

# Improved Risk Stratification for Breast Cancer Samples Based on the Expression Ratio of the Estrogen and Progesterone Receptor

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**Abstract.** *Background:* The receptors for estrogen (ESR1) and progesterone (PGR) are both part of the same signaling pathway and routinely used for breast cancer stratification. We tested the hypothesis if a coordinated analysis could add extra information for prognostic stratification. *Materials and Methods:* ESR1 and PGR gene expression was first investigated by quantitative reverse transcription polymerase chain reaction in fresh-frozen invasive ductal breast cancer samples (Hamburg collective, case-control, n=317). Our results were then tested using two datasets generated by different technical approaches: i) a public DNA-chip data set (GSE3494, n=251) and ii) semiquantitative protein expression data based on immunohistochemistry (Stuttgart collective, n=18,528). *Results:* The PGR/ESR1 gene-expression ratio was a prognostic indicator in those with ESR1/PGR-positive breast cancer (Hamburg collective), with a high PGR/ESR1 expression ratio indicating a favorable outcome. In all three collectives, the PGR/ESR1 mRNA ratio or its protein equivalent was a univariate prognostic factor and also a multivariate prognostic factor in the Hamburg and Stuttgart collectives. *Conclusion:* Calculation of the PGR/ESR1 gene-expression ratio and its immunohistochemical surrogate could

be a useful and simple addition to routine breast cancer diagnostics. A high PGR/ESR1 ratio could be indicative of a favorable clinical outcome.

Breast cancer (BC) is the most common malignant tumor in women. Its pathological routine diagnostics still depend on morphological and clinical parameters (1). The receptors for estrogen (ESR1) and progesterone (PGR) function as transcription factors and are known to play key roles in the life cycle and differentiation of epithelial breast cells (2). The corresponding proteins are well-established in routine immunohistochemistry (IHC) diagnostics defining BC subtypes with different prognosis and treatment options (3). However, the purpose of PGR testing has been questioned because most ESR1-negative tumors also do not express PGR (4). Novel approaches such as DNA chips enable definition of more BC subtypes, and several marker signatures with prognostic and predictive impact are commercially available (5), most of which comprise ESR1 and PGR. Some recent publications underline the high clinical impact of PGR. The loss of PGR seems to be a strong predictor of poor disease outcome or recurrence (6, 7). In this study, we hypothesized that a quantitative and coordinated hormone receptor expression analysis would reflect the related biology of ESR1 and PGR better than the simple positive/negative categories do and could reveal additional clinically relevant information.

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*Key Words:* Estrogen receptor, progesterone receptor, breast cancer, IHC, PGR/ESR1 ratio, prognosis.

## Materials and Methods

*Study collectives.* A total of 4673 patients with BC diagnosed at Pathologie Hamburg-West between 1995 and 2007 were asked for

their informed consent to participate in the study. 3488 samples were usable (2974 concordant, 514 deceased). 1332 tumors were available as fresh-frozen material, 314 of which (174 controls and 140 events (recurrence or death) were selected for *ESR1* and *PGR* expression analysis. Controls and event cases were matched as closely as possible with respect to main clinical features such as tumor type (invasive ductal carcinoma), grade, stage, receptor status, age at diagnosis. We excluded 97 samples (27 controls, 70 events) for the following reasons: age at diagnosis >80 years; tumor size >5 cm; unclear erb-b2 receptor tyrosine kinase 2 (ERBB2) status, follow-up (controls) <5 years; time to event >5 years. The remaining 217 samples (147 controls, 70 events) were used in the expression study. For the survival analysis all *ESR1*/*PGR*-negative cases were eliminated on the basis of their routine IHC results.

*ESR1* and *PGR* gene expression was first investigated by quantitative reverse transcription polymerase chain reaction (qRT-PCR) in fresh frozen invasive ductal breast cancer samples (Hamburg collective, n=217). The approach for sample selection in this case-control study is shown in Figure 1. Our gene expression results were then tested using two datasets generated by different technical approaches: i) a public DNA-chip gene expression data set (GSE3494, n=251 consecutive cases) and ii) semiquantitative protein expression data based on immunohistochemistry (Stuttgart collective, n=18,528). The criteria to exclude samples from the GSE 3494 data set were identical as mentioned above for the Hamburg data set. Clinical data for all three collectives are summarized in Table I.

**Histopathology and IHC.** Tumor classifications and IHC evaluations for the Hamburg collective were carried out by three experienced pathologists (SB, AD, AN) according to World Health Organization (WHO) and Union for International Cancer Control (UICC) guidelines (8, 9) during routine practice. Receptor status determination followed IHC standard methods using monoclonal antibodies against *ESR1* (clone 1D5) and *PGR* (clone Sp2) in a three-step procedure (DCS DuoVision Plus, DCS, Hamburg, Germany).

**RNA isolation and qRT-PCR.** Total RNA was extracted from fresh frozen material (three consecutive 5  $\mu$ m sections) using a QIAcube and the miRNeasy mini Kit (QIAGEN, Hilden, Germany) and stored at  $-80^{\circ}\text{C}$  until use. Tumor content was monitored in a stained adjacent section. Three independent two-step reverse transcription reactions (20  $\mu$ l, each containing approx. 300 ng total RNA) of each sample were synthesized according to the manufacturer's protocol (miScript Reverse Transcription Kit; QIAGEN), pooled and diluted 1:15 with water resulting in a total volume of 900  $\mu$ l. PCR analyses of cDNA were conducted in triplicates using the QuantiFast SYBR Green PCR Kit (QIAGEN) and a 7500 fast PCR machine (Applied Biosystems, Foster City, CA, USA). Each plate contained a dilution series (1:1, 1:6, 1:36, 1:216, 1:1296) of a 'calibrator' sample (*i.e.* cDNA from a pooled mix of 23 tumor samples) for relative quantification purposes and for determination of individual PCR run efficacy. Primer sequences for *ESR1*, *PGR* and for five housekeeper genes were taken from GeneGlobe (QIAGEN): Beta actin (*ACTB*), Glucuronidase (*GUS*), Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), Ribosomal protein lateral stalk subunit P0 (*RPLP0*), Transferrin receptor (TFRC) (10).

**qPCR data processing.** By using a calibrator at five concentrations in each run, triplicated ct values could be transformed into

triplicated relative mRNA amounts. Triplicated relative measurements of each sample were filtered and combined by averaging similar to the method of the RIKEN group (11) but calculations were made on the basis of relative quantities rather than ct values. Relative gene expression values were normalized using the geometric mean of five reference genes (10). Based on our quantitative PCR data, we stratified the Hamburg collective into three different risk groups (Figure 2B): high risk:  $PGR+ESR1 < 7.85$ ; intermediate risk:  $PGR+ESR1 > 7.85$  AND  $PGR/ESR1 < 1.5$ ; and low risk:  $PGR+ESR1 > 7.85$  AND  $PGR/ESR1 > 1.5$ .

**Validation collective 1 GSE 3494.** We used the public dataset GSE 3494 (12) for validation. *ESR1* and *PGR* expression was calculated from the two most reliable probe sets 205225\_at (*ESR1*) and 228554\_at (*PGR*). For the survival analysis, all patients with *ESR1*-negative or *PGR*-negative breast cancer by IHC or missing values were eliminated, leaving 127 patients in the study (94 controls and 33 events).

**Validation collective 2 (Stuttgart collective).** The Stuttgart collective comprises all breast cancer cases in the region of Stuttgart collected by Onkologischer Schwerpunkt Stuttgart (OSP) between 1989 and 2011 (N=18528) from five pathological institutions and has been used for breast cancer studies published elsewhere (13-15). All patients were asked for their informed consent. We excluded all *ESR1* and/or *PGR* negative cases (based on routine diagnosis) as well as missing values for *ESR1* and *PGR*. A total of 10,066 patients were used for the final analysis (Table II). Immunohistochemistry data based on routine practice were taken from the Stuttgart database. Remmele score cutoff values of 8 were defined for further stratification of *ESR1*- and *PGR*-positive cases. The receptor protein ratio was termed *PGR/ESR1*.

**Statistical calculations.** Statistical analyses were calculated using R v.3.1 (16). *p*-Values of less than 0.05 were regarded as significant and  $p < 0.0001$  as highly significant. We used the Chi-square test for categorical variables. The Kaplan-Meier and Cox regression methods were applied for survival analysis. Calculation of the *PGR/ESR1* ratio was as follows: the Remmele score (17) for *ESR1* and *PGR* is an ordinal scale with nine different levels. The IHC-based *PGR/ESR1* ratio (equivalent to  $PGR/ESR1$  by qRT-PCR) was calculated as:  $PGR/ESR1 = [\text{Remmele score (PGR)} + 1] / [\text{Remmele score (ESR1)} + 1]$ . We tested the hypothesis whether the  $PGR/ESR1$  ratio or its IHC equivalent was a predictor of disease outcome in the steroid-receptor positive invasive breast cancer subset. Models for disease outcome used the variables pT, pN, grade, pM (as available), age, and the *PGR/ESR1* or IHC ratio. If the Cox assumption was not valid, the Cox method was used with the stratification approach. Events were considered as death from any cause (overall survival, OS), recurrence (local or distant) and death in the disease free survival (DFS) analysis.

## Results

**Hamburg dataset.** Assuming a biological relation between *ESR1* and *PGR*, we plotted relative mRNA expression of both receptors against one another for 217 cases (Figure 2A) characterized as double-negative tumors (lower left quadrant): 10 controls, 16 events, event rate=0.62; *ESR1*+/*PGR*- (lower right quadrant): 15 controls and 11

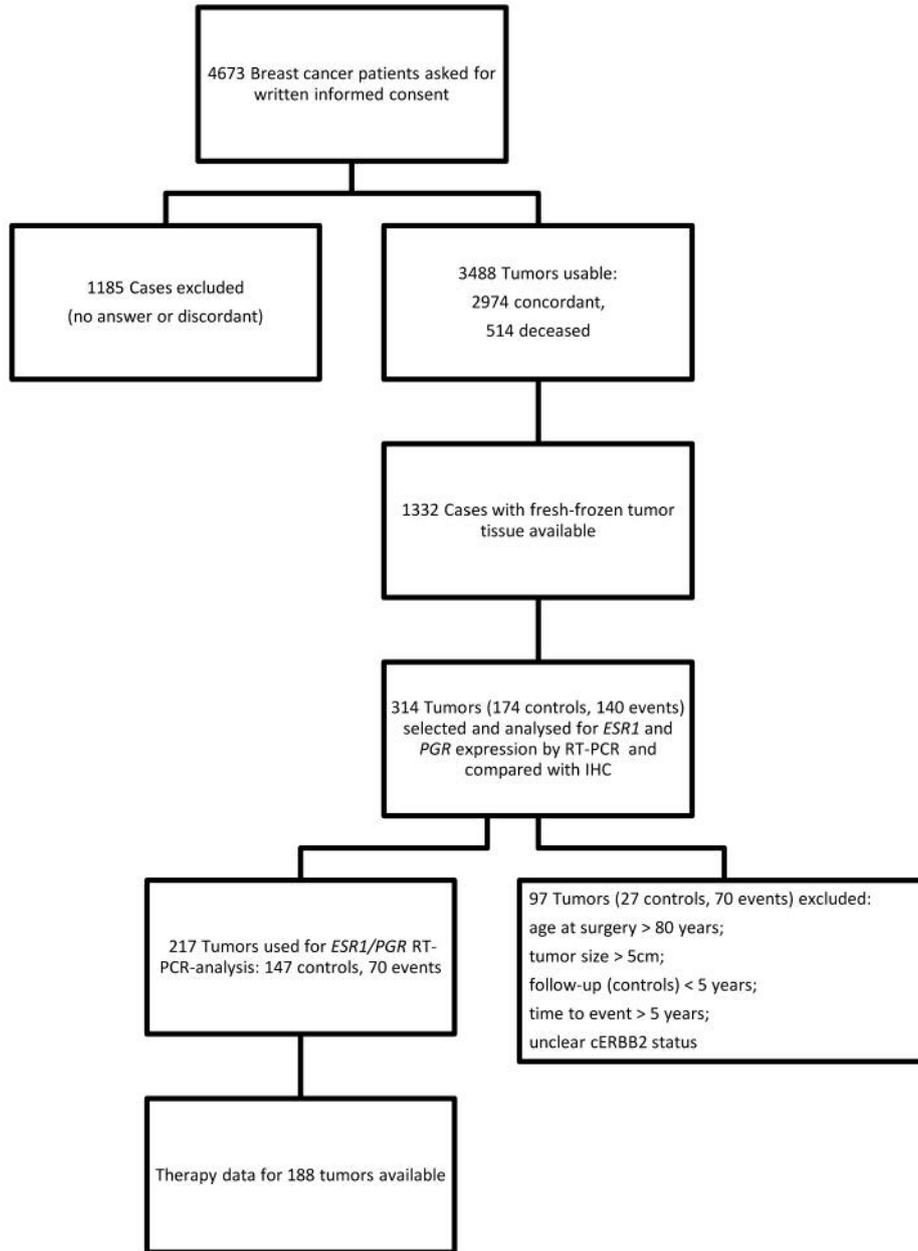


Figure 1. Selection criteria for the Hamburg dataset. PGR: progesterone receptor; ESR1: estrogen receptor; RT-PCR: reverse transcription polymerase chain reaction; IHC: immunohistochemistry; ERBB2: human epidermal growth factor receptor 2.

events, event rate=0.42;  $ESR1^-$  and  $PGR^+$  (upper left quadrant): 1 control, three events; double-positive tumors (upper right quadrant): (n=161) 121 controls, 40 events, event rate=0.25. The majority of control samples were located above the diagonal line shown in Figure 2A, defining an especially favorable subgroup ( $PGR > ESR1$ ).

The value of the receptor ratio is clearer in a plot of the sum of receptor expression ( $PGR + ESR1$ ) versus the ratio

( $PGR/ESR1$ ) shown in Figure 2B. The horizontal line ( $PGR/ESR1=1$ ) corresponds to the diagonal in Figure 2A. Cut-off values  $PGR + ESR1 = 7.85$  and  $PGR/ESR1 = 1.5$  define three different outcome groups. Firstly, a high-risk group of those with double-negative tumors on the left with  $PGR + ESR1 < 7.85$  (17 controls, 33 events, event rate=0.66). For this high-risk group, nodal status ( $p=0.018$ ), Nottingham prognostic index (NPI) ( $p < 0.001$ ) and ERBB2 status

Table I. Characteristics of study collectives.

| Source                 | Hamburg,                               |     | GSE3494,          |     | Stuttgart,        |     | p-Value  |
|------------------------|--|-----|-------------------|-----|-------------------|-----|----------|
|                        | n                                      | (%) | n                 | (%) | n                 | (%) |          |
| N                      | 4,673                                  |     | 255               |     | 18528             |     |          |
| Type of cohort         | Selection for prognosis (case control) |     | Consecutive cases |     | Whole population  |     |          |
| N                      | 217                                    |     | 156               |     | 18528             |     | 2.01     |
| Control cases          | 147 (67.7)                             |     | 111               |     | 11646 (7.0)       |     | 0.37     |
| Recurrences            | 14 (6.5)                               |     | No data           |     | 1301 (28.8)       |     |          |
| Death                  | 56 (25.8)                              |     | 45 (28.8)         |     | 5581 (30.1)       |     |          |
| Criteria for exclusion | PGR; ESR1; A                           |     | PGR; ESR1; A      |     | PGR; ESR1         |     |          |
| N after exclusion      | 167                                    |     | 127               |     | 10066             |     | 3.60     |
| Control cases          | 125 (74.9)                             |     | 94                |     | 7037 (69.9)       |     | 0.16     |
| Recurrences            | 12 (7.2)                               |     | 0                 |     | 608 (6.0)         |     |          |
| Death                  | 30 (17.9)                              |     | 33 (30.0)         |     | 2421 (24.1)       |     |          |
| Follow-up              |  |     |                   |     |                   |     | 105.6    |
| Mean±SD (years)        | 5.6±7.4                                |     | 9.2±3.4           |     | 6.8±5.2           |     | <0.00001 |
| Median                 | 6.0                                    |     | 10.6              |     | 5.6               |     |          |
| Mean age±SD (years)    | 57.1±10.9                              |     | 60.1±12.8         |     | 60.1±13.2         |     | 4.84     |
| Median                 | 59.5                                   |     | 60.1              |     | 60.2              |     | 0.09     |
| pT1                    | 87 (52.1)                              |     | 69 (59.8)         |     | 4836 (48.6)       |     | 29.8     |
| pT2                    | 79 (47.3)                              |     | 55 (43.7)         |     | 4009 (40.3)       |     | 0.0004   |
| pT3                    | 1 (0.6)                                |     | 2 (1.5)           |     | 506 (5.1)         |     |          |
| pT4                    | 0                                      |     | 0                 |     | 565 (5.7)         |     |          |
| Missing values         | 0                                      |     | 1                 |     | 150               |     |          |
| pN0                    | 64 (38.3)                              |     | 85 (66.9)         |     | 5905 (60.9)       |     | 62.0     |
| pN1/N1mic              | 94 (56.3)                              |     | 42 (33.1)         |     | 2970 (30.6)       |     | <0.00001 |
| pN2/3                  | 9 (5.4)                                |     | 0                 |     | 819 (8.5)         |     |          |
| Missing values         | 0                                      |     | 0                 |     | 370               |     |          |
| M0                     | no M1 cases                            |     | no M1 cases       |     | 9039 (95.5)       |     |          |
| M1                     |  |     |                   |     | 423 (4.5)         |     |          |
| Missing values         |  |     |                   |     | 604               |     |          |
| Grade 1                | 24 (14.4)                              |     | 46 (36.5)         |     | 947 (10.6)        |     | 99.2     |
| Grade 2                | 106 (63.4)                             |     | 70 (55.6)         |     | 5768 (64.8)       |     | <0.00001 |
| Grade 3                | 37 (22.2)                              |     | 10 (7.9)          |     | 2183 (24.5)       |     |          |
| Missing values         | 0                                      |     | 1                 |     | 1168              |     |          |
| ESR1+*                 | 167 (selection)                        |     | 127 (selection)   |     | 10066 (selection) |     |          |
| ESR1-*                 |  |     |                   |     |                   |     |          |
| Missing values         |  |     |                   |     |                   |     |          |
| PGR+*                  | 167 (selection)                        |     | 127 (selection)   |     | 10066 (selection) |     |          |
| PGR-*                  |  |     |                   |     |                   |     |          |
| Missing values         |  |     |                   |     |                   |     |          |
| cERBB2,* 0/1/2**       | 144 (86.2)                             |     | 116 (86.6)        |     | 4438 (91.0)       |     | 3.0      |
| cERBB2,* 3             | 23 (13.8)                              |     | 11 (13.4)         |     | 438 (9.0)         |     | 0.22     |
| Missing values         | 0                                      |     | 0                 |     | 5190              |     |          |

A: Additional exclusion criteria as outlined in the study collectives section and Figure 1; SD: standard deviation; ESR1: estrogen receptor; PGR: progesterone receptor; HER2: human epidermal growth factor receptor 2. \*Assessed by immunohistochemistry; \*\*missing values are included in the exclusion criteria.

( $p=0.006$ ) were still significant clinical parameters with respect to outcome, whereas tumor grade and size were not. There is also an intermediate-risk group (lower right, Figure 2B) with  $PGR+ESR1>7.85$  and  $PGR/ESR1<1.5$  (65 controls, 32 events, event rate=0.33). In this group, nodal status was the only significant clinical parameter ( $p=0.004$ ). A low-risk group was also found (upper right, Figure 2B) with

$PGR+ESR1>7.85$  and  $PGR/ESR1>1.5$  (65 controls, 6 events, event rate=0.085). ESR1 and PGR status by IHC, and NPI were still significant ( $p=0.006$ , 0.0014 and 0.03, respectively) in this group. Data are summarized in Figure 2B and Table II.

We looked at the distribution of the risk groups with regard to the different therapy regimens to be sure that our

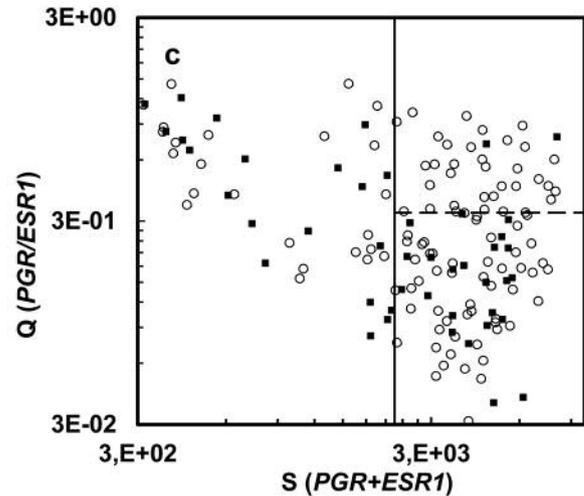
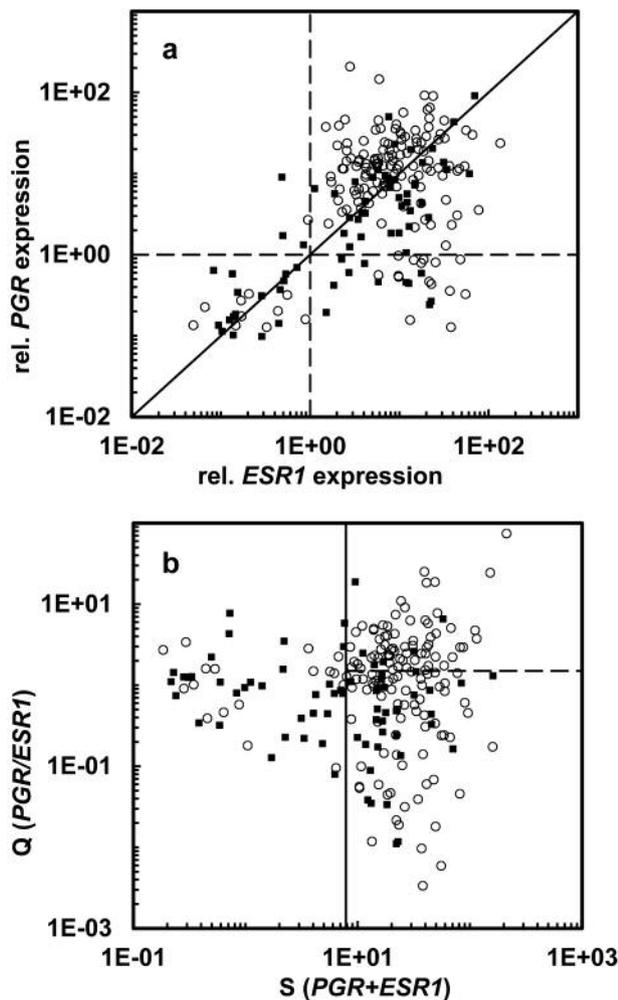


Figure 2. a: Plot of relative mRNA expression of progesterone receptor (PGR) versus estrogen receptor (ESR1) for the Hamburg collective (218 fresh-frozen BC: 147 controls, open circles; 71 events, filled squares) measured by quantitative reverse transcription polymerase chain reaction. The diagonal line indicates equal ESR1 and PGR expression. ESR1/PGR double-negative cases are located in the lower left quadrant. In the upper right quadrant (ESR1/PGR double-positive), most favorable clinical outcomes are found above the diagonal, with PGR exceeding ESR1. b: Plot of the sum of relative ESR1 and PGR expression ( $S$ ) versus the ratio of PGR and ESR1 expression ( $Q$ ) for the Hamburg collective. Most of those with favorable outcomes had a combined receptor expression greater than 7.85 (solid line) and a PGR/ESR1 ratio greater than 1.5 (dashed horizontal line), indicating the potential prognostic value of the PGR/ESR1 ratio. c: External validation using the GSE3494 dataset using the same  $S$  versus  $Q$  plot for 156 cases (111 controls and 45 events). Although scaled differently, ESR1 PGR double-positive cases ( $S > 2250$ , solid line) with a high PGR/ESR1 ratio ( $Q > 0.33$ , dashed line) mostly fall into the group with favorable clinical outcome.

observation was not restricted to a certain treatment. Treatment groups showing significant differences for the high-risk group were all related to anti-estrogenic treatment. Since the high-risk group is identical with hormone receptor-negative cases, it is no surprise to find many cases which did not receive anti-estrogenic treatment. With regard to treatment, the groups with intermediate and low risk do not show any significant differences. Regarding the three risk groups shown in Figure 2B for the Hamburg dataset (outcome: recurrence or death) we found a hazard ratio (HR) of 9.1 [95% confidence interval (CI) =3.59-23.05] for the high-risk  $ESR1^-/PGR^-$  as compared to the low-risk group and a HR of 3.63 (95% CI=1.46-9.08) for the intermediate-risk group.

*GSE 3494 dataset (evaluation dataset 1).* We used the publicly available chip dataset GSE 3494 (12) for validation. For sample selection we applied the same rules as for the

Hamburg collective including 156 samples (111 controls, 45 events, event rate=0.26). Although chip data are scaled differently, the same pattern is visible (Figure 2C): a double-negative high-risk group ( $PGR+ESR1 < 2250$ : 24 controls and 20 events, event rate=0.45), a medium-risk group ( $PGR+ESR1 > 2250$  and  $PGR/ESR1 < 0.33$ : 53 controls, 23 events, event rate=0.30) and a low-risk group with the most favorable outcome ( $PGR+ESR1 > 2250$  and  $PGR/ESR1 > 0.33$ : 34 controls, two events, event rate=0.056).

Significant clinical parameters for the intermediate-risk group were nodal status ( $p=0.0042$ ), tumor grade ( $p=0.012$ ) and size ( $p=0.01$ ). Data are summarized in Tables I and II. After eliminating all receptor-negative cases, 127 invasive steroid receptor-positive cases were used for the survival analysis (Figure 3). In a univariate analysis, patients with a high PGR/ESR1 ratio had a significantly better survival (Figure 3). However, this result was not confirmed in the multivariate Cox regression (Table II).

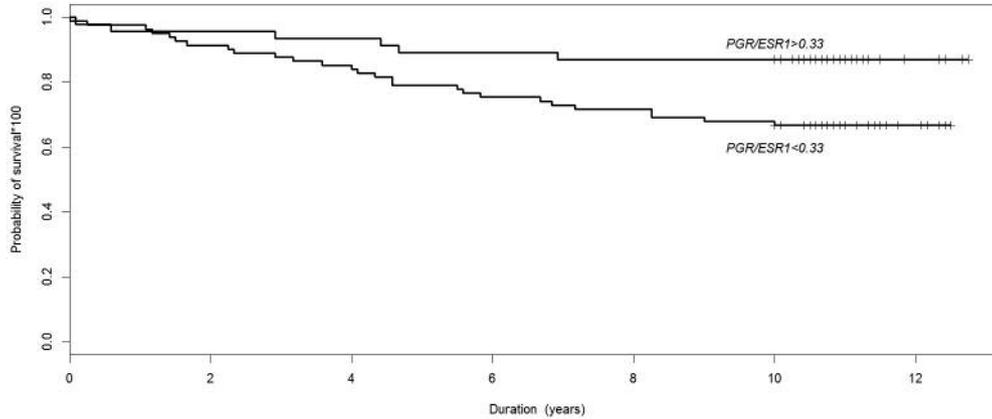


Figure 3. Kaplan–Meier plot of the GSE3494 dataset for 127 receptor-positive patients. The cut-off value for the ESR1/PGR mRNA ratio was 0.33.

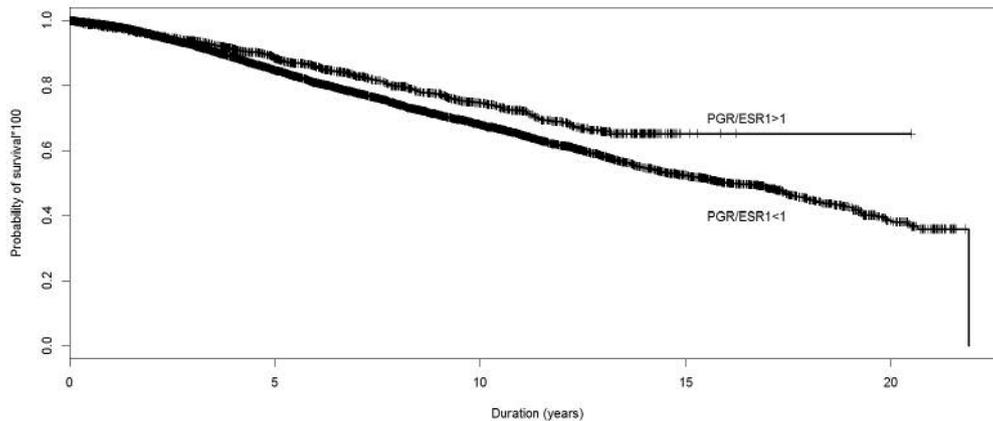


Figure 4. Kaplan–Meier plot for the Stuttgart dataset (N=10,066) based on the semiquantitative Remmele scores by immunohistochemistry. The cut-off value for the ESR1/PGR protein ratio was 1.

Survival analysis of the Stuttgart dataset (evaluation dataset 2). We used the IHC-based, semiquantitative Remmele scores for ESR1 and PGR to calculate the PGR+ESR1 ratio (equivalent to the qRT-PCR ratio). After defining a suitable cut-off (PGR/ESR1=1.0), we identified two significantly different populations with respect to disease outcome. After excluding all ESR1<sup>-</sup> and PGR<sup>-</sup> double-negative cases, we observed no statistical significance for the ESR1 (Remmele score cut-off=8,  $p=0.113$ ). However, PGR (cut-off=8,  $p=0.006$ ) and the PGR/ESR1 ratio ( $p=0.00002$ ) were still significantly correlated to disease outcome. In a Cox regression analysis of all three variables, the PGR/ESR1 ratio was significantly correlated with disease outcome in multivariate analysis (HR=0.82, 95% CI=0.70-0.95;  $p=0.01$ ) (Table II and Figure 4).

When we calculated three risk groups in a similar approach as with the PCR data, we found an HR of 1.4 (95%

CI=1.26-1.55) for the receptor-negative high-risk group compared to the low-risk group. Comparing the intermediate-risk group with the low-risk group, we found an HR of 1.30 (95% CI=1.16-1.66), confirming the impact of the PGR/ESR1 quotient for this IHC approach.

Comparison of the three collectives. The three collectives were not consistent with regard to survival ( $p=0.00028$ ). In the Hamburg collective, 5-year DFS was 80.3% (95% CI=74.7-86.4%, patients at risk=116), 5-year OS was 87.8% (95% CI=83.0-92.9%, patients at risk=116) and 10-year survival was not reliable (too few patients at risk, N=17). For the GSE3494 collective, 5-year survival was 82.7% (95% CI=76.4-89.5%, patients at risk=106) and the 10-year-survival was 74% (95% CI=interval 66.8-82.0%, patients at risk=95). In the Stuttgart collective, 5-year survival was

Table II. Comparison of cases double receptor-positive by quantitative reverse transcription polymerase chain reaction (Hamburg), Affymetrix chip (GSE3494) and immunohistochemistry (Stuttgart) with respect to estrogen receptor (ESR1), progesterone receptor (PGR) and their ratio as univariate or multivariate prognostic factors

| Study collective | Hamburg N=167 |           |           | GSE3494 N=127 |           |           | Stuttgart N=10066 |           |           |
|------------------|---------------|-----------|-----------|---------------|-----------|-----------|-------------------|-----------|-----------|
|                  | ESR1          | PGR       | PGR/ESR1  | ESR1          | PGR       | PGR/ESR1  | ESR1              | PGR       | PGR/ESR1  |
| Cutoff criteria  | >10.61        | >12.4     | >1.5      | >3680         | >925      | >0.33     | >8                | >8        | >1        |
| Controls>Cutoff  | 49            | 63        | 67        | 30            | 40        | 40        | 2890              | 2000      | 1142      |
| Events*>Cutoff   | 21            | 7         | 5         | 13            | 6         | 6         | 504               | 345       | 262       |
| Controls<Cutoff  | 76            | 62        | 58        | 64            | 54        | 54        | 4755              | 5645      | 6503      |
| Events*<Cutoff   | 21            | 35        | 37        | 20            | 27        | 27        | 1917              | 2076      | 2159      |
| Sum controls     | 125           | 125       | 125       | 94            | 94        | 94        | 7645              | 7645      | 7645      |
| Sum events*      | 42            | 42        | 42        | 33            | 33        | 33        | 2421              | 2421      | 2421      |
| Controls+events  | 167           | 167       | 167       | 127           | 127       | 127       | 10066             | 10066     | 10066     |
| Univariate HR    | 1.52          | 0.23      | 0.15      | 1.34          | 0.36      | 0.35      | 1.08              | 0.85      | 0.76      |
| 95% CI           | 0.83-2.79     | 0.10-0.53 | 0.06-0.38 | 0.67-2.70     | 0.15-0.87 | 0.15-0.86 | 0.98-1.12         | 0.76-0.95 | 0.67-0.86 |
| p-Value          | 0.17          | <0.001    | <0.001    | 0.41          | 0.023     | 0.02      | 0.113             | 0.006     | <0.001    |
| Multivariate HR  | 0.98          | 0.48      | 0.22      | 1.41          | 0.45      | 0.56      | 1.11              | 0.88      | 0.82      |
| 95% CI           | 0.51-1.89     | 0.19-1.23 | 0.07-0.65 | 0.67-2.93     | 0.16-1.29 | 0.20-1.60 | 0.99-1.25         | 0.76-1.02 | 0.70-0.95 |
| p-Value          | 0.95          | 0.13      | 0.007     | 0.36          | 0.14      | 0.28      | 0.08              | 0.1       | 0.01      |

HR: Hazard ratio; CI: confidence interval; \*Events: recurrence or death (Hamburg data set) or death (GSE3494 and Stuttgart datasets).

80.6% (95% CI=80.6-83.3%, patients at risk 4768) and the 10-year survival was 65.8% (95% CI=64.5-67.1%, patients at risk 1996). It was evident that the PGR/ESR1 or its IHC equivalent was a univariate prognostic factor for all three collectives (Table II). However, in a multivariate Cox analysis, we found the PGR/ESR1 by qRT-PCR and IHC to be independent prognostic factors only in the Hamburg and Stuttgart data sets ( $p=0.0007$  and  $0.01$ , respectively). For prediction of disease outcome, models with clinically relevant variables are mandatory. In the models we used for the Hamburg dataset (Table III), pT, pN and the PGR/ESR1 ratio (with or without stratification) were significant multivariate predictors of disease outcome. For the GSE dataset, the PGR/ESR1 quotient was a univariate, but not a multivariate predictor of disease outcome (Figure 3, Table III). For the Stuttgart dataset, tumor stage, grade and age (>50 years) and the IHC PGR/ESR1 quotient (Figure 4) (regardless of stratification for pT) were statistically significant predictors of OS.

## Discussion

In this study, we present data showing that the expression ratio of PGR/ESR1 by qRT-PCR and IHC could be a useful additional parameter for risk stratification within the receptor-positive subgroup of invasive breast cancer. This was first shown in a hypothesis-generating dataset of selected patients (Hamburg dataset). This new parameter is obviously strongest in the identification of low-risk patients: the majority of double-positive cases with a high PGR/ESR1

ratio had a good clinical outcome. We validated this observation in two other breast cancer datasets (GSE 3494 and Stuttgart collective). In both, the PGR/ESR1 ratio or its equivalent by IHC significantly predicted the disease outcome in univariate analysis (Table II, Figures 3 and 4). Even in multivariate analysis, the receptor ratio was a significant predictor of disease outcome ( $p<0.01$ ) in the Hamburg and Stuttgart collectives (Table II). This finding is in line with the hypothesis tested, namely that PGR (if enough ligand is available) drives BC tumor cells towards differentiation, whereas ESR1 (if enough ligand is available) drives tumor-cell proliferation (18). This hypothesis is based on the clinical observation of the effects ESR1 and PGR on endometrium and some cell-culture experiments, which demonstrate growth-inhibitory and differentiating effects of progesterone on breast cancer cells (18).

It should be noted, however, that not all chip datasets tested would support our findings. One possible reason could be a difference in mRNA stability of the two receptors. The Hamburg and the GSE3494 data were both generated using fresh-frozen tumor material with very limited mRNA decay. In our experience, PGR mRNA is much more susceptible to degradation than its ESR1 counterpart, which is especially true for formalin fixed paraffin embedded material (data not shown). Greater PGR mRNA degradation would reduce the PGR/ESR1 expression ratio, having a detrimental effect on this stratification marker.

To our knowledge, this report is only the second example for the prognostic/predictive value of a gene-expression ratio. The H:I index (19, 20) is based on the two apparently

Table III. Models for prediction of survival

| Variable                              | Coef. | (HR)    | 95% CI      | p-Value            |
|---------------------------------------|-------|---------|-------------|--------------------|
| Hamburg dataset                       |       |         |             |                    |
| pT2                                   | -0.05 | (0.95)  | 0.47-1.90   | 0.88               |
| pT3                                   | 2.94  | (20.00) | 2.06-175.45 | <b>0.009</b>       |
| pN1                                   | 0.19  | (1.21)  | 0.59-2.48   | 0.61               |
| pN2/3                                 | 2.02  | (7.55)  | 2.64-21.60  | <b>0.00016</b>     |
| Grade 2                               | 0.02  | (1.02)  | 0.34-3.03   | 0.98               |
| Grade 3                               | 0.02  | (0.97)  | 0.29-3.29   | 0.97               |
| Age >50 years                         | -0.25 | (0.78)  | 0.38-1.60   | 0.50               |
| PGR/ESR1 >1.5                         | -1.80 | (0.17)  | 0.06-0.44   | <b>0.00033</b>     |
| PGR/ESR1 >1.5*                        | -1.81 | (0.16)  | 0.06-0.44   | <b>0.00032</b>     |
| GSE3494 dataset                       |       |         |             |                    |
| pT2                                   | 1.26  | (3.52)  | 1.43-8.67   | <b>0.007</b>       |
| pT3                                   | 3.00  | (20.11) | 3.11-129.9  | <b>0.001</b>       |
| pN1/2/3 without further specification | 1.20  | (3.32)  | 1.46-7.51   | <b>0.004</b>       |
| Grade 2                               | 0.37  | (1.45)  | 0.53-3.99   | 0.48               |
| Grade 3                               | 0.74  | (2.10)  | 0.60-7.40   | 0.25               |
| Age >50 years                         | -0.19 | (0.83)  | 0.32-2.71   | 0.70               |
| PGR/ESR1 >0.33                        | -0.58 | (0.56)  | 0.20-1.52   | 0.26               |
| PGR/ESR1 >0.33*                       | -0.71 | (0.49)  |             | 0.19               |
| Stuttgart dataset                     |       |         |             |                    |
| Stage II**                            | 0.64  | (1.90)  | 1.68-2.14   | <b>&lt;0.00001</b> |
| Stage III**                           | 1.42  | (4.13)  | 3.58-4.77   | <b>&lt;0.00001</b> |
| Stage IV**                            | 2.61  | (13.66) | 11.60-16.09 | <b>&lt;0.00001</b> |
| Grade 2                               | 0.15  | (1.16)  | 0.99-1.37   | 0.07               |
| Grade 3                               | 0.33  | (1.39)  | 1.17-1.66   | <b>0.0003</b>      |
| Age >50 years                         | 0.10  | (1.11)  | 0.99-1.23   | 0.07               |
| IHC: PGR/ESR1 >1                      | -0.18 | (0.84)  | 0.73-0.97   | <b>0.018</b>       |
| IHC: PGR/ESR1 >1*                     | -0.20 | (0.87)  | 0.70-0.94   | <b>0.007</b>       |

ESR1: Estrogen receptor; PGR: progesterone receptor; IHC: immunohistochemistry; Coefficient; HR: hazard ratio; CI: confidence interval; \*Stratification for pT was necessary as the Cox assumption was not given for pT. \*\* Assessment of stage was only possible in the Stuttgart collective. Significant p-values are shown in bold.

unrelated genes homeobox B13 (*HOXB13*) and interleukin 17 receptor B (*IL17BR*) that exhibit opposite expression patterns with regard to clinical outcome. For the 'related' genes *PGR* and *ESR1*, the expression ratio might indeed reflect the underlying biology.

### Conclusion

Our data indicate that the expression ratio of the two well-established hormone receptors PGR and ESR1 is a useful parameter for further improving risk stratification in BC. This observation for the Hamburg data set was confirmed in two other datasets in univariate data analysis, and in one of the validation datasets, also in a multivariate data analysis with clinical relevant variables.

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