≥10 fmol/mg protein) and together with additional clinicopathological characteristics have been described before for each of the series (Bekhouche et al., 2011; Desmedt et al., 2007; Jansen et al., 2013; Kok et al., 2009; Loi et al., 2008; Reijm et al., 2014; Schmidt et al., 2008; Van der Auwera et al., 2010; Van Laere et al., 2007b; Wang et al., 2005).

The mRNA-datasets used in this study are presented in Table 1 and includes ER+ IBC-subsets (**I**–**II**), endocrine treated subsets (**III**–**VI**), and untreated lymph node-negative (LNN) patients (**VII**–**IX**).

The discovery and test phase incorporated 2 patient series with ER+ IBC: a) a discovery series (**I**) of 65 samples from patients with and without ER+ IBC retrieved from the TCRU (E-MTAB-1006) and b) an independent test series (**II**) of samples from 136 patients with and without ER+ IBC that received adjuvant treatment, retrieved from IPC. Samples from patients with ER+ IBC in series I (N = 22) and series II (N = 39) were selected by strictly adhering to the consensus diagnostic criteria (Dawood et al., 2010).

To evaluate discovered genes for their relationship with endocrine treatment outcome, 4 additional data sets (**III**–**VI**) were incorporated of patients with ER+ breast cancer treated with endocrine therapy for primary and advanced disease: 3 data sets (**IV**–**VI**) of 411 metastatic breast cancer patients in total from EMC, NKI and TRCU; and 1 data set with primary breast cancers of 250 patients (**III**). The data set of 411 patients with advanced ER+ breast cancer treated with first-line therapy contained three subsets, one of 96 patients treated with tamoxifen (**IV**) and one of 84 patients treated with aromatase inhibitors (AIs) (**V**), and one of 231 patients treated with tamoxifen and profiled using RT-qPCR for dedicated genes (VI). The RT-qPCR data set was used as an independent validation series for the genome-wide expression series. All samples in these cohorts were classified according to the Recurrence Score (RS) (24), the Genomic Grade Index (GGI) (25). For each of these patient series tumor size and histological grade were recorded, in addition to age and menopausal status at start of therapy, dominant site of relapse and disease-free interval for the RT-qPCR data set. To assess the prognostic value of the discovered genes, we incorporated also 3 series (**VII-IX**) of ER+ tumors from 517 LNN breast cancer patients, who did not receive any type of adjuvant systemic therapy. Details regarding the application of the above classifiers are provided in the Supplementary data file, Tables A.1 and A.2.

The discovered predictive genes were also evaluated for their protein expression pattern in a tissue microarray including cores of ER-positive primary tumor specimens from a cohort of advanced breast cancer patients who have been treated with first-line tamoxifen previously described (Reijm et al., 2014). A subset of 110 ER-positive tumors were explored for their protein staining, i.e. the number of positive cells and the staining intensity, and a staining IHC-score was calculated to evaluate the relationship between IHC-score and progression-free survival.

2.2. *Methods*

2.2.1. *RNA isolation and (genome-wide) expression profiling*

RNA isolation for the samples retrieved from each of the participating centers (TCRU, EMC, NKI and IPC) and quality control was done as described before (Bekhouche et al., 2011; Jansen et al., 2005, 2013; Kok et al., 2009; Van Laere et al., 2007a). Genome-wide expression profiles were available from Affymetrix HGU133A or HGU133plus2 platforms (**I**–**III**, **VII**–**IX**) and 44k mRNA oligoarrays of Agilent Technologies (**IV**–**V**).

Expression analyses were verified by RT-qPCR (series **VI**) and were performed for the "IBC-like" genes (i.e. *ABAT*, *ADAMDEC1*, *CLEC7A*, *ETS1*, *ITK* and *STC2*) to discriminate between

Table 1 – Datasets. Detailed information on the datasets used in this study. The datasets are numbered as series I to IX and the subset provides information about its study subject (IBC, treatment outcome, clinical outcome). For each dataset the author and array reference are presented next to the array-platform.

Information of datasets used in this study

Series	Analysis	Subset	Reference	GEO/ArrayExpress	Platform	Total number of cases
I	IBC	Discovery	Van Laere et al.	E-MTAB-1006	Affymetrix	65
II		Validation	Bertucci et al.	NA	Affymetrix	136
III	Treatment Outcome	Tamoxifen Adjuvant	Loi et al.	GSE6532	Affymetrix	250
IV		Tamoxifen Advanced	Kok et al.	NA	Agilent	96
V		Aromatase Inhibitors	Jansen et al. (2013)	GSE41994	Agilent	84
VI		Tamoxifen Advanced	Jansen et al. (2007)	NA	qRT-PCR	231
VII	Clinical Outcome	LNN ER+, untreated	Wang et al.	GSE2034	Affymetrix	221
VIII		LNN ER+, untreated	Desmedt et al.	GSE7390	Affymetrix	134
IX		LNN ER+, untreated	Schmidt et al.	GSE11121	Affymetrix	162

IBC and nIBC, for the Recurrence Score genes (i.e. *AURKA*, *BAG1*, *BCL2*, *BIRC5*, *CCNB1*, *CD68*, *CTSL2*, *ERBB2*, *ESR1*, *GRB7*, *GSTM1*, *MKI67*, *MMP11*, *MYBL2*, *PGR* and *SCUBE2*) and for a panel of reference genes (i.e. *HMBS*, *HPRT1*, *TBP* and *B2M*). Assay details are provided in Supplementary Table A.1. The cDNA synthesis, quantification and the methodology to ensure PCR specificity have been described previously (Sieuwerts et al., 2005; van Agthoven et al., 2009). RT-qPCR was performed in a Mx3000P™ Real-Time PCR System (Agilent, Amsterdam, The Netherlands) using the TaqMan-based gene expression assays from Applied Biosystems/Life Technologies and SYBR-based intron-spanning forward and reverse primer combinations for the other genes. Levels of the target genes, expressed relative to the reference genes were quantified as follows: mRNA target $= 2^{(\text{mean Ct reference genes}-\text{mean Ct target genes})}$.

Expression levels of each series (Supplementary Table A.2) were normalized and subsequently harmonized for cross-platform evaluation and robust regression analyses. To accomplish harmonization of series, Hampel'S M-Estimators were calculated in SPSS (version 20) for all series and applied to establish the harmonization factor for the genes in each series when using series I as reference (Supplementary Table A.3).

2.2.2. *Comparative analysis or ER+ IBC and nIBC expression profiles*

Global differences in gene expression between samples from patients with and without ER+ IBC in the discovery series were analyzed using the global test (Goeman et al., 2004) and Principal Component Analysis (PCA). Using the PAM50-algorithm, each sample in the discovery series was classified according to the molecular subtypes, ER activity, and Risk-Of-Relapse (ROR) models based on the molecular subtypes alone (ROR-S) or in combination with cell proliferation (ROR-P), as described before (Ellis et al., 2011). In addition, the Recurrence Score (RS) (Paik et al., 2004) and the *HOXB13/IL17RB* gene expression ratio (Jansen et al., 2007; Ma et al., 2006) were calculated.

2.2.3. *Biomarker discovery analysis*

We performed Prediction Analysis of Microarrays (PAM) (Tibshirani et al., 2002) to identify a series of biomarkers able to discriminate ER+ IBC from ER+ nIBC samples. The discovery series were randomly divided into training sets of 40 samples and test sets of 25 samples to obtain 10 gene signatures, which were compared to identify common classifiers. In total six classifier genes were shared between these 10 gene signatures, i.e. *ABAT, ADAMDEC1*, *CLEC7A*, *ETS1*, *ITK* and *STC2*. PCA was performed onto the ER+ IBC discovery and validation series to evaluate the discriminatory performance of all genes and of the six common classifier genes together. Mutual relationships between these common classifier genes and ER were investigated using the Ingenuity Pathway Analysis (IPA) software.

2.2.4. *Construction of an ER+ IBC-like metagene*

The thus identified six genes *ABAT, ADAMDEC1*, *CLEC7A*, *ETS1*, *ITK* and *STC2* were combined into an ER+ IBC-like metagene. The regression coefficients of each of the genes obtained within the discovery series were used to calculate a score for the metagene in all other series. This metagene score was evaluated as biomarker representing the signature of above six genes.

2.2.5. *Diagnostic evaluation of biomarkers and classifiers*

The IBC discovery and test sets (series **I** & **II**) were used to assess the predictive potential of the biomarkers to identify IBC and nIBC. The biomarkers were evaluated as continuous variables with Receiver Operator Characteristic (ROC) analyses using the STATA statistical package. The ROC analyses were performed to define Area Under Curves (AUC) and assess the discriminatory potential of the biomarkers. Next, ROC analyses were used to select cutoffs with optimal sensitivity and specificity. These cutoffs generated dichotomized biomarkers which were subsequently explored as classifiers using distribution dot-plots created in STATA and evaluated for their diagnostic effectiveness by SISA (http://www.quantitative-skills.com/sisa/). The distribution dot-plots illustrated the performance and the number of false positives/negatives when applying the cutoffs. The SISA tool established for the classifiers their accuracy, sensitivity, specificity and Youden's Index of the predictions.

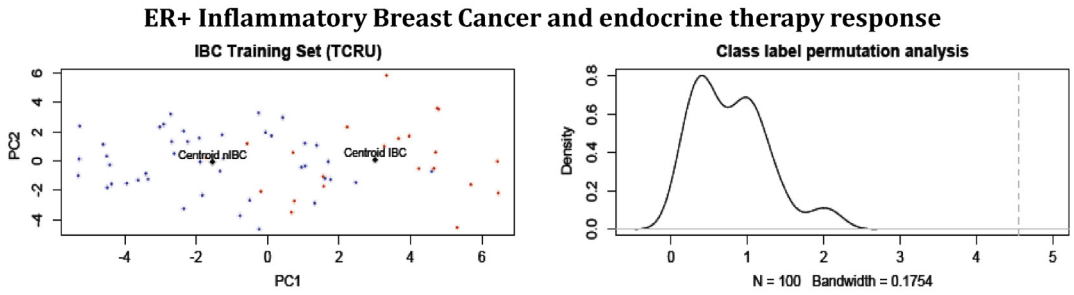
2.2.6. *Survival analysis of biomarkers and classifiers*

The biomarkers were evaluated for their relationship with survival using Cox regression analyses in two ways: as a continuous variable or dichotomized to a threshold as classifier to distinguish "IBC-like" and "nIBC-like". For the assessment of the relationship with first-line therapy outcome in advanced disease, Progression-Free Survival (PFS), defined as the time elapsed between initiation of endocrine therapy and the first detection of disease progression, was considered as endpoint. PFS was censored at 36 months. For the assessment of the relationship with prognosis and adjuvant therapy in early disease, Metastasis-Free Survival (MFS) was used as endpoint and defined as the time elapsed between the date of diagnosis and the date of distant metastatic relapse. Multivariate Cox regression analyses were performed on each of the endocrine treated advanced disease subsets for PFS (series **IV**–**VI**). The models included the biomarkers as continuous variable on the one hand and the published signatures for the Recurrence Score or the GGI on the other hand. Additional multivariate analyses were performed with the base model of clinic-pathological factors including age and menopausal status at start of therapy, dominant site of relapse, disease free interval (DFI), and the mRNA expression levels of ER (*ESR1*), PR (*PGR*), and HER2 (*ERBB2*). All data computations were done with the STATA statistical package version 12.0. All P-values are two-sided and $P < 0.05$ was considered statistically significant.

2.2.7. *Tissue microarrays and immunohistological and evaluation*

Tissue microarrays (TMAs) of formalin-fixed, paraffin-embedded primary breast tumor specimens were prepared and immunohistochemically stained according to the procedures described previously (Reijm et al., 2014). The staining was performed with the primary monoclonal antibody against ABAT (HPA041690) and STC2 (HPA045372; Atlas Antibodies AB,

A



B

Factor	IBC	nIBC	P-value
PAM50: Luminal A	13%	51%	0.002
PAM50: ER activity	-0.46	0.64	0.001
PAM50: ROR-S	47.90	29.25	0.007
PAM50: ROR-P	47.31	28.85	0.017
Recurrence Score	39.81	19.00	0.002
HOXB13/IL17RB	0.67	0.49	0.047

C

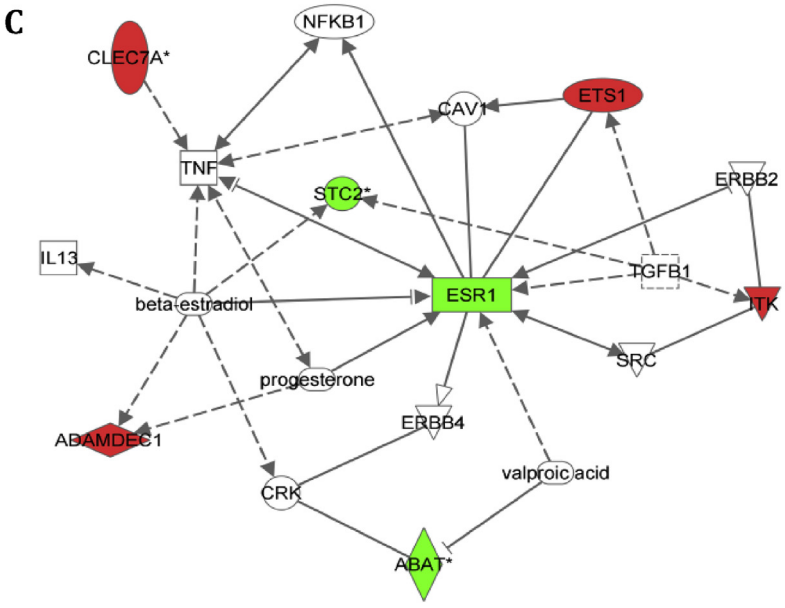


Figure 1 – IBC discriminatory genes. Evaluation of IBC and nIBC in the discovery series I (Figure 1A–B) and of ER+ IBC discriminatory genes (*ABAT*, *ADAMDEC1*, *CLEC7A*, *ETS1*, *ITK*, *STC2*) (Figure 1C). Figure 1A illustrates the Principle Component Analyses of the tumor samples by their gene expression profiles. Red dots denote ER+ IBC samples, blue dots denote ER+ nIBC samples. The centroids for both tumor phenotypes are indicated in black and labeled respectively "Centroid IBC" and "Centroid nIBC". PCA for the common 6 classifier genes showed an expected segregation of ER+ samples from patients with and without IBC on the 2D scatter plot representation of the 1st (X-axis) and the 2nd (Y-axis) principal component. Class label permutation analyses (applying 100 class label permutations) demonstrated that the centroids of the ER+ samples from patients with and without IBC are significantly segregated (Observed Euclidean distance = 4.555, average expected Euclidean distance = 0.890; $P < 0.010$). Figure 1B presents the results on PAM50 analyses, Recurrence score, and HOXB13/IL17RB. For PAM50, the percentage Luminal A-type tumors in IBC and nIBC is provided in addition to the ROR-S, ROR-P and ER activity scores. The ER activity score ranges from negative to positive, with negative values indicating repressed ER activity. In addition, the RS and the HOXB13/IL17RB gene expression ratio are provided for both tumor types. The reported P-values result from the comparison of the IBC and nIBC groups with respect to these variables. Figure 1C depicts the network obtained for the 6 IBC discriminatory genes together with the estrogen receptor-α (ESR1) when evaluated with Ingenuity Pathway Analyses. This exploratory analysis revealed interactions with hormone receptor signaling, inflammation, cell survival, epidermal growth factor signaling, stem cell signaling and TGFβ signaling, indicating a potential involvement for each of these biological features in endocrine resistance. The molecules are color-coded red if the corresponding gene is overexpressed in ER+ IBC samples and green if the corresponding gene is repressed in ER+ IBC samples. Uncolored nodes are added by the software. Solid lines signify direct gene–gene interactions, whereas broken lines represent indirect relationships that may require secondary effectors not depicted in the network. All connections are supported by at least one published report or from canonical information stored in the Ingenuity Pathway Knowledge Base.

Stockholm, Sweden). The antibodies were incubated for 1 h (1:100 dilution) after 20 min antigen retrieval at pH6.0. Subsequently, the TMA-slides were incubated with a secondary antibody and staining was visualized using diaminobenzidine (DAB). ABAT and STC2 protein staining was scored for quantity and intensity. Staining was grouped into standardized categories for the percentage of staining positive cells (0%, 1–20%, 21–50%, 51–75%, or 76–100% of positive cells) and for staining intensity (negative, weak, moderate, strong). Subsequently, the scores for quantity and intensity were multiplied to generate a staining IHC-score. Based on the IHC-score, a specimen was classified as negative or positive for ABAT and STC2 protein expression.

3. Results

3.1. *Comparative analysis of ER+ IBC and nIBC expression profiles*

Using global test analysis and PCA, we observed that ER+ samples from patients with and without IBC exhibited significant differences in their expression profiles that led to segregation of IBC and nIBC samples in the PCA plot of the discovery series (Figure 1A). Evaluation of the Recurrence Score, the HOXB13/IL17RB expression ratio and PAM50-derived scores for molecular subtypes, ER activity, risk of relapse models (ROR-S and ROR-P), indicated decreased ER signaling and sensitivity to endocrine treatment for ER+ samples from IBCs compared to those of nIBCs (Figure 1B).

3.2. *Biomarker discovery analysis*

As shown above, ER+ IBC samples exhibited molecular characteristics of resistance to endocrine therapy, making their gene expression profiles a potential source for biomarker discovery. Using PAM on 10 alternatively composed training sets of 40 randomly selected ER+ samples from the discovery set (series **I**), we generated 10 distinct gene signatures distinguishing IBC from nIBC samples. Application of these gene signatures onto corresponding series of the 25 left-out samples revealed an average sensitivity of 89% (range 71%–100%), specificity of 80% (range 67%–100%) and test error rate of 18% (range 0%–28%). These 10 gene signatures had 6 overlapping genes: *ABAT*, *ADAMDEC1*, *CLEC7A*, *ETS1*, *ITK1* and *STC2*. Relative to nIBC, *ABAT* and *STC2* expression levels were decreased in IBC whereas the levels of the remaining 4 genes were increased (Table 2 and Figure 2). Exploratory IPA analysis was performed to investigate mutual relationships between the 6 classifier genes and ER (Figure 1C) and suggested their potential involvement in endocrine therapy resistance.

3.3. *Diagnostic effectiveness of biomarkers and classifiers*

Next, these six genes were evaluated for their potential to distinguish IBC from nIBC in an independent subset of patients with and without IBC (series **II**). Only the AUCs for the metagene score, *ABAT, and STC2* were discriminatory and comparable in both the discovery and test cohort (Figure 2). The ROC AUCs of these three biomarkers were further explored to establish optimal classifier cutoffs. Distribution dot-plots in the discovery cohort were used to verify the ER+ IBC classification thresholds, which were set for the metagene score at ≥0.0 and for both *ABAT* and *STC2* at ≤ 10.0 (Figure 3A, C and E). The classifiers, however, exhibited moderate diagnostic effectiveness with regard to ER+ IBC prediction, since only a maximum of 64% accuracy was achieved within the test (Figure 3B, D and F).

Table 2 – ER+ IBC discriminatory genes. The expression levels in IBC and nIBC, significance and regression coefficients for the genes identified in the biomarker discovery analysis.

ER+ IBC-Metagene				
Median expression level				
Genes	nIBC (N = 43)	IBC (N = 22)	P-value	Regression coefficient
ABAT	10.63	8.64	9.19E-08	−0.295
ADAMDEC1	6.26	9.07	1.31E-03	0.566
CLEC7A	7.24	8.12	9.55E-03	0.270
ETS1	4.09	4.99	7.09E-05	0.112
ITK	5.27	7.09	2.49E-04	0.332
STC2	10.8	7.64	1.70E-04	−0.630

3.4. *Biomarkers in early disease: prognosis and adjuvant tamoxifen*

The metagene score, *ABAT*, and *STC2* were subsequently evaluated as continuous variable for their relationship with MFS with regard to prognosis and outcome after adjuvant tamoxifen (Table 3). The prognostic value was determined on three series of in total 517 (neo)adjuvant systemic treatment naïve patients with ER+ LNN breast cancer (series **VII–IX**). None of the biomarkers, assessed by microarrays, were prognostic when evaluated for all 517 patients. The biomarkers were also evaluated in 250 ER+ patients treated with adjuvant tamoxifen (series **III**). Decreased expression of *ABAT* (HR = 0.73; 95% CI = 0.61–0.87; P = 0.001) showed a significant correlation with poor MFS in these tamoxifen treated patients, whereas *STC2* and the metagene score had no association with MFS after tamoxifen.

3.5. *Biomarkers and first-line endocrine therapy for advanced disease*

The metagene score, *ABAT*, and *STC2* were also evaluated for their relation with PFS on microarray based series of patients with advanced disease treated with first-line tamoxifen (**IV**, N = 96) or aromatase inhibitors (**V**, N = 84), and validated with RT-qPCR on an independent series of patients treated with first-line tamoxifen (**VI**, N = 231). As continuous variables, increased metagene scores and decreased expression of *ABAT* and *STC2* were correlated with poor treatment outcome in all three series of patients, except for *STC2* in series **IV** (Table 3). As classifiers, apart from *STC2* in series **IV**,

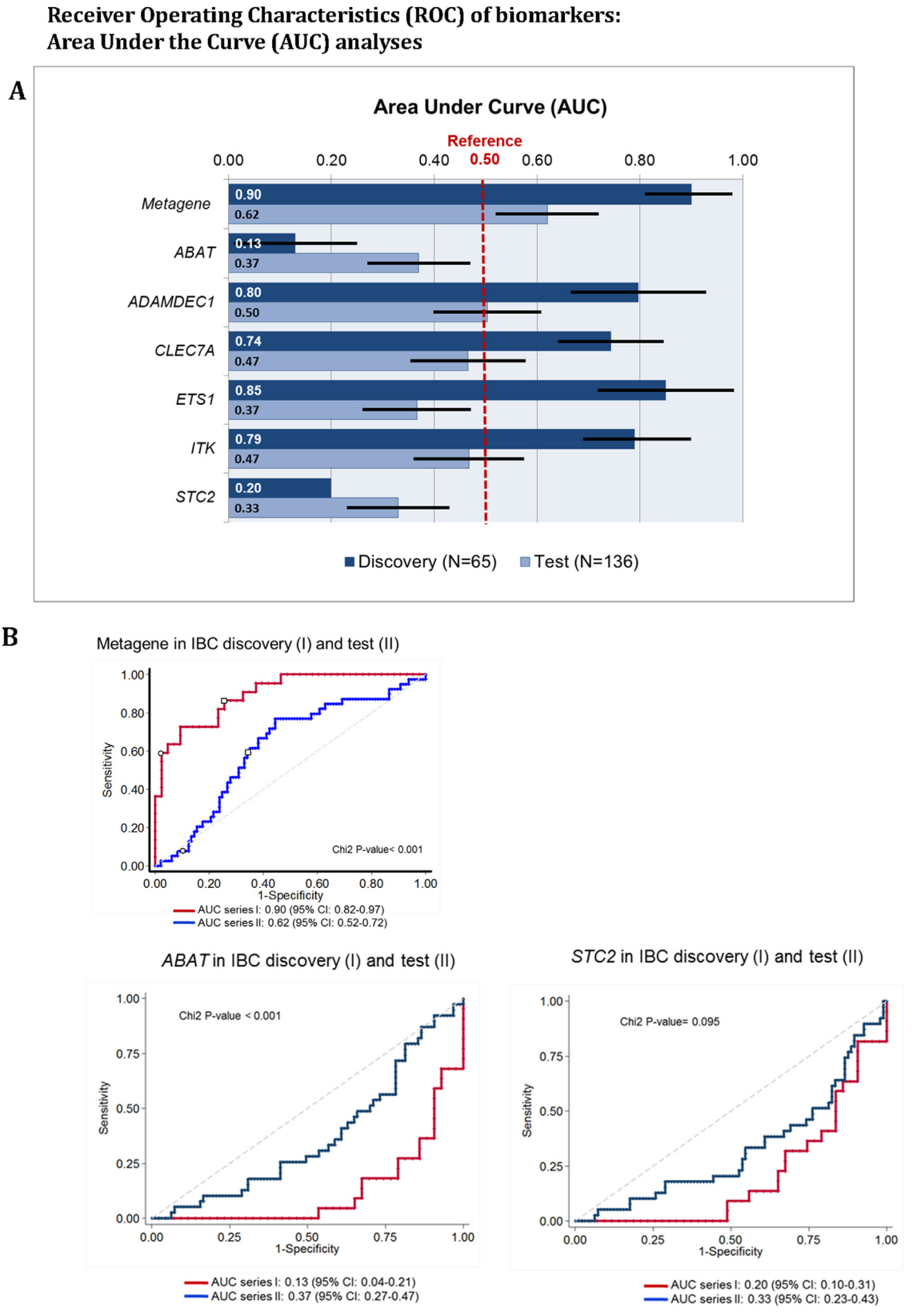


Figure 2 – Receiver Operator Characteristics (ROC) Analyses. The ROC-analyses generate Area Under Curve (AUC) values presented in Figure 2A as measure for the discriminatory potential of the individual genes to predict IBCs and nIBCs correctly within the test (series II) compared to the discovery (series I). Factors with AUC (or their intervals) value 0.5 are not informative. The results show as illustrated with ROC plots in Figure 2B that AUCs for only the metagene, *ABAT* and *STC2* are discriminatory and comparable for the discovery and test.

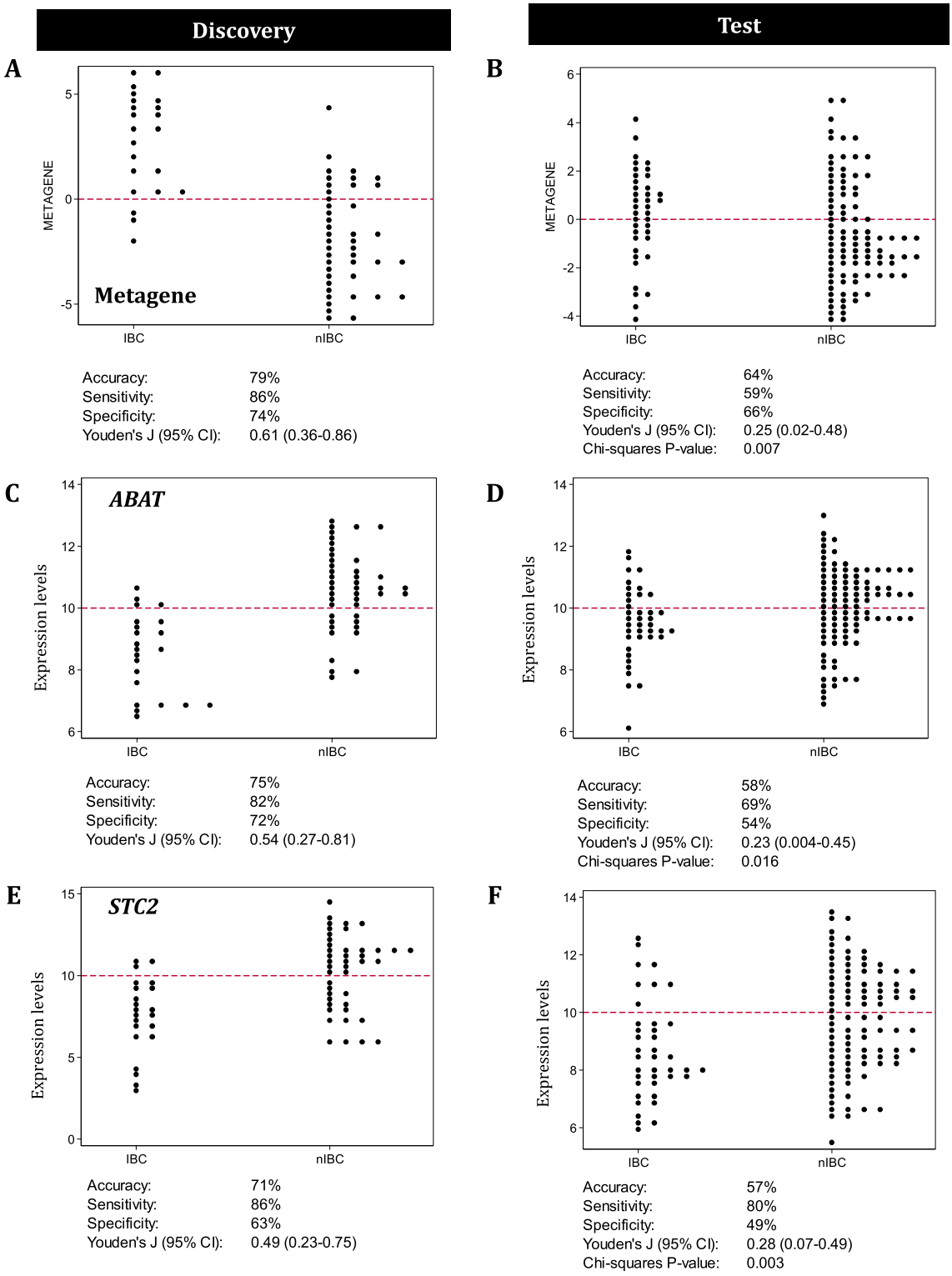


Figure 3 – Dot-plots and diagnostic effectiveness. This figure represents dot-plots and the diagnostic performance of the biomarkers in the discovery and test series for IBC and nIBC (series I and II). The metagene scores and expression levels of ABAT and STC2 measured in the discovery (series I) were evaluated in dot-plots to explore the defined thresholds that classify samples as IBC-like or nIBC-like. The diagnostic effectiveness of the biomarker IBC classification were evaluated in the independent test (series II).

Table 3 – Biomarkers and outcome. This table provides the results of the univariate Cox regression analyses performed for MFS and PFS to determine the prognostic and predictive value of the metagene, *ABAT* and *STC2* in the different patient series.

Clinical setting	Series	N	outcome[a]	Biomarkers as continuous variable:					
				Metagene score		*ABAT*		*STC2*	
				HR (95%CI)[b]	P	HR (95%CI)	P	HR (95%CI)	P
Early Disease									
Prognosis	VII–IX	517	MFS	0.99 (0.92–1.08)	0.967	0.93 (0.82–1.06)	0.298	1.01 (0.92–1.11)	0.867
Adjuvant tamoxifen	III	250	MFS	1.09 (0.99–1.20)	0.084	0.73 (0.61–0.87)	0.001	0.93 (0.83–1.04)	0.216
Advanced Disease									
First-line tamoxifen	IV	96	PFS	1.13 (1.03–1.25)	0.012	0.80 (0.69–0.93)	0.004	0.90 (0.79–1.03)	0.117
First-line aromatase inhibitors	V	84	PFS	1.28 (1.13–1.45)	<0.001	0.74 (0.60–0.91)	0.004	0.76 (0.66–0.88)	<0.001
First-line tamoxifen	VI	231	PFS	1.09 (1.04–1.14)	<0.001	0.85 (0.80–0.92)	<0.001	0.93 (0.88–0.98)	0.004

a Outcome defined by metastasis free survival (MFS) or by progression free survival (PFS).
b Hazard Ratio (HR) and its 95% confidence interval (CI) for MFS in early disease and PFS in advanced disease.

all three biomarkers showed significant associations with PFS for all series in the Kaplan–Meier survival analyses (Figure 4).

3.6. Biomarkers and published signatures

The biomarkers were compared for their relation with PFS in advanced disease (series **IV, V, VI**) with published signatures for Recurrence Score and GGI (Table 4). When compared to Recurrence Score and GGI only *ABAT* remained significantly associated with PFS in all series (except for GGI in series V). In contrast, the metagene score and *STC2* were only independent from Recurrence Score and GGI in AI-treated patients (series **V**). In summary, especially *ABAT* expression levels were independently associated with PFS when compared to published signatures separately and validated with RT-qPCR.

3.7. Biomarkers and clinicopathological predictors

Multivariate analyses were performed by adding the biomarkers separately to a base model of traditional clinicopathological factors for endocrine therapy in advanced disease (Table 5). The model included age, menopausal status, dominant site of relapse, disease-free interval and mRNA expression levels for *ESR1*, *PGR*, and *ERBB2*. These multivariate analyses showed that low *ABAT* levels were significantly related with poor PFS in the series of 96 patients treated with tamoxifen (series **IV** (HR = 0.78, P = 0.027)), whereas high metagene scores (HR = 1.24, P = 0.005) and low *STC2* levels (HR = 0.79, P = 0.011) were associated with poor PFS in the series of 84 patients treated with AI (series **V**). All three biomarkers were independently related with PFS in the RT-qPCR validation series of 231 patients treated with tamoxifen (series **VI**).

3.8. Biomarkers and protein expression

In an exploratory study, ABAT and STC2 protein expression were examined in 110 ER-positive primary breast cancer specimens (Figure 5A). Evaluation of quantity and intensity separately showed for both proteins no significant relationships with PFS (Supplementary Figure 1). The quantity and intensity scores were multiplied to generate an IHC-score for the staining and classified 77 specimens (69%) as ABAT-positive and 78 specimens (70%) as STC2-positive (Figure 5B). These dichotomized IHC-scores were not related with PFS, i.e. not for ABAT (HR = 0.79; 95% CI = 0.51–1.23; P = 0.30) and not for STC2 (HR = 0.93; 95% CI = 0.60–1.44; P = 0.74).

4. Discussion

Inflammatory breast cancer is a rare (~5%) but highly aggressive form of locally advanced breast cancer with an elevated invasive and metastatic potential. It is characterized by clinical and pathological characteristics atypical for breast cancer in general, amongst others a low frequency of ER positivity. In the past, we showed that the molecular portrait of IBC indeed contained fingerprints of aggressive tumor cell behavior in breast cancer in general (Van Laere et al., 2008). Patients with IBC bearing ER expressing tumor cells constitute approximately 30% of all IBC cases and endocrine treatment in these patients is observed to be poorly effective. The molecular profile of samples from patients with ER+ IBC could provide additional hints towards unraveling the molecular biology associated with resistance to endocrine treatment. In this study, we demonstrate that, at least at the molecular level, ER+ IBC is characterized by features associated with endocrine resistance. For instance, the recurrence score and the HOXB13/IL17RB gene expression ratio are both significantly elevated in ER+ IBC compared with ER+ nIBC. Several studies have shown that elevated levels for both parameters are highly predictive of endocrine therapy resistance (Dowsett et al., 2010; Jansen et al., 2007; Ma et al., 2004; Paik et al., 2004). In addition, application of the PAM50-algorithm (Tibshirani et al., 2002) revealed a remarkably low frequency of Luminal A-type samples, which are shown to be more frequently responsive to endocrine treatment compared with their Luminal B-type counterparts. This hypothesis is supported by the observation that samples from tumors with a Luminal B-phenotype frequently exhibit high Recurrence Scores (Fan et al., 2006).

Using repetitive prediction analysis to obtain robust predictors, we identified a metagene of six genes consisting of *ABAT*, *ADAMDEC1*, *CLEC7A*, *ETS1*, *ITK* and *STC2* to discriminate

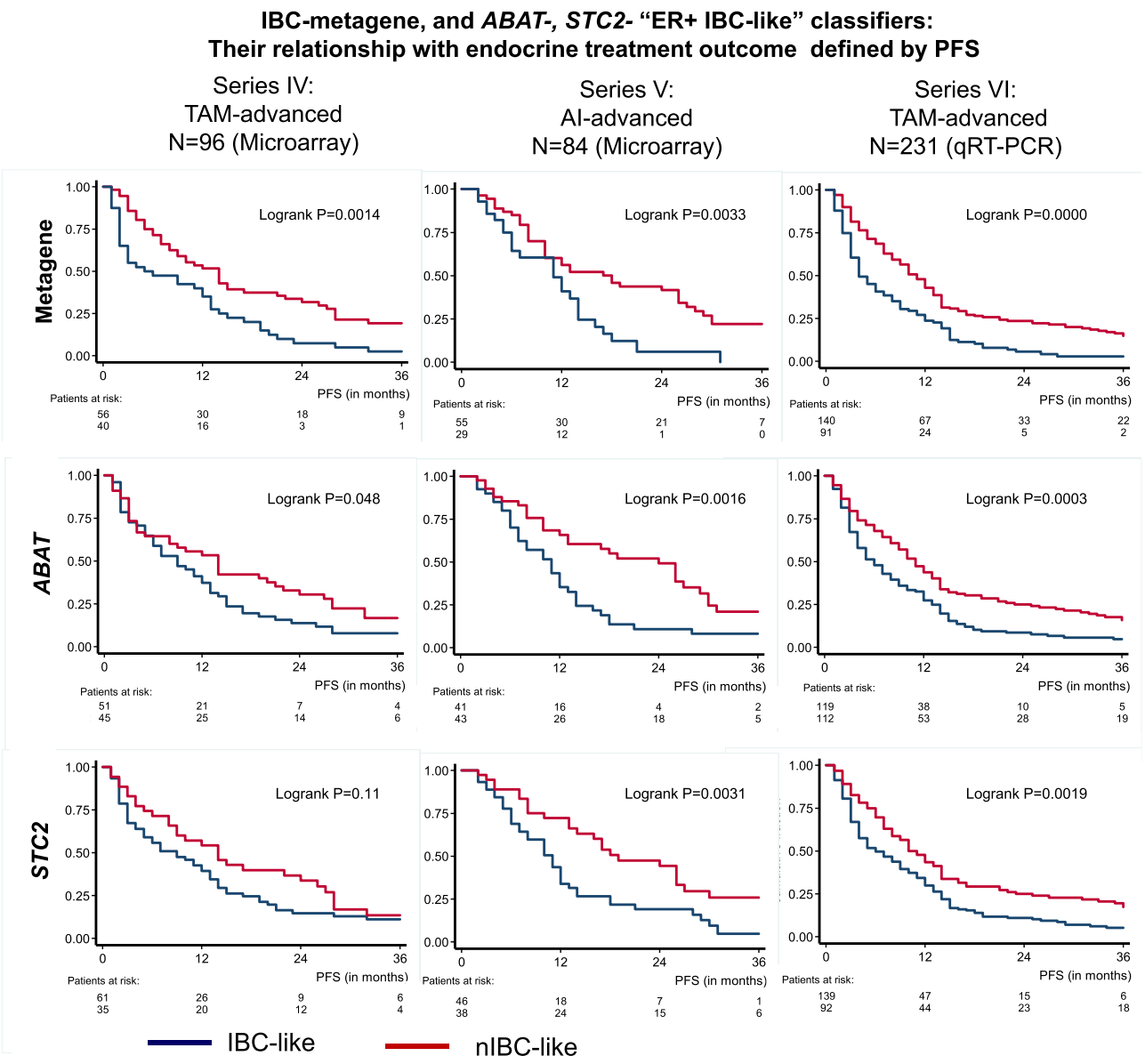


Figure 4 – Kaplan–Meier Analyses for outcome after endocrine treatment The metagene, *ABAT* and *STC2* as IBC/nIBC classifiers and their relation with PFS as measure for treatment outcome in advanced disease after first-line tamoxifen (series IV and VI) and aromatase inhibitors (series V).

ER+ IBC from ER+ nIBC samples within the discovery series. These biomarkers were each verified and demonstrated that only the metagene and the genes *ABAT* and *STC2* remained predictive in the test series. The metagene is a slightly better predictor than the single genes, however, its performance in the other series was largely dictated by *ABAT* and *STC2*. It is intriguing that *ABAT* and *STC2*, as 2 genes not yet linked to inflammation, discriminate between ER+ IBC and nIBC. A role in the inflammatory response, however, is less likely to be established since both *ABAT* and *STC2* are down-regulated in IBC compared to nIBC.

Recently, both *ABAT* and *STC2* were described in the 100 rules used in the Absolute Intrinsic Molecular Subtyping (AIMS) (Paquet and Hallett, 2015). AIMS enables subtyping from gene expression profile at mRNA expression levels of an individual sample without the need of large, diverse, and normalized datasets. These findings indicate that both genes are highly relevant in molecular subtyping. Moreover, the molecular subtyping of breast cancer becomes more and more important in the clinical management of patients. The results of our study may therefore contribute with regard to endocrine treatment decision making.

Although our study demonstrated only that both *ABAT* and *STC2* are just biomarkers, literature suggests for both a role in ER signaling. *ABAT* (MIM: 137150) has been identified as a luminal-like gene with an *ER*-binding site within 20 kb distance from the transcription start site (Krijgsman et al., 2011). This gene is incorporated in Agendia's BluePrint assay, an 80-gene molecular subtyping profile developed in 200 breast cancer specimens and validated in four independent

Table 4 – Biomarkers and published signatures in advanced disease. The metagene, *ABAT* and *STC2* were compared with the published signatures for Recurrence Score and Genomic Grade Index (GGI) for their relationship with PFS in advanced disease after treatment with tamoxifen (series IV and VI) or aromatase inhibitors (series V).

Univariate				Bivariate (biomarker together with published signature evaluated)					
				Metagene score		*ABAT*		*STC2*	
Signature	N	HR (95% CI)	P	HR (95% CI)	P	HR (95% CI)	P	HR (95% CI)	P
Series IV. Tam (N = 96)									
Recurrence Score									
low	35	1.00		1.11 (0.99–1.24)	0.085	0.84 (0.71–0.99)	0.036	0.94 (0.82–1.09)	0.430
moderate	12	1.01 (0.49–2.08)	0.977						
high	49	1.61 (1.00–2.60)	0.050						
GGI	96	0.99 (0.90–1.10)	0.899	1.14 (1.03–1.26)	0.010	0.80 (0.69–0.93)	0.005	0.90 (0.78–1.03)	0.113
TAM78	62	2.34 (1.34–4.11)	0.003	1.11 (0.98–1.27)	0.102	0.86 (0.71–1.04)	0.130	0.94 (0.79–1.11)	0.455
ROR-S									
low	33	1.00		1.10 (0.97–1.24)	0.141	0.85 (0.72–1.01)	0.065	0.96 (0.83–1.10)	0.534
moderate	30	1.42 (0.82–2.46)	0.216						
high	33	1.93 (1.14–3.25)	0.014						
ROR-P									
low	23	1.00		1.13 (1.01–1.26)	0.039	0.80 (0.67–0.96)	0.018	0.91 (0.78–1.06)	0.230
moderate	37	0.93 (0.53–1.65)	0.816						
high	36	1.34 (0.77–2.33)	0.307						
Series V. AI (N = 84)									
Recurrence Score									
low	18	1.00		1.23 (1.08–1.42)	0.003	0.77 (0.62–0.95)	0.013	0.79 (0.67–0.93)	0.004
moderate	7	1.85 (0.64–5.36)	0.259						
high	59	2.86 (1.34–6.07)	0.006						
GGI	84	3.64 (1.87–7.08)	<0.001	1.19 (1.03–1.38)	0.019	0.84 (0.67–1.05)	0.124	0.84 (0.71–0.99)	0.043
TAM78	84	1.80 (1.08–3.00)	0.023	1.26 (1.11–1.44)	0.001	0.74 (0.60–0.93)	0.008	0.76 (0.65–0.88)	<0.001
ROR-S									
Low	24	1.00		1.19 (1.03–1.37)	0.016	0.82 (0.66–1.03)	0.087	0.83 (0.71–0.97)	0.018
moderate	31	1.20 (0.61–2.36)	0.603						
high	29	3.20 (1.65–6.21)	0.001						
ROR-P									
low	18	1.00		1.19 (1.03–1.38)	0.017	0.80 (0.64–1.00)	0.050	0.82 (0.71–0.96)	0.015
moderate	38	1.79 (0.84–3.82)	0.132						
high	28	3.94 (1.81–8.59)	0.001						
Series VI. Tam (N = 231), qRT-PCR									
Recurrence Score									
low	65	1.00		1.05 (0.99–1.11)	0.134	0.88 (0.80–0.97)	0.011	0.96 (0.90–1.02)	0.196
moderate	13	1.90 (1.00–3.60)	0.048						
high	131	2.39 (1.70–3.37)	<0.001						
GGI	226	1.53 (1.29–1.81)	<0.001	1.06 (1.01–1.12)	0.032	0.89 (0.81–0.97)	0.009	0.94 (0.89–1.01)	0.057

cohorts. *ABAT* encodes for 4-aminobutyrate aminotransferase, an enzyme responsible for the catabolism of gamma-aminobutyric acid (*GABA*), which might be involved in the hormonal regulation and pathogenesis of breast cancer (Opolski et al., 2000). Moreover, comparative metabolomics demonstrated alterations in glutamine and beta-alanine metabolism along with low ABAT expression with shortened survival in ER+ and ER– breast cancer (Budczies et al., 2013).

Bouras and colleagues have shown that *STC2* (MIM: 603665) as an estrogen responsive gene is co-expressed with ER (Bouras et al., 2002). In fact, independent studies have indeed shown that *STC2* is a dynamic marker of estrogen-driven pathway activation and that constitutive expression after serum withdrawal negatively affects breast cancer cell growth, cell viability and cell migration (Raulic et al., 2008; Urruticoechea et al., 2008)). Therefore, reduced expression of *STC2* in breast cancer cells enables survival and cell growth in the absence of estrogen, thereby contributing to endocrine treatment resistance. Of note, in a recent effort to redefine the molecular portraits of IBC on an extended series of 137 samples, repressed STC2 expression levels were observed in IBC in a molecular subtype-independent manner (Van Laere et al., 2010). Future studies are needed to provide functional evidence that *ABAT* and *STC2* are mechanistically involved in endocrine therapy response.

Based upon above considerations and findings we evaluated *ABAT* and *STC2* further in different datasets to determine their relationship with prognosis and treatment outcome to adjuvant and first-line endocrine therapy (i.e. tamoxifen and aromatase inhibitors). Both biomarkers were not prognostic, whereas only decreased levels of *ABAT* were associated with

Table 5 – Biomarkers and clinico-pathological factors in advanced disease. This table provides the results of the uni- and multivariate Cox regression analyses for PFS performed on the advanced disease patient series (series IV–VI). The biomarkers were separately added to the base model of clinico-pathological factors in multivariate analysis.

Clinicopathological factors	Series IV. Tam (N = 96)					Series V. AI (N = 84)					Series VI. Tam (N = 231), qRT-PCR				
	N	Univariate (N = 96)		Multivariate (N = 91)		N	Univariate (N = 84)		Multivariate (N = 82)		N	Univariate (N = 231)		Multivariate (N = 231)	
		HR (95% CI)	P	HR (95% CI)	P		HR (95% CI)	P	HR (95% CI)	P		HR (95% CI)	P	HR (95% CI)	P
Age															
<55	34	1.00		1.00		18	1.00		1.00		91	1.00		1.00	
56–70	43	0.75 (0.46–1.22)	0.250	1.09 (0.43–2.79)	0.855	32	1.07 (0.56–2.05)	0.839	0.86 (0.36–2.02)	0.727	81	0.78 (0.57–1.07)	0.123	0.83 (0.53–1.31)	0.427
>70	19	0.90 (0.49–1.65)	0.742	1.21 (0.44–3.38)	0.712	34	0.75 (0.38–1.46)	0.390	0.59 (0.23–1.48)	0.260	59	0.69 (0.48–0.98)	0.038	0.69 (0.43–1.12)	0.134
Menopausal status															
Premenopausal	29	1.00		1.00		7	1.00		1.00		61	1.00		1.00	
Postmenopausal	67	0.81 (0.51–1.30)	0.388	0.99 (0.39–2.54)	0.988	76	0.99 (0.43–2.30)	0.978	1.29 (0.41–4.04)	0.662	170	0.80 (0.59–1.09)	0.155	1.14 (0.72–1.80)	0.586
Dominant site of relapse															
Bone	41	1.00		1.00		46	1.00		1.00		120	1.00		1.00	
LRR or viscera	53	0.81 (0.52–1.26)	0.345	0.78 (0.46–1.32)	0.357	38	0.79 (0.48–1.31)	0.367	0.86 (0.49–1.49)	0.586	111	0.91 (0.69–1.20)	0.518	0.93 (0.70–1.23)	0.600
Disease free interval															
<1 year	17	1.00		1.00		14	1.00		1.00		65	1.00		1.00	
2–3 years	24	0.43 (0.22–0.84)	0.013	0.43 (0.21–0.88)	0.021	28	0.64 (0.32–1.27)	0.204	0.84 (0.39–1.79)	0.650	100	0.58 (0.42–0.80)	0.001	0.55 (0.40–0.77)	<0.001
>3 years	52	0.36 (0.20–0.64)	0.001	0.33 (0.18–0.63)	0.001	41	0.38 (0.19–0.75)	0.005	0.44 (0.21–0.91)	0.027	66	0.47 (0.32–0.68)	<0.001	0.46 (0.32–0.67)	<0.001
mRNA levels as continuous variable:															
ESR1	96	0.84 (0.73–0.95)	0.007	0.81 (0.68–0.96)	0.016	84	0.67 (0.32–1.40)	0.285	1.03 (0.46–2.35)	0.935	231	0.91 (0.85–0.97)	0.003	0.93 (0.86–1.00)	0.037
PGR	95	0.94 (0.86–1.03)	0.217	0.99 (0.88–1.10)	0.807	84	0.55 (0.37–0.80)	0.002	0.61 (0.40–0.94)	0.026	231	0.90 (0.84–0.97)	0.004	0.92 (0.85–1.00)	0.043
HER2	96	1.05 (0.89–1.25)	0.550	1.13 (0.94–1.37)	0.198	84	1.17 (0.69–1.98)	0.563	1.40 (0.79–2.50)	0.253	231	1.13 (1.00–1.27)	0.044	1.07 (0.95–1.20)	0.279
				Added to the model					Added to the model					Added to the model	
Metagene score	96	1.29 (1.13–1.46)	<0.001	1.09 (0.95–1.24)	0.210	84	1.28 (1.13–1.45)	<0.001	1.24 (1.07–1.44)	0.005	231	1.09 (1.04–1.14)	<0.001	1.08 (1.02–1.14)	0.009
ABAT	96	0.80 (0.69–0.94)	0.005	0.78 (0.63–0.97)	0.027	84	0.74 (0.60–0.91)	0.004	0.83 (0.65–1.05)	0.127	231	0.85 (0.80–0.92)	<0.001	0.87 (0.79–0.96)	0.004
STC2	96	0.90 (0.79–1.03)	0.117	0.99 (0.83–1.18)	0.903	84	0.76 (0.66–0.88)	<0.001	0.79 (0.67–0.95)	0.011	231	0.93 (0.88–0.98)	0.004	0.93 (0.87–0.99)	0.017

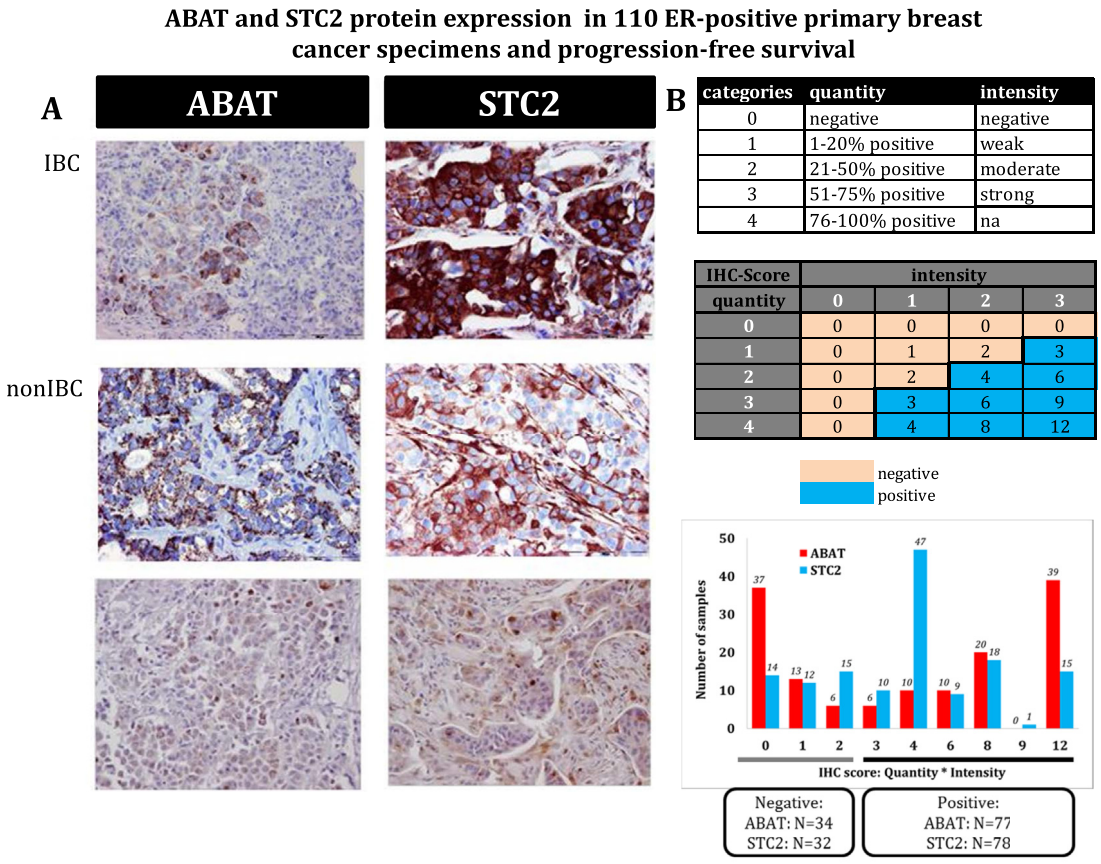


Figure 5 – ABAT and STC2 protein expression The expression of ABAT and STC2 protein was evaluated with immunohistochemistry in 110 ER-positive primary tumor specimens of advanced breast cancer patients treated with first-line tamoxifen. In Figure 5A representative samples are shown for ABAT and STC2 staining in IBC and nIBC patients. Figure 5B demonstrates the staining categories for quantity, intensity, and IHC-scores, and the distribution of IHC-scores for ABAT and STC2. The IHC-scores were dichotomized into positive and negative scores, identifying 77 ABAT-positive and 78 STC2-positive specimens. Both ABAT and STC2 protein expression had no relationship with progression free survival.

shorter MFS after adjuvant tamoxifen. In the advanced disease setting, decreased expression of *ABAT* and *STC2* characterized patients with reduced PFS under either tamoxifen- or AI-based endocrine therapy. Particularly in patients treated with first-line tamoxifen these "ER+ IBC-like" predictors were associated with sensitivity to endocrine treatment in an independent data set profiled with an alternative technology. The latter is important as it proves that determination of *ABAT* and *STC2* expression levels is also applicable with standard PCR technologies, making the "bench-to-bedside" transition more feasible. We also explored whether ABAT and STC2 protein expression might be applicable as predictive biomarkers in immunohistochemical assays, however, our findings showed no relationship with tamoxifen outcome.

Combining these biomarkers with clinico-pathological factors in multivariate analyses demonstrated that *ABAT* remained significantly associated with response to tamoxifen in two independent patient series, whereas the metagene and *STC2* was only independent within the qRT-PCR validation. Moreover, only *ABAT* remained independent predictive for tamoxifen in both patient series when combined with the published signatures Recurrence Score and GGI. All these multivariate analyses on different patient series summarized indicate *ABAT* as a robust predictor for the response to tamoxifen.

In conclusion, this study has identified an increased metagene score and decreased expression of *ABAT* and *STC2* in IBC, and correlated the metagene and low expression of the genes with poor tamoxifen treatment outcome in the advanced setting as shown with qRT-PCR in an independent validation. ABAT and STC2 protein expression were not informative with regard to treatment outcome. Further studies on the classifier genes are needed to elucidate the mechanism of therapy resistance.

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Conflict of interest

None of the authors has a conflict of interest.

Authors' contributions

LS and MJ contributed equally. MJ, LS, AS and SvL designed the study. FB, IS, PR, SL, PvD, MK, CvD, JM, PV, JF, LD, and EB participated in the study design, provided tissues and clinical follow-up data and helped to draft the manuscript. AS, CvL, DRA, ML, CvD, MT and KR carried out experiments and performed (statistical) data analyses. MJ, LS, AS, EB and SvL prepared the manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.molonc.2015.02.006.

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