Development and validation of a gene profile predicting benefit of post-mastectomy radiotherapy in high risk breast cancer patients: A study of gene expression in the DBCG82bc cohort

Trine Tramm[1*], Hayat Mohammed[2*], Simen Myhre[3,4,5], Marianne Kyndi[1], Jan Alsner[1], Anne-Lise Børresen-Dale[3,4], Therese Sørlie[3,4], Arnoldo Frigessi[2^], Jens Overgaard[1^]

* joint first authorship

^ joint last authorship

1: Dept. of Experimental Clinical Oncology, Aarhus University Hospital, Denmark

2: Dept. of Biostatistics, Institute of Basic Medical Sciences, University Oslo, Norway

3: Dept. of Genetics, Institute of Cancer Research, Oslo University Hospital, Radiumhospitalet, Norway

4: K-G. Jebsen Center for Breast Cancer Research, Institute of Clinical Medicine, Faculty of Medicine, University Oslo, Norway

5: Atlantis Medical University College, Oslo, Norway

Corresponding author: Trine Tramm, Dept. of Experimental Clinical Oncology,
Aarhus University Hospital**,** Nørrebrogade 44, building 5, 8000 Aarhus C, Denmark
E-mail: tramm@oncology.dk
Telephone number: +45 78 46 26 20
Fax number: +45 86 19 71 09

Number of pages: 28

Number of tables: 2

Number of figures: 2

Running head: Gene profile predictive of postmastectomy radiotherapy

Keywords: Breast cancer; Gene expression profiling; post-mastectomy radiotherapy; prediction; prognosis

Statement of Translational relevance:

Post-mastectomy radiotherapy (PMRT) is currently administered according to a clinico-pathological estimation of the patient's risk of local recurrence (LRR). The presented DBCG-RT gene profile consistently predicts LRR in patients with early breast cancer and predicts benefit from PMRT. The DBCG-RT profile identifies a subgroup of patients with low risk of LRR, who from traditional clinico-pathological risk-estimation would be considered to have a high risk of LRR and as such eligible for PMRT. These patients defined by the DBCG-RT profile as having a low risk of LRR and no additional benefit from PMRT in terms of increased local control may potentially be spared the risk of radiation-induced morbidity and secondary cancer.

The DBCG-RT profile works on routinely processed formalin fixed, paraffin embedded tissue and is potentially applicable in a daily clinical setting.

Abstract

Purpose: To identify genes predicting benefit of radiotherapy in high-risk breast cancer patients treated with systemic therapy and randomized to receive or not receive post-mastectomy radiotherapy (PMRT).

Experimental Design: The study was based on the Danish Breast Cancer Cooperative Group (DBCG82bc) cohort. Gene-expression analysis was performed in a training set of frozen tumor-tissue from 191 patients. Genes were identified through the Lasso method with the endpoint being loco-regional recurrence (LRR). A weighted gene-expression index (DBCG-RT profile) was calculated and transferred to quantitative Real-Time Polymerase Chain Reaction (RT-qPCR) in corresponding formalin-fixed, paraffin-embedded tissue (FFPE), before validation in FFPE from 112 additional patients.

Results: Seven genes were identified, and the derived DBCG-RT profile divided the 191 patients into a "high LRR risk" and "low LRR risk" group. PMRT significantly reduced risk of LRR in "high LRR risk" patients, whereas "low LRR risk" patients showed no additional reduction in LRR rate. Technical transfer of the DBCG-RT profile to FFPE/RT-qPCR was successful, and the predictive impact was successfully validated in another 112 patients.

Conclusions: A DBCG-RT gene profile was identified and validated, identifying patients with very low risk of LRR and no benefit from PMRT. The profile may provide a method to individualize treatment with PMRT.

Introduction

Radiotherapy is known to improve loco-regional control, disease free survival (DFS) and to have a long-term improvement on overall survival (OS) in high-risk patients suffering from breast cancer (1). Post-mastectomy radiotherapy (PMRT) is currently administered according to clinico-pathological criteria, defining the patient's *a priori* risk of subsequent loco-regional recurrence (LRR), and not according to individual prediction of the likelihood of benefit from radiotherapy.

Results from the randomized trials, Danish Breast Cancer Cooperative Group (DBCG) protocol 82bc and the British Columbia Randomized Radiation trial, showed a substantial OS benefit after PMRT in patients with 1-3 positive nodes (2-4), and this has been supported by the Early Breast Cancer Trialists´ Collaborative Group (EBCTCG) 2014 overview (5) as well. Recommendation for PMRT is especially consolidated in patients estimated to have a high risk of LRR (e.g. involvement of ≥4 lymph nodes or tumorsize > 5 cm) (6, 7). The most recent St. Gallen Consensus (2011) (8) further supports the administration of PMRT to patients < 45 years of age with 1-3 positive nodes, and to patients of all ages if 1-3 positive lymph nodes are accompanied by evidence of lympho-vascular invasion by histology.

The beneficial effect of PMRT is, however, suspected to be more heterogeneous than the conventional clinico-pathological parameters are capable of describing. Gene expression profiles predictive of response to e.g. docetaxel (9), and prognostic in terms of OS and distant metastasis (DM) have been published (10-14), but currently no validated biomarkers or molecular profiles are available to assist the radiation oncologist in stratifying a more individualized approach to adjuvant radiotherapy.

In a study of biological parameters in 1001 patients from the DBCG82bc cohort (15), four subgroups approximating the intrinsic subtypes of breast cancer described by Perou and Sørlie (12, 13) was constructed from immunohistochemical information for estrogen receptor (ER), progesterone receptor (PR) and HER2/neu-receptor (HER2). Triple negativity or having a HER2-like tumor (ER-/PR-/HER2+) was associated with significantly increased risk of LRR in a

multivariate analysis, but the largest absolute reduction in LRR rate and largest translation of LRR rate reduction into survival benefit were observed among patients with the most advantageous prognostic features (ER/PR+ and HER2-). The studies on the DBCG82bc cohort (2, 3, 15, 16) have, however, not been able to localize any subgroups lacking benefit from PMRT in terms of LRR.

Preventing LRR is of high priority with respect to the distressful situation for the patient, as well as in the sense that a LRR can act as nidus for subsequent DM. Radiotherapy is at the same time associated with early and late side-effects leading to morbidity of possible considerable consequences for the patient (17-20).

It would, therefore, be desirable if a more refined partitioning of patients likely to benefit from PMRT could be established, and it can be hypothesized that a molecular signature predictive of LRR and radio-therapeutic outcome could add more specific and individualized information than the current clinico-pathological risk estimation.

In the present study, we describe a gene-profile associated with risk of LRR in patients not receiving PMRT, and predictive of benefit from adjuvant PMRT in a cohort of high-risk breast cancer patients (DBCG82bc) treated with systemic treatment and randomized to receive or not to receive PMRT.

Material and Methods

Patients

The DBCG82bc cohort has been described in detail elsewhere (2, 3, 21, 22). In brief, 3083 high-risk breast cancer patients (< 70 years of age) treated with mastectomy and partial axillary dissection were included in the period 1982-1990. All patients were randomized to PMRT. Pre-menopausal women (DBCG82b) were treated with cyclophosphamide, methotrexate, and fluorouracil (CMF), and post-menopausal women (DBCG82c) were treated with Tamoxifen. Radiotherapy was delivered as an anterior photon field against the supraclavicular, infraclavicular and axillary lymph nodes, and an anterior electron field against the chest wall and intramammary lymph nodes. Intended dose was 50 Gy/25 fractions/ 5 weeks, or 48 Gy/ 22 fractions/ 5 ½ weeks (2, 3, 23). A median of 7 axillary lymph nodes were removed.

Methods

Fresh frozen tumor (FFT) samples were available from 273 DBCG82bc patients. Extraction of mRNA from FFT, and microarray analysis was performed as described in detail in Myhre *et al.* (24). Microarray analysis was successful in 70% (191/273) of the frozen samples, containing > 5% invasive carcinoma (Supplementary Figure 1), and these constituted the training set. The microarray data has previously been published and submitted to GEO with accession number GSE24117 (24).

To identify a gene profile that is predictive of benefit from adjuvant PMRT, genes whose expression levels interacted with PMRT on the association with LRR, were first identified through a two-step Cox Proportional Hazard model with Lasso penalty (25), and a weighted index (CVSI), based on the expression levels of the identified genes, was calculated as described in detail in Supplementary Document 1. This was performed in all 191 patients of the training set. The number of patients receiving CMF in the group of patients randomized to PMRT (46/97, 47%) was not statistically significant from non-irradiated patients receiving CMF (41/94, 44%) (p=0.66).

Secondly, the prognostic value of the gene profile was tested in the subset of 94/191 non-irradiated patients.

From the rest of the DBCG82bc cohort, only FFPE samples were available. Therefore, a technical transfer of the identified genes and the derived index to RT-qPCR and FFPE was needed in order to proceed to validation. The technical transfer was carried out in 146/191 patients of the training set, from whom corresponding FFPE samples with >5% invasive carcinoma were available (Supplementary Figure 1). Details on transfer of technology and recalculation of the index in order to make it independent of the training set is described in Supplementary Document 2.

The DBCG82bc cohort has previously been criticized for a limited axillary surgical procedure, potentially influencing the rate of local recurrences in the cohort. Therefore, the validation set was chosen to originate from the subgroup of 1001 DBCG82bc patients with the most extensive axillary surgery (>7 lymph nodes removed) and with FFPE with histologically verified tumor content available (Supplementary Figure 2), previously included in the study by Kyndi *et al* (15). The more extensive surgical procedure in the validation set was expected to be associated with a lower LRR rate in this group of patients.

Extraction of mRNA from FFPE using the Tissue Preparation System together with VERSANT Tissue Preparation Reagents (Siemens Healthcare Diagnostics, Tarrytown, NY), and subsequent RT-qPCR using the Fluidigm Biomark 96.96 dynamic gene expression system, preceded by a pre-amplification step, was carried out as previously described (26-29). Inventoried TaqMan gene expression assays (Applied Biosystems, Foster City, USA) were used if available (Supplementary Table 2). ER- and HER2–receptor status was available from IHC analyses on Tissue Micro Arrays (TMA) (30). If missing IHC values, ER was supplemented with original biochemical analyses (31) retrieved from clinical records due to missing IHC results (15% = 29/191 patients), and HER2 with gene expression-derived *ERBB2* status (16% =31/191 patients), both known to correlate well to the IHC status (27). All assays were performed blinded to the study endpoint.

Statistical analysis:

The endpoint considered was LRR. In agreement with the original publications on the cohort (2, 3), LRR was defined as the appearance of local or regional disease (chestwall, axilla, supra/infraclavicular) occurring as an isolated event, or at least 1 month before DM, or simultaneously with DM within +/- 1 month. LRR occurring more than 1 month after DM was censored at time to DM, and did not count as a LRR. Patients with DM and no LRR were censored at DM-time, and patients with neither DM nor LRR were censored at last date of vital-status/follow-up (2, 3). The date for assessment of recurrence and vital status was January 1st 2012.

The Fisher exact test was used for testing relationship between categorical variables as well as between the CVSI index calculated from FFT and FFPE in the training set. A competing risk model was used for calculating cumulative incidence with inclusion of death before LRR or development of DM as competing events. Cumulative incidence probability curves were plotted and tested for differences (Wald test). Cox uni- and multivariate regression analyses were performed, and assumptions of proportional hazards were tested graphically using log-minus-log plots. Level of significance was 5%, and all estimated p-values were two-sided. Statistical calculations were performed using STATA version 11.2 (StataCorp, College Station, TX) and R (Development Core Team (2011) (32).

Results

Clinical characteristics and outcome description

In the training set, 53/191 patients with FFT available experienced a LRR, and 40/146 patients included in the technical transfer experienced a LRR. In the validation set, 20/112 patients experienced a LRR. The difference in number of events in the training set and validation set was not significant ($p > 0.05$). Median follow-up time was 25.1 years for patients in the training set, and 24.6 years for patients in the validation set. The median age of the patients included in the validation set was lower than in the training set (Table 1 and Suppl. Table 1) with a higher fraction of pre-menopausal women in the validation set ($p=0.006$). The validation set further included a higher fraction of patients with small tumors (< 2 cm) ($p=0.001$) and HER2 negative tumors ($p<0.0001$), but the distribution of other clinical parameters were similar.

Median tumor area fraction was 50% (range: 5-85%) in the 191 frozen samples of the training set, 60% in the corresponding 146 FFPE samples (range: 5-100%), and 60% (range: 5-100%) in the 112 FFPE samples of the final validation set.

Development of a radiation profile in the training set:

From the microarray data, 7 probes whose transcripts interact with PMRT to modify the hazard of LRR were identified (*HLA-DQA, RGS1, DNALI1, hCG2023290, IGKC, OR8G2, ADH1B*). A weighted CVSI index based on the 7 probes was calculated (Supplementary document 1), and the 191 patients were ranked according to the size of the index. Based on a cumulated odds-ratio plot, a cut off was chosen that separated the patients of the training set into two groups: “low LRR risk” (high index) and “high LRR risk" (low index) (for details, see Supplementary Document 1). The cut off coincided with the upper quartile of the CVSI index. In both risk prediction groups, the patients were well-distributed between the two randomization arms for all evaluated clinico-pathological parameters. A “low LRR risk” group could be identified among all clinico-pathological subgroups, even in subgroups with tumorsize > 5 cm or ≥ 4 positive lymph nodes (Table 1).

There was no statistically significant difference in the distribution of clinico-pathological parameters between the two groups, except for ER.

Prognostic impact in the non-irradiated group of patients in the training set:

The prognostic value of the gene-profile (DBCG-RT profile) was evaluated in the non-irradiated subgroup of the training set. Among the 94 patients treated with systemic treatment alone, the "high LRR risk" patients was shown to have significantly higher risk of LRR as compared to the patients with "low LRR risk" (57% *vs.* 8% at 20 years; p<0.0001; unadjusted hazard ratio [HR] = 0.09; 95% confidence intervals [CI], 0.02 to 0.36) (Figure 1). The DBCG-RT profile remained a significant prognostic factor for local failure in a multivariate Cox regression analysis, when adjusting for lymph node status and locally advanced disease (tumorsize > 5 cm or skin- or fascia invasion) (p<0.0001, adjusted HR = 0.07 [0.02-0.30]).

Predicting benefit of PMRT in the training set:

When analyzing the two risk-groups separately in the training set of 191 patients and stratifying them according to PMRT, a predictive value can be seen in Figure 2A-B. Radiotherapy significantly improved local control rates in "high LRR risk" patients (57% *vs.* 12% at 20 years; p<0.0001; adjusted HR = 0.17; 95%CI, 0.08 to 0.34) (Figure 2A); equalizing the rate to "low LRR risk" patients, who showed no additional improvement of local control by PMRT (8% *vs.* 9% at 20 years; p=0.93; adjusted HR = 1.13; 95%CI, 0.14-9.15) (Figure 2B). Unadjusted hazard ratios can be seen in Table 2. The DBCG-RT profile identified a subset of patients with a "low LRR risk" profile even among subgroups traditionally considered to have a high risk of recurrence, and benefit from PMRT was, on the other hand, found for "High LRR risk" patients even when having small tumors (< 2 cm) (Supplementary Figure 3A) or 1-3 positive lymph nodes (Supplementary Figure 3B). Benefit from PMRT was also observed for patients with a "High LRR risk" regardless of ER status (Supplementary Figure 3C), menopausalstatus, or age < or ≥ 50 years (data not shown). When combining tumorsize and number of positive lymph nodes into subgroups of clinical relevance for

current treatment decision making, benefit from PMRT could also be found for patients with small tumors and few involved lymph nodes (T1-2, 1-3 positive lymph nodes) (Supplementary Figure 3D).

The technical transfer of the DBCG-RT profile to RT-qPCR was successfully carried out in 146/191 patients (For details, see Supplementary Document 2). The 146 patients were representative for the training set in terms of clinico-pathological variables (Supplementary Table 3), and did not differ statistically significantly from the 45 patients excluded (Supplementary Table 4). The DBCG-RT profile was modified according to availability of suitable PCR assays, and success-rate of detecting the genes in FFPE. Therefore, the FFPE/RT-qPCR modified DBCG-RT profile was based on only 4 of the 7 interaction genes as described in Supplementary Document 2. The gene expression levels of these 4 genes (*IGKC, RGS1, ADH1B, DNALI1*) correlated significantly with the expression levels measured by array technology on the corresponding FFT. There was also a significant correlation between the original FFT/array based classification of the 146 patients and classification based on corresponding FFPE samples with RT-qPCR ($p < 0.0001$). The FFPE/RT-qPCR based classification provided the same predictive value, when stratified according to PMRT (Figure 2 C, D and Table 2) as the FFT/array based classification (Figure 2 A,B). The FFPE/RT-qPCR index cut-off value for dividing patients into "high LRR risk" and "low LRR risk" patients, respectively, was -1.1.

Validation of the predictive impact in the DBCG82bc cohort:

Detection of the 4 genes in the FFPE/RT-qPCR modified DBCG-RT profile was successful in 112 validation samples (Supplementary Figure 2). Using the defined FFPE/RT-qPCR index cut-off value of -1.1 as determined in the technical transfer part of the analysis, 22 patients (20%) were designated as "low LRR risk" and 90 patients (80%) as "high LRR risk". The predictive value was validated in the 112 patients, and a significant benefit from radiotherapy in terms of local control could be seen in the "high LRR risk" patients (30% *vs.* 7%; p=0.003, adjusted HR = 0.09; 95% CI,

0.02 to 0.31) (Figure 2E), whereas "low LRR risk" patients did not show benefit (8% *vs.* 0%; p=0.30, adjusted HR not estimated) (Figure 2F) (See Table 2 for unadjusted hazard ratios).

There was no statistically significant difference in the distribution of clinico-pathological variables between the two risk-groups, except for HER2 (p=0.04) (Supplementary Table 1).

When combining tumor size and number of positive lymph nodes into subgroups, there was no statistically significant difference in the distribution of the two risk groups as determined by the DBCG-RT profile for any of the three patient cohorts (Supplementary Table 5).

Discussion

The present study is the first to define and validate a gene-profile associated with risk of LRR in patients not receiving PMRT, and predictive of benefit from adjuvant PMRT in a cohort of high-risk breast cancer patients (DBCG82bc) treated with adjuvant systemic treatment and randomized to receive or not to receive PMRT.

Prognostic gene-expression profiles predicting risk of LRR after breast conserving therapy (BCT) (33-36) and after mastectomy (37) has been published previously, but none of them have been successfully validated. The study by Cheng *et al.* (37) identified 34 genes with significant association with LRR. There is no overlap between the 34 genes and the genes identified in this study. Neither was there any overlap with the genes in a 10-gene signature predictive of cellular radiosensitivity that has been developed from microarray analysis on 35 cell lines (38), and subsequently clinically validated, primarily in terms of risk of DM, in two breast cancer data set (39). Two studies by Nuyten *et al.* and Kreike *et al.* (33, 40), did not identify a specific gene-set predicting risk of recurrence after BCT, though a gene-profile based on the wound response signature was described as being of independent prognostic value. Later, the same group developed a 111-gene signature (35), but it did not show independent prognostic value in multivariate analysis, and lost prognostic impact when tested in independent cohorts (34, 41). The gene-profile by Niméus-Malmström *et al.* (34), aiming to identify patients developing LRR despite of RT after BCT, was also unable to be validated in other cohorts (35, 36, 41), and the most recent study by Sabatier *et al*. (36) did not succeed in determining a robust prognostic signature of LRR.

The two LRR risk-groups in the present study could be identified among all clinico-pathological subgroups, and even among patients traditionally considered to have a high risk of LRR (e.g. tumorsize > 5 cm of ≥ 4 positive lymph nodes) the DBCG-RT profile identified patients with a very low risk of LRR. Equally, “high LRR risk” patients could be found among patients with 1-3 positive lymph nodes or tumorsize < 2 cm, and benefit from PMRT was observed in these patient

groups also. Indication for PMRT could be found even in the subgroup of patients with a combination of small/intermediate tumor size and few involved lymph nodes (T1-2 and 1-3 positive lymph nodes), where indication for PMRT has been controversial and a subject for substantial discussion over the last decade. This indicates that the presented DBCG-RT profile adds information to the conventional parameters used for present day treatment decision making. The distribution of the risk groups suggests that the ER-negativity adds to the poor prognostic impact of the "high LRR risk" group, contributing to the fact that this study finds a greater benefit from PMRT in ER-negative patients as compared to the preceding study of this cohort (15). The DBCG-RT profile has in a related study (42) been found to be independent of the intrinsic subtypes as determined by either immunohistochemistry (e.g. ER, PR and HER2) or gene expression profiling, and a "Low LRR risk" and a "High LRR risk" group was in this study found among all subtypes, even among the Luminal A subtype.

Of the 7 probes identified from the analysis of FFT, four (*IGKC, RGS1, ADH1B, DNALI1*) were successfully transferred to corresponding FFPE. One of the probes (*hCG2023290*) did not recognize a known gene, and a suitable assay could not be found for *OR8G2*. Of the 5 genes measured in FFPE, one (*HLA-DQA*) could only be detected in 10% of the samples and was omitted for further analysis. Explanations to the low success rate of detecting the four genes of the FFPE/RT-qPCR modified DBCG-RT profile in FFPE samples of the validation set could be *e.g.* low yields of mRNA caused by age of the paraffin block or technical issues. The identification of genes in FFPE is dependent on gene expression level as well as factors related to handling of the paraffin block. The age of the blocks is of great influence, since mRNA degrades with a half-life of 4.6 years (26) implying that the absolute expression values decreases with age of the blocks. This potentially leads to low detection rates further accentuated by low expressed genes. The initial selection of the genes based on FFT in this study did not take into account that the genes should be highly expressed. The patients were, however, reliably assigned to the two risk-groups based on the reduced set of 4 genes.

One of the genes included in the gene-profile (*IGKC*) has previously been reported as prognostic in terms of DM in breast cancer and able to predict response to antracycline-based neoadjuvant chemotherapy (43). *IGKC* is expected to be derived from mature tumor-infiltrating plasma cells, and as such related to a humoral immune response of presumably protective nature (43). The gene has also been reported as a prognostic marker for DFS and OS in node negative breast cancer patients (44). Increased expression of IGKC protein based on immunohistochemistry in non-small lung cancer has also been associated with improved outcome (45). Elevated expression of this gene might indicate an increased immune-response favorable in cancer treatment, and interestingly, among the top correlated genes to several of the seven identified genes, immune response and lymphocyte activation was found to be enriched, when analyzed by the DAVID and Reactome analysis tools. This suggests a potential impact of tumor infiltrating immune cells on the effect of PMRT on LRR.

RGS1 encodes a regulator of G protein signaling, and the gene has found to be overexpressed in melanoma (46). Furthermore, *RGS1* has in combination with two other genes (*NCOA3, SPP1)* been described as an independent predictor of DFS in malignant melanoma (47) but not in breast cancer. The expression of *RGS1* has further been studied in relation to lymphomas and hypoxia (48), and has been described to be associated with *HIF1α*. The association with hypoxia, known to be a factor related to radioresistance, is consistent with the present findings that tumors with a "low LRR risk" is not showing benefit from PMRT. Both *IGKC* and *RGS1* are included in the FFPE/RT-qPCR modified DBCG-RT profile. None of the 5 remaining genes have been described in association with intrinsic radiosensitivity, hypoxia or cell proliferation commonly considered influential in terms of radioresistance.

The increasing incidence of breast cancer patients and increasing ratio of breast conserving therapy (BCT) *vs.* mastectomies subsequently leads to an increasing number of patients who, now and in the future, will be offered adjuvant radiation therapy. The LRR rate has been observed to decrease, and the strategies for administration of RT must carefully counterbalance the benefits in terms of

increased survival and decreased risk of LRR against the risk of secondary morbidity, including ischemic heart disease and secondary cancer. The capability of the presented DBCG-RT profile in identifying patients likely to benefit from PMRT/RT needs to be examined in a prospective study in order to evaluate the prognostic and predictive impact in patients treated with present day systemic adjuvant treatment. Whether the prognostic and predictive impact of the DBCG-RT profile is applicable to BCT or not also needs testing in a prospective study.

In conclusion, we have identified and validated a DBCG-RT profile attaining prognostic and predictive impact in relation to adjuvant radiotherapy after mastectomy. The gene-profile allowed the identification of patients not benefitting from radiotherapy in terms of LRR, and describes characteristics not already embraced by clinico-pathological variables.

Ethical considerations: The study of the DBCG82bc cohort has been approved by the Regional Ethical Committee (Journal number 20030263)

Reporting Recommendation for Tumor Marker Prognostic studies (REMARK) criteria were adhered to, when reporting the results of the study (49).

Conflict of Interest Statement: All authors, except Marianne Kyndi, holds a patent on the presented gene-profile (international patent publication no. WO 2013/132354 A2)

Acknowledgements

We thank Prof. Jahn Nesland at Division of Pathology, Norwegian Radium Hospital, Oslo University Hospital for performing the initial histopathological evaluation of the frozen samples. We further thank Brita Singer Sørensen for assistance during processing of the raw data from RT-qPCR.

The study was financially supported by CIRRO - The Lundbeck Foundation Center for Interventional Research in Radiation Oncology and The Danish Council for Strategic Research; The Danish Council for Independent Research | Medical Sciences; The Danish Cancer Society; and Aarhus University; the Norwegian Research Council; Statistics for Innovation and the University of Oslo, Norwegian Cancer Society and Health region South East. The funding sources of the study have played no role in the design of the study design; in collection, analysis or interpretation of the data; in the writing of the manuscript or in the decision to submit the manuscript for publication.

Figure legends

Figure 1. Plot of cumulative incidence proportion of loco-regional recurrence (LRR) risk showing a prognostic impact of the identified DBCG-RT profile. LRR risk in the subgroup of 94 patients in the training set of high-risk breast cancer patients who were treated with systemic treatment alone and did not receive post-mastectomy radiotherapy (PMRT), is plotted as a function of two risk groups ("high LRR risk" *vs.* "low LRR risk"). 20-year actuarial recurrence probabilities are stated in the figure. 95% CIs are presented for hazard ratios (HR) adjusted for lymph nodal status and advanced local disease. P-values are tested by log-rank test.

Figure 2. Predictive impact of the identified DBCG-RT profile is presented in the training set of 191 patients (A,B), in the subset of 146 patients from the training set, where FFPE was available (C,D), and in 112 high-risk breast cancer patients constituting the validation set (E,F). Patients are divided according to risk group, and cumulative incidence proportions of loco-regional recurrence (LRR) plotted as a function of PMRT in "high LRR risk" groups (A,C,E) and "low LRR risk" groups (B,D,F). 20-year actuarial recurrence probabilities are stated in the figures. 95% CIs are presented for hazard ratios adjusted for lymph nodal status and advanced local disease. P-values are tested by log-rank test.

Table 1. Distribution of clinico-pathological parameters among 191 patients included in the training set, randomly assigned to receive post-mastectomy radiotherapy (PMRT) or not receive PMRT (No PMRT), and stratified according to the DBCG-RT profile ("low LRR risk" *vs.* "high LRR risk") determined from microarray analysis based on fresh frozen tissue.

Training set	All patients (n=191)		"Low LRR risk" (n=48)				"High LRR risk" (n= 143)				P*
			No PMRT (n= 25)		PMRT (n=23)		No PMRT (n= 69)		PMRT (n=74)		
Patient/tumor data	*n*	*(%)*	*n*	*(%)*	*n*	*(%)*	*n*	*(%)*	*n*	*(%)*	
Age (years)											0.29
Median	55		61		53		56		55		
Range	(30-68)		(36-68)		(39-68)		(31-68)		(30-68)		
< 50 years	63	*33*	9	*36*	10	*43*	23	*33*	21	*28*	
≥ 50 years	128	*67*	16	*64*	13	*57*	46	*67*	53	*72*	
Menopausal status											1.00
Pre-menopausal	87	*46*	9	*36*	13	*57*	32	*46*	33	*45*	
Post-menopausal	104	*54*	16	*64*	10	*43*	37	*54*	41	*55*	
Tumour size											0.44
< 20 mm	56	*29*	6	*24*	5	*22*	19	*28*	26	*35*	
21-50mm	107	*56*	15	*60*	16	*70*	37	*54*	39	*53*	
> 50 mm	28	*15*	4	*16*	2	*9*	13	*19*	9	*12*	
Malignancy grade											0.12
Grade I	41	*21*	8	*32*	6	*26*	16	*23*	11	*15*	
Grade II	97	*51*	13	*52*	13	*57*	32	*46*	39	*53*	
Grade III	45	*24*	4	*16*	4	*17*	17	*25*	20	*27*	
Unknown	8	*4*	0	*0*	0	*0*	4	*6*	4	*5*	
Histology type											0.95
Ductal carcinoma	160	*84*	21	*84*	21	*91*	54	*78*	64	*86*	
Lobular carcinoma	24	*13*	3	*12*	2	*9*	11	*16*	8	*11*	
Other carcinomas	6	*3*	1	*4*	0	*0*	4	*6*	1	*1*	
Unknown	1	*1*	0	*0*	0	*0*	0	*0*	1	*1*	
Estrogen receptor status†											<0.001
Negative	53	*28*	1	*4*	3	*13*	26	*38*	23	*31*	
Positive	138	*72*	24	*96*	20	*87*	43	*62*	51	*69*	
HER2 status‡											0.13
Negative	143	*75*	22	*88*	18	*78*	49	*71*	54	*73*	
Positive	48	*25*	3	*12*	5	*22*	20	*29*	20	*27*	
Positive nodes											0.45
None	11	*6*	1	*4*	2	*9*	5	*7*	3	*4*	
1-3	98	*51*	15	*60*	13	*57*	32	*46*	38	*51*	
≥ 4	82	*43*	9	*36*	8	*35*	32	*46*	33	*45*	

* Fisher exact test for comparison between "low LRR risk" *vs.* "high LRR risk".

† Estrogen receptor status determined by immunohistochemistry (10% cut off).
Supplemented with status determined from biochemistry measurements, if missing values (29 pts.).

‡ HER2 status determined by immunohistochemistry (HercepTest) and FISH if equivocal (2+).
Supplemented with status determined from gene expression, if missing values (31 pts.).

Table 2. Hazard ratios (HR) for patients treated with or without postmastectomy radiotherapy (PMRT) in "low LRR risk" and "high LRR risk" groups. Presented unadjusted and adjusted for lymph node status and locally advanced disease, with 95% confidence intervals (CI)

	All patients HR (95% CI)	"Low LRR risk" HR (95% CI)	"High LRR risk" HR (95% CI)
*Training set, FFT**	n=191	n=48	n=143
PMRT, unadjusted	0.23 (0.12-0.44)	1.20 (0.17-8.52)	0.17 (0.09-0.35)
PMRT, adjusted	0.20 (0.10-0.38)	1.13 (0.14-9.15)	0.17 (0.08-0.34)
Training set, FFPE†	n=146	n=37	n=109
PMRT, unadjusted	0.26 (0.13-0.53)	0.61 (0.10-3.72)	0.22 (0.10-0.48)
PMRT, adjusted	0.23 (0.11-0.48)	0.58 (0.09-3.83)	0.20 (0.09-0.45)
Validation set, FFPE	n=112	n=22	n=90
PMRT, unadjusted	0.13 (0.04-0.46)	n.e.[#]	0.13 (0.04-0.46)
PMRT, adjusted	0.08 (0.02-0.29)	n.e.	0.09 (0.02-0.31)

* Fresh frozen tissue (FFT)

† Formalin fixed, paraffin embedded (FFPE)

[#] Not estimated (n.e.)

References

1. Clarke M, Collins R, Darby S, Davies C, Elphinstone P, Evans E, et al. Effects of radiotherapy and of differences in the extent of surgery for early breast cancer on local recurrence and 15-year survival: An overview of the randomised trials. Lancet 2005;366(9503):2087-106.

2. Overgaard M, Hansen PS, Overgaard J, Rose C, Andersson M, Bach F, et al. Postoperative radiotherapy in high-risk premenopausal women with breast cancer who receive adjuvant chemotherapy. danish breast cancer cooperative group 82b trial. N Engl J Med 1997;337(14):949-55.

3. Overgaard M, Jensen MB, Overgaard J, Hansen PS, Rose C, Andersson M, et al. Postoperative radiotherapy in high-risk postmenopausal breast-cancer patients given adjuvant tamoxifen: Danish breast cancer cooperative group DBCG 82c randomised trial. Lancet 1999;353(9165):1641-8.

4. Ragaz J, Olivotto IA, Spinelli JJ, Phillips N, Jackson SM, Wilson KS, et al. Locoregional radiation therapy in patients with high-risk breast cancer receiving adjuvant chemotherapy: 20-year results of the british columbia randomized trial. J Natl Cancer Inst 2005;97(2):116-26.

5. EBCTCG. Early Breast Cancer Trialists' Collaborative Group. Effect of radiotherapy after mastectomy and axillary surgery on 10-year recurrence and 20-year breast cancer mortality: Meta-analysis of individual patient data for 8135 women in 22 randomised trials. Lancet. Epub 2014 Mar 19.

6. The national institutes of health consensus development conference: Adjuvant therapy for breast cancer. Bethesda, Maryland, USA. November 1-3, 2000. Proceedings. J Natl Cancer Inst Monogr 2001;(30):1-152.

7. Recht A, Edge SB, Solin LJ, Robinson DS, Estabrook A, Fine RE, et al. Postmastectomy radiotherapy: Clinical practice guidelines of the american society of clinical oncology. J Clin Oncol 2001;19(5):1539-69.

8. Goldhirsch A, Wood WC, Coates AS, Gelber RD, Thurlimann B, Senn HJ, et al. Strategies for subtypes--dealing with the diversity of breast cancer: Highlights of the st. gallen international expert consensus on the primary therapy of early breast cancer 2011. Ann Oncol 2011;22(8):1736-47.

9. Chang JC, Wooten EC, Tsimelzon A, Hilsenbeck SG, Gutierrez MC, Elledge R, et al. Gene expression profiling for the prediction of therapeutic response to docetaxel in patients with breast cancer. Lancet 2003;362(9381):362-9.

10. van de Vijver MJ, He YD, van't Veer LJ, Dai H, Hart AA, Voskuil DW, et al. A gene-expression signature as a predictor of survival in breast cancer. N Engl J Med 2002;347(25):1999-2009.

11. van 't Veer LJ, Dai H, van de Vijver MJ, He YD, Hart AA, Mao M, et al. Gene expression profiling predicts clinical outcome of breast cancer. Nature 2002;415(6871):530-6.

12. Sørlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, et al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci U S A 2001;98(19):10869-74.

13. Perou CM, Sørlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, et al. Molecular portraits of human breast tumours. Nature 2000;406(6797):747-52.

14. Wang Y, Klijn JG, Zhang Y, Sieuwerts AM, Look MP, Yang F, et al. Gene-expression profiles to predict distant metastasis of lymph-node-negative primary breast cancer. Lancet 2005;365(9460):671-9.

15. Kyndi M, Sørensen FB, Knudsen H, Overgaard M, Nielsen HM, Overgaard J, et al. Estrogen receptor, progesterone receptor, HER-2, and response to postmastectomy radiotherapy in high-risk breast cancer: The danish breast cancer cooperative group. J Clin Oncol 2008;26(9):1419-26.

16. Overgaard M, Nielsen HM, Overgaard J. Is the benefit of postmastectomy irradiation limited to patients with four or more positive nodes, as recommended in international consensus reports? A subgroup analysis of the DBCG 82 b&c randomized trials. Radiother Oncol 2007;82(3):247-53.

17. Grantzau T, Mellemkjaer L, Overgaard J. Second primary cancers after adjuvant radiotherapy in early breast cancer patients: A national population based study under the danish breast cancer cooperative group (DBCG). Radiother Oncol 2013;106(1):42-9.

18. McGale P, Darby SC, Hall P, Adolfsson J, Bengtsson NO, Bennet AM, et al. Incidence of heart disease in 35,000 women treated with radiotherapy for breast cancer in denmark and sweden. Radiother Oncol 2011;100(2):167-75.

19. Højris I, Andersen J, Overgaard M, Overgaard J. Late treatment-related morbidity in breast cancer patients randomized to postmastectomy radiotherapy and systemic treatment versus systemic treatment alone. Acta Oncol 2000;39(3):355-72.

20. Højris I, Overgaard M, Christensen JJ, Overgaard J. Morbidity and mortality of ischaemic heart disease in high-risk breast-cancer patients after adjuvant postmastectomy systemic treatment with or without radiotherapy: Analysis of DBCG 82b and 82c randomised trials. radiotherapy committee of the danish breast cancer cooperative group. Lancet 1999;354(9188):1425-30.

21. Nielsen HM, Overgaard M, Grau C, Jensen AR, Overgaard J. Loco-regional recurrence after mastectomy in high-risk breast cancer--risk and prognosis. an analysis of patients from the DBCG 82 b&c randomization trials. Radiother Oncol 2006;79(2):147-55.

22. Danish Breast Cancer Cooperative Group, Nielsen HM, Overgaard M, Grau C, Jensen AR, Overgaard J. Study of failure pattern among high-risk breast cancer patients with or without postmastectomy radiotherapy in addition to adjuvant systemic therapy: Long-term results from the danish breast cancer cooperative group DBCG 82 b and c randomized studies. J Clin Oncol 2006;24(15):2268-75.

23. Overgaard M, Christensen JJ. Postoperative radiotherapy in DBCG during 30 years. techniques, indications and clinical radiobiological experience. Acta Oncol 2008;47(4):639-53.

24. Myhre S, Mohammed H, Tramm T, Alsner J, Finak G, Park M, et al. In silico ascription of gene expression differences to tumor and stromal cells in a model to study impact on breast cancer outcome. PLoS One 2010;5(11):e14002.

25. Tibshirani R. Regression shrinkage and selection via lasso. Journal of Royal Statistical Society 1996;58:267.

26. Tramm T, Sørensen BS, Overgaard J, Alsner J. Optimal reference genes for normalization of qRT-PCR data from archival formalin-fixed, paraffin-embedded breast tumors controlling for tumor cell content and decay of mRNA. Diagn Mol Pathol 2013;22(3):181-7.

27. Tramm T, Hennig G, Kyndi M, Alsner J, Sørensen FB, Myhre S, et al. Reliable PCR quantitation of estrogen, progesterone and ERBB2 receptor mRNA from formalin-fixed, paraffin-embedded tissue is independent of prior macro-dissection. Virchows Arch 2013;463(6):775-86.

28. Toustrup K, Sørensen BS, Lassen P, Wiuf C, Alsner J, Overgaard J, et al. Gene expression classifier predicts for hypoxic modification of radiotherapy with nimorazole in squamous cell carcinomas of the head and neck. Radiother Oncol 2012;102(1):122-9.

29. Müller BM, Kronenwett R, Hennig G, Euting H, Weber K, Bohmann K, et al. Quantitative determination of estrogen receptor, progesterone receptor, and HER2 mRNA in formalin-fixed

paraffin-embedded tissue--a new option for predictive biomarker assessment in breast cancer. Diagn Mol Pathol 2011;20(1):1-10.

30. Kyndi M, Sørensen FB, Knudsen H, Overgaard M, Nielsen HM, Andersen J, et al. Tissue microarrays compared with whole sections and biochemical analyses. A subgroup analysis of DBCG 82 b&c. Acta Oncol 2008;47(4):591-9.

31. Andersen J, Bentzen SM, Poulsen HS. Relationship between radioligand binding assay, immunoenzyme assay and immunohistochemical assay for estrogen receptors in human breast cancer and association with tumor differentiation. Eur J Cancer Clin Oncol 1988;24(3):377-84.

32. R Development Core Team. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing. Vienna, Austria; 2011.

33. Nuyten DS, Kreike B, Hart AA, Chi JT, Sneddon JB, Wessels LF, et al. Predicting a local recurrence after breast-conserving therapy by gene expression profiling. Breast Cancer Res 2006;8(5):R62.

34. Niméus-Malmström E, Krogh M, Malmstrom P, Strand C, Fredriksson I, Karlsson P, et al. Gene expression profiling in primary breast cancer distinguishes patients developing local recurrence after breast-conservation surgery, with or without postoperative radiotherapy. Breast Cancer Res 2008;10(2):R34.

35. Kreike B, Halfwerk H, Armstrong N, Bult P, Foekens JA, Veltkamp SC, et al. Local recurrence after breast-conserving therapy in relation to gene expression patterns in a large series of patients. Clin Cancer Res 2009;15(12):4181-90.

36. Sabatier R, Finetti P, Cervera N, Tallet A, Benchalal M, Houvenaeghel G, et al. Gene expression profiling and its utility in prediction of local relapse after breast-conserving therapy in early breast cancer. Cancer Genomics Proteomics 2011;8(4):199-209.

37. Cheng SH, Horng CF, West M, Huang E, Pittman J, Tsou MH, et al. Genomic prediction of locoregional recurrence after mastectomy in breast cancer. J Clin Oncol 2006;24(28):4594-602.

38. Torres-Roca JF, Eschrich S, Zhao H, Bloom G, Sung J, McCarthy S, et al. Prediction of radiation sensitivity using a gene expression classifier. Cancer Res 2005;65(16):7169-76.

39. Eschrich SA, Fulp WJ, Pawitan Y, Foekens JA, Smid M, Martens JW, et al. Validation of a radiosensitivity molecular signature in breast cancer. Clin Cancer Res 2012;18(18):5134-43.

40. Kreike B, van Kouwenhove M, Horlings H, Weigelt B, Peterse H, Bartelink H, et al. Gene expression profiling and histopathological characterization of triple-negative/basal-like breast carcinomas. Breast Cancer Res 2007;9(5):R65.

41. Servant N, Bollet MA, Halfwerk H, Bleakley K, Kreike B, Jacob L, et al. Search for a gene expression signature of breast cancer local recurrence in young women. Clin Cancer Res 2012;18(6):1704-15.

42. Tramm T, Kyndi M, Myhre S, Nord S, Alsner J, Sørensen F, et al. Relationship between the prognostic and predictive value of the intrinsic subtypes and a validated gene profile predictive of loco-regional control and benefit from post-mastectomy radiotherapy in high risk breast cancer patients. Acta Oncol 2014; (In press)

43. Schmidt M, Hellwig B, Hammad S, Othman A, Lohr M, Chen Z, et al. A comprehensive analysis of human gene expression profiles identifies stromal immunoglobulin kappa C as a compatible prognostic marker in human solid tumors. Clin Cancer Res 2012;18(9):2695-703.

44. Chen Z, Gerhold-Ay A, Gebhard S, Boehm D, Solbach C, Lebrecht A, et al. Immunoglobulin kappa C predicts overall survival in node-negative breast cancer. PLoS One 2012;7(9):e44741.

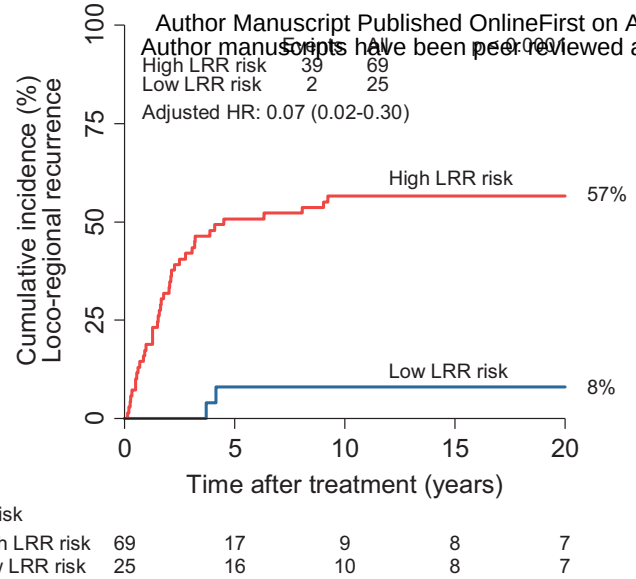
45. Lohr M, Edlund K, Botling J, Hammad S, Hellwig B, Othman A, et al. The prognostic relevance of tumour-infiltrating plasma cells and immunoglobulin kappa C indicates an important role of the humoral immune response in non-small cell lung cancer. Cancer Lett 2013;333(2):222-8.

46. Rangel J, Nosrati M, Leong SP, Haqq C, Miller JR,3rd, Sagebiel RW, et al. Novel role for RGS1 in melanoma progression. Am J Surg Pathol 2008;32(8):1207-12.

47. Kashani-Sabet M, Venna S, Nosrati M, Rangel J, Sucker A, Egberts F, et al. A multimarker prognostic assay for primary cutaneous melanoma. Clin Cancer Res 2009;15(22):6987-92.

48. Piovan E, Tosello V, Indraccolo S, Masiero M, Persano L, Esposito G, et al. Differential regulation of hypoxia-induced CXCR4 triggering during B-cell development and lymphomagenesis. Cancer Res 2007;67(18):8605-14.

49. Altman DG, McShane LM, Sauerbrei W, Taube SE. Reporting recommendations for tumor marker prognostic studies (REMARK): Explanation and elaboration. PLoS Med 2012;9(5):e1001216.



At risk

High LRR risk	69	17	9	8	7
Low LRR risk	25	16	10	8	7

Figure 1

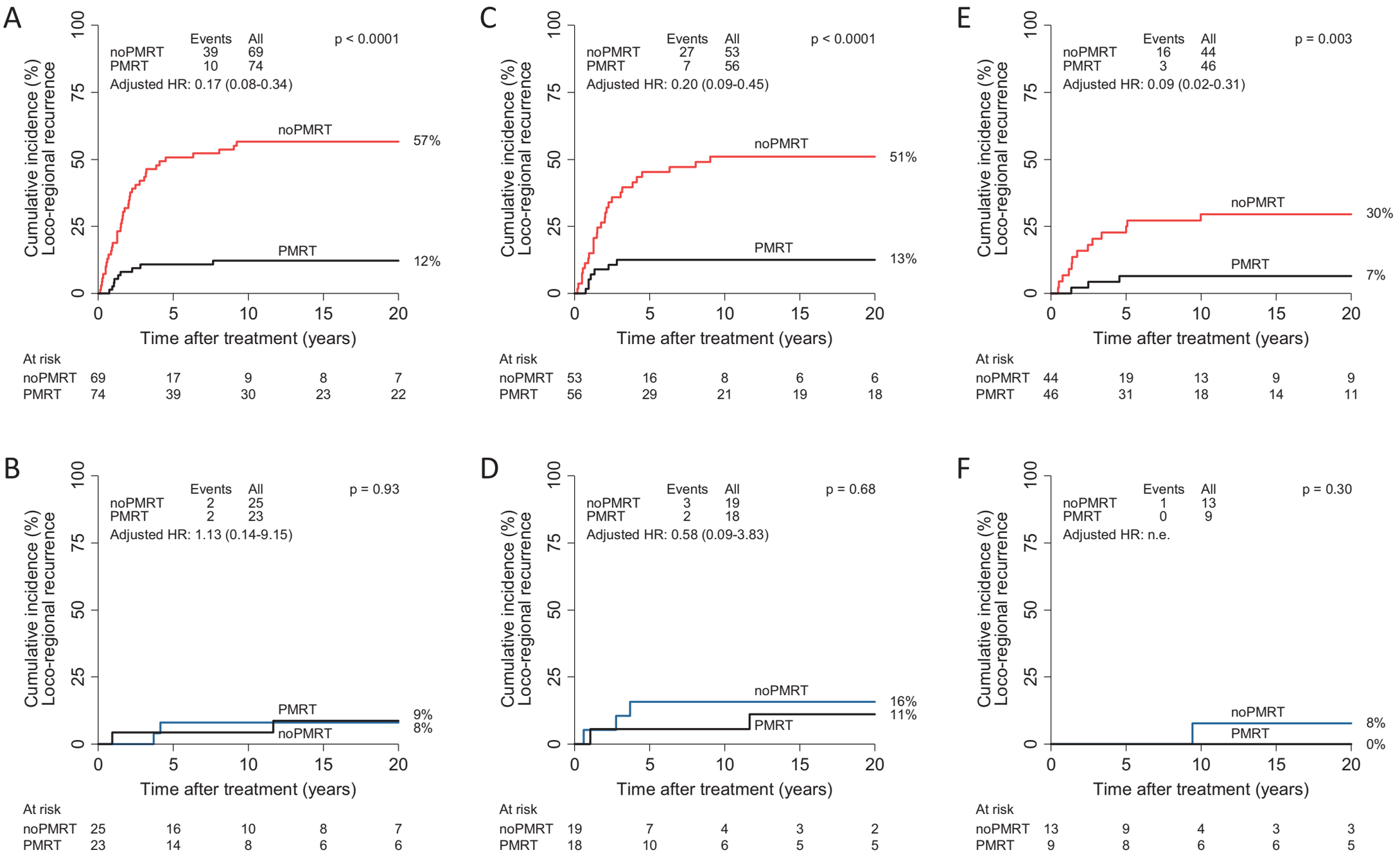


Figure 2

AACR American Association for Cancer Research

Clinical Cancer Research

Development and validation of a gene profile predicting benefit of post-mastectomy radiotherapy in high risk breast cancer patients: A study of gene expression in the DBCG82bc cohort

Trine Tramm, Hayat Mohammed, Simen Myhre, et al.

Clin Cancer Res Published OnlineFirst August 22, 2014.