Low residual proliferation after short-term letrozole therapy is an early predictive marker of response in high proliferative ER-positive breast cancer

Philippe L Bedard*, Sandeep K Singhal1*, Michail Ignatiadis1, Ian Bradbury2,3, Benjamin Haibe-Kains1,4,8, Christine Desmedt1, Sherene Loi1, Dean B Evans5, Stefan Michiels1, J Michael Dixon6, William R Miller7, Martine J Piccart1 and Christos Sotiriou1

Division of Medical Oncology and Hematology, Department of Medicine, Princess Margaret Hospital, University of Toronto, Toronto, Ontario, Canada
1Breast Cancer Translational Research Laboratory JC Heuson, Jules Bordet Institute, Université Libre de Bruxelles, Rue Heger-Bordet 1, 1000 Brussels, Belgium
2Frontier Science (Scotland) Ltd, Inverness-shire, UK
3School of Medicine, Royal Victoria Hospital, Queen’s University Belfast, Belfast, UK
4Machine Learning Group, Université Libre de Bruxelles, Brussels, Belgium
5Novartis Institutes for BioMedical Research, Basel, Switzerland
6Breakthrough Breast Research Group, Edinburgh, UK
7University of Edinburgh, Edinburgh, UK
8Department of Biostatistics and Computational Biology, Dana-Farber Cancer Institute, and Department of Biostatistics, Harvard School of Public Health, Boston, Massachusetts, USA

(Correspondence should be addressed to C Sotiriou; Email: christos.sotiriou@bordet.be)

*(P L Bedard and S K Singhal contributed equally to this work)

Abstract

The gene expression grade index (GGI) is a 97-gene algorithm that measures proliferation and divides intermediate histological grade tumors into two distinct groups. We investigated the association between early changes in GGI and clinical response to neoadjuvant letrozole and compared this to Ki67 values. The paired gene expression data at the beginning and after 10–14 days of neoadjuvant letrozole treatment were available for 52 post-menopausal patients with estrogen receptor (ER)-positive breast cancer. Baseline values and changes in GGI, Ki67, and RNA expression modules representing oncogenic signaling pathways were compared to sonographic tumor volume changes after 3 months of treatment in the subsets of patients defined by high and low baseline GGI. The clinical response was observed in 80% genomic low-grade (24/30) and 59% genomic high-grade (13/22) tumors (P=0.10). Low residual proliferation after 10–14 days of neoadjuvant letrozole therapy, measured by either GGI or Ki67, was associated with sonographic response in genomic high-grade (GGI, P=0.003; Ki67, P=0.017) but not genomic low-grade (GGI, P=0.25; Ki67, P=1.0) tumors. The analysis of expression modules suggested that sonographic response to letrozole in genomic high-grade tumors was associated with an early reduction in IGF1 signaling (unadjusted P=0.018). The major conclusion of this study is that the early assessment of proliferation after short-term endocrine therapy may be useful to evaluate endocrine responsiveness, particularly in genomic high-grade ER-positive breast cancer.

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Introduction

Histological grade is a powerful prognostic factor for early-stage breast cancer (Rakha et al. 2008). The gene expression grade index (GGI) is a 97-gene algorithm that separates intermediate histological grade tumors into low genomic grade and high genomic grade tumors, with long-term outcomes that resemble low and high histological grade tumors in the absence of systemic therapy (Sotiriou et al. 2006). At least, four distinct clinically relevant molecular subtypes of breast cancer have consistently been identified (luminal A, luminal B, HER2 like, and basal like; Perou et al. 2000, Sorlie et al. 2001). Previously, when we subdivided estrogen receptor-positive (ER+) tumors into a high and low proliferative group using a binary GGI division, we observed that ER-positive, HER2-negative, high genomic grade and luminal B tumors are highly concordant, as are ER-positive, HER2-negative, low genomic grade and luminal A tumors (Loi et al. 2007). High genomic grade tumors are more likely to achieve pathological complete response (pCR) with neoadjuvant chemotherapy than low genomic grade tumors, although their long-term survival remains inferior (Liedtke et al. 2009).

High genomic grade is associated with poor disease-free survival for patients treated with adjuvant tamoxifen therapy (Loi et al. 2007). Recently, aromatase inhibitors have been shown to be marginally superior to tamoxifen as adjuvant therapy for post-menopausal hormone receptor-positive breast cancer (Coombes et al. 2004, Jakesz et al. 2005, Forbes et al. 2008, Mouridsen et al. 2009). Neoadjuvant endocrine therapy refers to the administration of endocrine therapy before breast cancer surgery, with the goal of improving operability and/or breast conservation. Although baseline assessment of the proliferation antigen Ki67 by immunohistochemistry was not associated with relapse-free survival (RFS) in the IMPACT trial of neoadjuvant endocrine therapy, a low absolute value of Ki67 after 2 weeks of treatment (<2.7%) was predictive of better long-term RFS (Dowsett et al. 2007). The utility of Ki67 as a predictive biomarker of response to endocrine therapy is limited by intrapatient and interlaboratory variability (Mengel et al. 2002, Assersohn et al. 2003). Multi-gene expression predictors may be more reproducible markers of treatment effect that can be applied to clinical decision making (Anderson et al. 2006).

In this study, we hypothesized that residual proliferation after short-term endocrine treatment would be a more clinically important biomarker of treatment effect especially in high genomic grade tumors at baseline compared with low genomic grade tumors and therefore reanalyzed publicly available gene expression data of a neoadjuvant phase II study of letrozole.

Materials and methods

Patients: immunohistochemistry, genomic, and response assessment

Consecutive post-menopausal patients with ER-positive breast cancer presenting to the Edinburgh Breast Unit were prospectively recruited to a phase II study neoadjuvant study with letrozole (2.5 mg daily) as described previously (Forouhi et al. 1994, Miller et al. 2009). Briefly, patients had tumor biopsies performed before and after 10–14 days of treatment, along with breast sonography before and after 3 months of treatment performed by a single operator (J M D). Clinical response was based on ≥50% reduction in tumor volume as determined from sonographic measurements over the 3-month treatment period, confirmed by corresponding changes in caliper and mammographic measurement. Immunohistochemistry for ER and PR status was performed as described previously (Miller et al. 2006). Ki67 immunostaining was conducted using an antibody to the MIB1 (Ki67) antigen (Goying 1994). RNA was extracted from snap-frozen tumor biopsies, amplified, and hybridized on high-density Affymetrix HG-U133A chips.

Microarray analysis

For each pre-treatment biopsy, a GGI score was assigned using the previously published algorithm (Sotiriou et al. 2006). GGI was used as a dichotomized value and tumors were assigned to either high genomic grade or low genomic grade categories using a cutoff representing the midpoint between the mean values associated with high histological grade and low histological grade tumors from this cohort, as in the original publication (Sotiriou et al. 2006) and as performed by Loi et al. (2007). GGI scores at day 10/14 and changes from baseline to day 10/14 were assessed as a continuous variable to explore the relationship between GGI modulation and clinical response following 3 months of letrozole therapy.

Other reported prognostic gene expression algorithms, such as the 21-gene recurrence score (Paik et al. 2004; GENE21) and the 70-gene signature (van ’t Veer 2002; GENE70), were computed as described previously (Desmedt et al. 2008). Gene expression modules based on genes associated with specific
biological processes in breast cancer were calculated for each tumor sample as

\[ \text{Module score} = \frac{\sum |W_i|}{\sum |W_i|} \]

where \( X_i \) is the expression of a gene in the module that is present in the data set platform and \( W_i \) is either +1 or −1 depending on the sign of the association with the biological process. In the event that multiple probes were associated with a single gene, we selected the probe with the greatest variance in the data set. The gene module weighing algorithm gives preference to probe sets that have higher average signal intensities.

Robust scaling was done on each gene expression module score to have the interquartile range from −1 to 1 with the median equal to 0, allowing for a straightforward comparison between module scores. Additionally, we used the 50-gene set intrinsic subtype classifier PAM50 for subdivide into the luminal A and luminal B subtypes at baseline and estimated the risk of relapse score for each sample using correlation to the subtype alone (ROR-S) as follows: \( \text{ROR-S} = 0.05 \times \text{Basal} + 0.12 \times \text{HER2} + (−0.34) \times \text{LumA} + 0.23 \times \text{LumB} \) (Parker et al. 2009). Robust scaling was also performed on ROR-S (Parker et al. 2009). Microarray data analyses were performed using R software version 2.9.2 (www.r-project.org).

**Statistical analysis**

The primary objective was to explore the relationship between GGI values at day 10/14 and sonographic response under letrozole treatment in the two groups of tumors of high and low baseline GGI. No formal sample size calculation was performed because no prior data on day 10/14 GGI values were available. The same analysis was repeated for day 10/14 Ki67 values and changes from baseline to day 10/14 values.

Secondary objectives were to explore the association between biological pathways and sonographic response. Both a gene enrichment analysis (see below) and an analysis based on the expression modules from above were applied. The false discovery rate (FDR) criterion was used to adjust for multiple testing of both. The predictive performance of the various gene signatures was evaluated by the area under the receiver operating characteristics (ROC) curves (AUC) with associated 95% confidence intervals (CIs) using the gene expression signature score as a continuous variable. The asymptotic significance level for the AUC was considered \( P < 0.05 \).

Associations between categorical variables were assessed using the \( \chi^2 \) test. The Pearson correlation test was used to assess association between two continuous variables and the Mann–Whitney \( U \) test was used to compare group medians. All statistical analyses were performed using SPSS version 15.0 (SPSS, Inc., Chicago, IL, USA) and R software.

**Gene set enrichment analysis**

Gene set enrichment analysis (GSEA) was performed to evaluate whether gene sets were enriched in GGI high-grade non-responding tumors compared with GGI high-grade responding tumors at baseline (Subramanian et al. 2005). An initial GSEA screen (GSEA v 2.04) was carried out using the C1 (positional) and C2 (curated) gene sets exported from the Molecular Signature Database (MSigDB, version 2.5).

**Results**

**Patient characteristics**

Paired gene expression profiles at baseline and after 10–14 days of letrozole therapy were available from 52 post-menopausal patients (Table 1). The median age was 78 years (range 61–88) and all tumors were ER positive by immunostaining (IHC) at baseline. Histological grade was considered low in six tumors (12%), intermediate in 34 tumors (65%), and high in nine tumors (17%). Based on the GGI cutoff defined for this series, 30 patients (58%) were classified as low genomic grade and 22 patients (42%) as high genomic grade at baseline (Table 1). There was a strong correlation between histological grade and genomic grade (\( P = 0.001 \)). Using PAM50, we found 23 luminal A and 29 luminal B tumors at baseline. The misclassification rate between GGI and PAM50 was 15 out of 52 (29%).

**Association between baseline genomic grade and response to neoadjuvant letrozole therapy**

Low genomic grade was associated with a non-statistically significant trend toward a higher sonographic response rate after 3 months of letrozole therapy compared with high genomic grade (80 vs 59%; \( P = 0.10 \); Table 1). The observed response rate of low (83%) and intermediate (76%) histological grade tumors was greater than high (56%) histological grade tumors, although this was not statistically significant (\( P = 0.28 \)).

**Letrozole treatment reduces GGI in all patients**

GGI was significantly reduced after 10–14 days of neoadjuvant letrozole (\( P < 0.001 \)) in the overall set of patients and in both the low genomic grade (\( P < 0.001 \)).
and high genomic grade tumors separately \((P < 0.001; \text{Fig. 1A})\). The day 10/14 GGI of high genomic grade tumors was similar to the baseline GGI of low genomic grade tumors (Fig. 1B).

**Early assessment of GGI values is predictive of response**

Although the continuous value of GGI at baseline was not predictive of sonographic response to neoadjuvant letrozole \((P = 0.28)\), tumors that achieved sonographic response had lower GGI values after 10–14 days of letrozole \((P = 0.002)\) in the overall set of patients (Fig. 1C). As far as our primary objective was concerned, when tumors were divided by baseline genomic grade, GGI after 10/14 days of neoadjuvant letrozole was only associated with response in tumors with a high genomic grade \((P = 0.003)\) but not in tumors with a low genomic grade at baseline \((P = 0.25; \text{Fig. 1D})\). Similarly, the ROR-S score after 10/14 days of neoadjuvant letrozole was borderline significantly associated with response in luminal B tumors defined by PAM50 \((P = 0.059)\) but not in luminal A at baseline \((P = 0.38)\).

Then, we focused on the change in GGI from baseline to day 10/14 and its association with response. Tumors that achieved sonographic response demonstrated a greater change in GGI from baseline to day 10/14 \((P = 0.062)\). This difference in GGI by sonographic response was similar for low genomic grade \((P = 0.029)\) and genomic high-grade \((P = 0.066)\) tumors.

**Modulation of other prognostic gene expression profiles with letrozole**

Since GGI and other prognostic gene expression profiles include many genes associated with proliferation, we hypothesized that other first-generation prognostic gene expression profiles, such as the 70-gene signature (GENE70), the 21-gene recurrence score (GENE21), and the risk of relapse score (ROR-S), would show similar patterns of change with letrozole therapy. Correlation values between changes in the four proliferation modules were moderate and ranged from 0.50 to 0.69, with the highest correlation values between GGI and ROR-S \((r = 0.69)\) and between GGI and GENE70 \((r = 0.68)\). The values of GENE70 \((P < 0.001)\), GENE21 \((P = 0.13)\), and ROR-S \((P < 0.001)\) profile scores were reduced after 10–14 days of letrozole therapy, but only significantly for GENE70 and ROR-S (Fig. 2).
Comparison of day 10/14 GGI with Ki67 immunostaining

Previously, low residual proliferation after short-term neoadjuvant endocrine therapy assessed by the Ki67 immunostaining assay has been shown to be predictive of long-term recurrence-free survival (Dowsett et al. 2007). In our study, low values of Ki67 immunostaining after 10–14 days of letrozole therapy were associated with sonographic response ($P=0.047$), although there was no association between baseline Ki67 immunostaining and clinical response ($P=0.72$). Similar to GGI, low day 10/14 Ki67 was predictive of clinical response for high genomic grade tumors (AUC = 0.82, 95% CI 0.64 to 1.0) but not low genomic grade tumors (AUC = 0.5, 95% CI 0.23 to 0.77; Fig. 3). The value of day 10/14 GGI was moderately correlated with day 10/14% Ki67 immunostaining ($r = 0.64$, $P<0.001$). When Ki67 at day 10/14 was dichotomized using a previously published cutoff (Baselga et al. 2009), low genomic grade tumors were more likely to achieve a day 10/14 < 2.7% than high genomic grade tumors ($P=0.027$; Table 1).

Early changes in oncogenic signaling pathways associated with clinical response in genomic high-grade tumors

To search for oncogenic signaling pathways that are associated with treatment resistance, we performed GSEA using the MSigDB database of curated gene sets. Of the 1868 gene sets analyzed, none were enriched or depleted in non-responding genomic low-grade tumors at baseline with a FDR < 0.25. In genomic high-grade tumors, we did not find any gene sets that were significantly depleted in non-responding tumors. The only gene set that was significantly enriched in responding tumors at baseline was BRCAX_DN, a nine-gene set differentially expressed in two groups of non-BRCA1/2-mutated familial breast cancer of unknown biological significance (Hedenfalk et al. 2003).

Figure 1 Cumulative frequency plots of GGI at baseline and after 10/14 days of letrozole therapy. (A) GGI was significantly reduced after 10–14 days after neoadjuvant letrozole therapy ($P<0.001$). (B) The day 10/14 GGI of high genomic grade tumors was similar to the baseline GGI of low genomic grade tumors. (C) There was no difference in GGI at baseline between responding and non-responding tumors ($P=0.28$); however, day 10/14 GGI was lower in responding tumors ($P=0.002$). (D) When tumors were divided according to baseline genomic grade, day 10/14 GGI was only associated with response in high genomic grade ($P=0.003$) but not low genomic grade tumors ($P=0.25$). (Note: all $P$ values are based on the Kruskal–Wallis test).
In order to evaluate whether early changes in oncogenic signaling pathways are associated with response to letrozole therapy, we explored the change in expression of several gene expression modules that reflect important biological processes in breast cancer from baseline to day 10/14 by clinical response category. Similar to previous reports using this data set (Miller et al. 2009, Miller & Larionov 2011), we also observed a marked reduction in the expression of the estrogen receptor gene (ESR1) and a gene expression module reflecting ER signaling (Desmedt et al. 2008) after 10–14 days of letrozole therapy ($P < 0.0001$). Interestingly, there was no difference in the change of ESR1 expression or ER module expression (ESR1) according to clinical response in both high genomic grade ($P = 0.33$ and $P = 0.57$ respectively) and low genomic grade tumors ($P = 0.84$ and $P = 0.72$). The reduction in gene expression module scores associated with wound response (Chang 2005; $P = 0.012$), insulin-like growth factor 1 (IGF1; Creighton et al. 2008; $P = 0.018$), and growth factor signaling (Loboda et al. 2009; $P = 0.021$) from baseline to day 10/14 was greater in high genomic grade tumors that achieved response compared with those that did not respond to 3 months of letrozole with an FDR < 25%.

Since many oncogenic pathway gene expression signatures are heavily influenced by the expression of proliferation-related genes, we evaluated the association between changes in oncogenic pathway signatures in high genomic grade breast cancers after short-term letrozole therapy. As expected, the change in GGI, a known proliferation-dependent gene expression profile, was strongly correlated with a reduction in the wound response ($r = 0.63$, $P = 0.002$) and growth factor signaling modules ($r = 0.83$, $P < 0.001$), but only moderately correlated with a change in the IGF1 signaling module ($r = 0.42$, $P = 0.05$). This suggests that modulation of the IGF1 signaling pathway may be important to achieve sonographic response in high genomic grade tumors.

**Discussion**

In this study, we examined the association between sonographic response to neoadjuvant letrozole and GGI, a multi-gene surrogate of histological grade. Our data indicate that GGI may be a useful predictive biomarker of response to neoadjuvant anti-estrogen therapy in post-menopausal ER-positive breast cancer. Post-menopausal women with low genomic grade were more likely to respond to 3 months of neoadjuvant letrozole therapy with an odds ratio (OR) of borderline statistical significance (OR 2.77; 95% CI 0.81–9.52). For all patients, GGI values were significantly reduced after 10–14 days of neoadjuvant letrozole ($P < 0.001$), and also within the high and low baseline genomic grade subsets (both $P < 0.001$). High genomic grade has previously been shown to be an unfavorable prognostic marker for patients who did not receive any chemotherapy or endocrine therapy and for
In the IMPACT study, low tumor Ki67 immunostaining after 2 weeks of endocrine therapy was predictive of improved long-term RFS (Dowsett et al. 2007). In our study, low residual proliferation after 10–14 days of letrozole therapy, assessed by either Ki67 immunostaining or GGI, was associated with sonographic response to letrozole. Tumor shrinkage after neoadjuvant endocrine therapy is not as robust an endpoint for responsiveness to therapy as pCR with neoadjuvant chemotherapy for ER-negative disease (Liedtke et al. 2008). Tumor measurement by physical examination demonstrates variable inter-rater reliability and poor correlation with pathological tumor size. Clinical response assessment by sonographic tumor volume reduction (Forouhi et al. 1994), as performed by single experienced operator in our study, is more closely associated with pathological tumor size and may be a more accurate measure of treatment effect (Dixon et al. 2000). The overall response rate observed in our study (71%) is higher than previous studies of pre-operative endocrine therapy (Eiermann et al. 2001, Smith et al. 2005, Baselga et al. 2009). This may be due to differences in study population, method of sonographic assessment, or response criteria.

There is substantial molecular heterogeneity in ER-positive breast cancer. The performance of gene expression signatures is largely driven by an improved quantification of proliferation in ER-positive breast cancer (Wirapati et al. 2008). In this study, low levels of residual proliferation after 10–14 days of letrozole therapy, measured by multi-gene algorithms, such as GGI, and tumoral Ki67 immunostaining were associated with sonographic response in high genomic grade tumors. Paradoxically, although residual proliferation after short-term endocrine therapy remains higher in tumors that are highly proliferative prior to treatment, early suppression of proliferation may be a better early read-out of treatment effect in high genomic grade tumors. It should be noted that measures of proliferation decreased after short-term endocrine treatment in both high genomic grade and low genomic grade tumors. The lack of association observed between residual proliferation and response in low genomic grade tumors in our study may be due to insufficient power. Additional studies are required to validate these preliminary findings, including correlation with long-term clinical outcome.

The ongoing Pre-Operative Endocrine Treatment for Individualized Care (POETIC) study will randomize 4000 post-menopausal women with ER-positive breast cancer to 2 weeks of neoadjuvant aromatase inhibitor therapy vs no systemic therapy prior to surgical resection of their primary tumor to validate Ki67 immunostaining after short-term endocrine therapy as a predictive marker of long-term outcome (UKCRN trial id 4023). Based on our data, we hypothesize that markers of low residual proliferation after short-term endocrine treatment in both high genomic grade and low genomic grade tumors. The lack of association observed between residual proliferation and response in low genomic grade tumors in our study may be due to insufficient power. Additional studies are required to validate these preliminary findings, including correlation with long-term clinical outcome.

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the prediction of sonographic response after 3 months of letrozole therapy. In spite of longstanding efforts to elucidate de novo predictive biomarkers of endocrine responsiveness, only ER status is widely used clinically. Early on-treatment measurements may be more effective predictors of treatment efficacy (Dowsett & Dunbier 2008). In the future, the evaluation of residual proliferation after short-term treatment with endocrine therapy in patients with high genomic grade ER-positive breast cancer may distinguish patients who are adequately treated with adjuvant endocrine therapy alone from those who require additional systemic therapy, such as chemotherapy and/or targeted therapy.

The assessment of residual proliferation by GGI after 10/14 days of letrozole was similar to Ki67 immunostaining for the prediction of response for high genomic grade tumors. However, Ki67 immunostaining was performed by a single breast cancer pathologist in a high-volume academic laboratory (Miller et al. 2006). Other investigators have demonstrated high inter-laboratory variability in Ki67 immunostaining due to differences in antigen retrieval or staining techniques that might limit its utility as a predictive biomarker for clinical decision making (Mengel et al. 2002). GGI and other multi-gene expression signatures may be more reproducible measures of residual proliferation that can be used for clinical decision making.

Although ER-positive high genomic grade tumors are more likely to achieve pCR after neoadjuvant chemotherapy, their long-term outcome is suboptimal (Liedtke et al. 2008). Further information with regard to the underlying biological mechanisms that contribute to their proliferation phenotype may help guide drug development for these breast cancer patients. We did not find gene sets associated with known oncogenic signaling pathways whose expression was increased in non-responding tumors prior to the start of letrozole therapy. This highlights the difficulty of identifying predictive markers of response to endocrine therapy prior to the initiation of treatment. With only 22 high genomic grade tumors included, our analysis have not been adequately powered to identify gene sets that are enriched in non-responding high genomic grade tumors. The analysis of early changes in oncogenic signaling pathways, measured by differences in the expression of signaling pathway genes from baseline to day 10/14, suggests that an early reduction in wound response, IGF1, and growth factor signaling may be associated with response but came with relatively high FDR. Importantly, the reduction in IGF1 signaling only moderately correlated with the change in GGI. There is extensive cross talk between the IGF1 and ER pathways (Kahlert et al. 2000, Gee et al. 2005). Our findings suggest that ER-positive tumors that are able to shut down the IGF1 signaling axis with letrozole therapy are more likely to achieve sonographic response. It may be that ER-positive tumors that have other mechanisms of IGF1 activation besides ER, such as the insulin receptor pathway or other growth factors pathways that interact with IGF1, are not adequately treated with letrozole therapy alone and require additional growth factor pathway modulation. This preliminary finding supports studies of IGF1 pathway inhibition combined with endocrine therapy in high genomic grade ER-positive breast cancer, although additional evidence is needed to confirm the relevance of this putative therapeutic target in high genomic grade ER-positive breast cancer.

In summary, our study suggests that an early dynamic change GGI is associated with response to neoadjuvant letrozole in post-menopausal ER-positive breast cancer and demonstrates that residual proliferation measured by multi-gene expression signatures or Ki67 immunostaining is an early marker of treatment response in high genomic grade ER-positive breast cancer. Evaluation of residual proliferation after therapy with short-term neoadjuvant endocrine treatment may be a useful strategy to prioritize promising novel targeted treatments for further development in high genomic grade ER-positive breast cancer. These hypothesis-generating findings require further validation in independent data sets.

Declaration of interest
C Sotiriou and M Piccart are co-inventors of the GGI. The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement
Lorna Renshaw recruited patients and collected the clinical samples from the Breakthrough Breast Research Group in Edinburgh, UK. Dr Tom Anderson performed histological assessment of the tumors included in this study.
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