Assessment of an RNA interference screen-derived mitotic and ceramide pathway metagene as a predictor of response to neoadjuvant paclitaxel for primary triple-negative breast cancer: a retrospective analysis of five clinical trials

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Summary

Background Addition of taxanes to preoperative chemotherapy in breast cancer increases the proportion of patients who have a pathological complete response (pCR). However, a substantial proportion of patients do not respond, and the prognosis is particularly poor for patients with oestrogen-receptor (ER)/progesterone-receptor (PR)/human epidermal growth factor receptor 2 (HER2; ERBB2)-negative (triple-negative) disease who do not achieve a pCR. Reliable identification of such patients is the first step in determining who might benefit from alternative treatment regimens in clinical trials. We previously identified genes involved in mitosis or ceramide metabolism that influenced sensitivity to paclitaxel, with an RNA interference (RNAi) screen in three cancer cell lines, including a triple-negative breast-cancer cell line. Here, we assess these genes as a predictor of pCR to paclitaxel combination chemotherapy in triple-negative breast cancer.

Methods We derived a paclitaxel response metagene based on mitotic and ceramide genes identified by functional genomics studies. We used area under the curve (AUC) analysis and multivariate logistic regression to retrospectively assess the metagene in six cohorts of patients with triple-negative breast cancer treated with neoadjuvant chemotherapy: two cohorts treated with paclitaxel (n=27, 30) and four treated without paclitaxel (n=88, 28, 48, 39).

Findings The metagene was associated with pCR in paclitaxel-treated cohorts (AUC 0.79 [95% CI 0.53–0.93], 0.72 [0.48–0.90]) but not in non-paclitaxel treated cohorts (0.53 [0.31–0.77], 0.59 [0.22–0.82], 0.53 [0.36–0.71], 0.64 [0.43–0.81]). In multivariate logistic regression, the metagene was associated with pCR (OR 19.92, 2.62–151.57; p=0.0039) with paclitaxel-containing chemotherapy.

Interpretation The paclitaxel response metagene shows promise as a paclitaxel-specific predictor of pCR in patients with triple-negative breast cancer. The metagene is suitable for development into a reverse transcription-PCR assay, for which clinically relevant thresholds could be established in randomised clinical trials. These results highlight the potential for functional genomics to accelerate development of drug-specific predictive biomarkers without the need for training clinical trial cohorts.

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Introduction Despite the use of modern cytotoxic agents, the proportion of patients who achieve a complete pathological response (pCR) to preoperative chemotherapy remains low, at 15–25% for all breast-cancer histopathological subtypes. Rates of pCR in sporadic oestrogen-receptor (ER)/progesterone-receptor (PR)/human epidermal growth factor receptor 2 (HER2; ERBB2)-negative (triple-negative) breast cancer range from 12% for taxane monotherapy to 45% with combination neoadjuvant chemotherapy regimens. Patients who achieve a pCR after chemotherapy have excellent disease-free and overall survival. The outcome for patients who do not achieve a pCR varies, and the prognosis for patients with triple-negative cancers and residual disease after preoperative chemotherapy is particularly poor. Better understanding of breast-cancer biology is likely to expand the list of potentially effective chemotherapeutic agents in the neoadjuvant setting, and will help identify tailored chemotherapy schedules for distinct patient cohorts based on tumour molecular characterisation. If it can be reliably established that patients resistant to one type of therapy are sensitive to a different agent, then robust predictors of chemotherapeutic response will have an essential role in selecting the optimum treatment in the neoadjuvant setting. The identification of patients with disease that is resistant to...
conventional chemotherapy combinations is the first step in this process.

Several predictive biomarkers have been discovered using associative-learning strategies. Predictive gene-expression signatures derived from an associative analysis approach are susceptible to chance associations which lead to overestimation of true clinical accuracy; therefore, two separate trial cohorts are required to train and validate the predictive signature.\textsuperscript{14} Associative strategies developed from genomics signatures that are predictive of drug response in cell lines might circumvent the need for training and validation of trial cohorts.\textsuperscript{7} Quantifying distinct biological processes within gene-expression datasets, instead of a gene-by-gene based associative analysis, may further avoid these problems and accelerate biomarker development.\textsuperscript{8} However, potentially relevant biological processes or functional modules must be identified in advance for such an analysis to be possible.

**RNA interference (RNAi)** functional screening may be an applicable method to identify biological processes relevant to drug response in cancer medicine. To test this hypothesis, we revisited results of our previous study\textsuperscript{9} of an RNAi drug-resistance screen across three cancer cell lines, including a triple-negative breast-cancer cell line, MDA-MB-231. In this screen, we identified two distinct gene sets regulating sensitivity to paclitaxel.\textsuperscript{7} The first set of genes is involved in mitosis and the mitotic spindle assembly checkpoint (SAC), and the second set is involved in metabolism of the proapoptotic lipid, ceramide. The involvement of both of these gene sets is consistent with the current biological understanding of the mechanism of action of paclitaxel. An activated SAC orchestrates a paclitaxel-induced mitotic arrest, and in our RNAi screen, silencing several genes implicated in SAC control impaired the accumulation of cells in mitosis and subsequent cell death in response to paclitaxel. Identification of ceramide pathway genes as regulators of paclitaxel sensitivity\textsuperscript{9} is consistent with published evidence that overexpression of glucosylceramide synthase (UGCG) promotes resistance to paclitaxel and repression promotes paclitaxel sensitivity.\textsuperscript{10–12}

We created a **metagene** using established methodology\textsuperscript{8,11} to quantify the activity of these two biological pathways identified by our RNAi screen, and tested its paclitaxel-predictive value in patients with triple-negative breast cancer who were treated in clinical trials with either a paclitaxel-containing combination regimen, T-FAC (paclitaxel followed by fluorouracil, doxorubicin, and cyclophosphamide) or by regimens without paclitaxel.

**Methods**

**Patients and procedures**

Gene-expression and treatment-response data were retrieved from six cohorts in five neoadjuvant clinical trials, referred to in this study as MDA1, MDA/MAQC-II, TOP, EORTC\textsubscript{FEC}, EORTC\textsubscript{TET}, and DFCI. In all trials, pCR was determined at the time of surgery (no evidence of residual invasive cancer in the breast or lymph nodes at time of study). Number of patients (133-102), median age (28-79), range), treatment (T-FAC, Epirubicin, and Cisplatin), and biomarker development (MDA1, MDA/MAQC-II, TOP, DFCI, EORTC10944).

**Table 1:** Clinical and histopathological characteristics of the cohorts analysed

| Treatment | MDA1 | MDA/MAQC-II | TOP | DFCI | EORTC10944 |
|-----------|------|-------------|-----|------|-------------|
| Microarray platform | U133A | U133A | U133 Plus 2.0 | U133 Plus 2.0 | X3P | X3P |
| Treatment | T-FAC | T-FAC | Epirubicin | Cisplatin | FEC | TET |
| Number of patients | 133 | 100 | 120 | 28 | 102 | 58 |
| Median age (years; range) | 51 (28-79) | 50 (26-73) | 47 (22-68) | 50 (29-69) | 49 (26-70) | 49 (34-70) |
| Histology, n (%) | IDC 130 (98) | 93 (93) | 112 (93) | 28 (100) | 52 (51) | 56 (97) |
| N0 | 1 (1) | 7 (7) | 1 (1) | 0 (0) | 4 (4) | 2 (3) |
| Other | 2 (1) | 0 (0) | 7 (6) | 0 (0) | 7 (7) | 0 (0) |
| Unknown | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 39 (38) | 0 (0) |
| Stage, n (%) | T1 13 (10) | 10 (10) | 17 (14) | 1 (4) | 2 (2) | 1 (2) |
| T2 | 70 (52.5) | 62 (62) | 83 (69) | 22 (79) | 63 (62) | 37 (64) |
| T3 | 22 (16.5) | 13 (13) | 5 (4) | 4 (14) | 34 (33) | 20 (34) |
| T4 | 28 (21) | 15 (15) | 15 (13) | 0 (0) | 0 (0) | 0 (0) |
| Unknown | 0 (0) | 0 (0) | 0 (0) | 1 (4) | 3 (3) | 0 (0) |
| No. of patients | SLN positive | ND | ND | ND | 13 (46) | ND |
| N2 | 40 (30) | 27 (27) | 55 (46) | 15 (54) | 37 (36) | 22 (38) |
| N1 | 62 (47) | 47 (47) | 60 (50) | ND | 55 (54) | 31 (53) |
| N3 | 14 (11) | 13 (13) | 3 (2) | ND | 7 (7) | 5 (9) |
| N0 | 17 (13) | 13 (13) | 2 (2) | ND | 0 (0) | 0 (0) |
| Unknown | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 3 (3) | 0 (0) |
| Grade, n (%) | 1 | 2 (1) | 11 (11) | 2 (2) | 0 (0) | 2 (2) | 0 (0) |
| 2 | 54 (41) | 42 (42) | 20 (17) | 0 (0) | 21 (21) | 16 (28) |
| 3 | 77 (58) | 47 (47) | 92 (77) | 28 (100) | 31 (30) | 37 (64) |
| Unknown | 0 (0) | 0 (0) | 6 (5) | 0 (0) | 48 (47) | 5 (9) |
| Oestrogen receptor status, n (%) | Positive | 82 (62) | 61 (61) | 1 (1) | 0 (0) | 37 (36) | 0 (0) |
| Negative | 51 (38) | 39 (39) | 119 (99) | 28 (100) | 65 (64) | 58 (100) |
| Progesterone receptor status, n (%) | Positive | 75 (56) | 51 (51) | 1 (1) | 0 (0) | 25 (25) | 0 (0) |
| Negative | 55 (41) | 49 (49) | 75 (62) | 28 (100) | 75 (74) | 58 (100) |
| Unknown | 3 (2) | 0 (0) | 44 (37) | 0 (0) | 2 (2) | 0 (0) |
| HER2 status, n (%) | Amplified | 33 (25) | 7 (7) | 32 (27) | 0 (0) | 26 (25)* | 19 (32)* |
| Not amplified | 99 (74) | 93 (93) | 61 (52) | 28 (100) | 76 (75)* | 39 (67)* |
| Unknown | 1 (1) | 0 (0) | 27 (23) | 0 (0) | 0 (0)* | 0 (0)* |
| Total triple-negative, n (%) | 27 (20) | 30 (30) | 88 (73)** | 28 (100) | 48 (47) | 39 (67) |
| Response, n (%) | pCR | 34 (26) | 15 (15) | 17 (14) | 5 (18) | 39 (38) | 26 (45) |
| No pCR | 99 (74) | 85 (85) | 101 (84) | 21 (75) | 63 (62) | 52 (85) |
| Non-evaluable | 0 (0) | 0 (0) | 2 (2) | 2 (2) | 0 (0) | 0 (0) |

T stage was measured before treatment. Nodal status was confirmed at surgery for MDA1, MDA/MAQC-II, TOP, and EORTC. Nodal status for DFCI was assessed before treatment with SLN biopsy. T-FAC = paclitaxel, fluorouracil, doxorubicin, and cyclophosphamide. FEC = fluorouracil, epirubicin, and cyclophosphamide. TET = docetaxel followed by epirubicin and docetaxel. IDC = invasive ductal carcinoma. ILC = invasive lobular carcinoma. SLN = sentinel lymph node. ND = not determined. pCR = pathological complete response. *Receptor status inferred by gene expression. **Triple-negative breast cancer subtype inferred by expression when insufficient data by immunohistochemistry/FISH.

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RNA interference
A mechanism by which small double-stranded RNAs can reduce expression of any mRNA having a similar sequence. RNAi can be applied to genome-wide high-throughput screens to identify genes essential for a particular pathway or function.

Metagene
A set of genes whose combined expression represents the activity of a specific cellular pathway.

See Online for webappendix

Figure 1: Process to derive the paclitaxel response metagene
(A) Flow chart describing the derivation of the gene modules and the paclitaxel response metagene. UGCG was identified from the literature as a gene in the same pathway that promotes paclitaxel sensitivity when repressed. (B) Proximal regulators of the prosapoptic lipid, ceramide, that alter paclitaxel sensitivity through conversion to sphingomyelin (via COL4A3BP ceramide transporter) or glucosylceramide (UGCG glucosylceramide synthase) and conversion of glucosylceramide to ceramide (GBA1 and GBA3 beta-glucosidase). Genes for which repression promotes paclitaxel sensitivity are shown in green and for which repression promotes paclitaxel resistance are shown in red. RNAi=RNA interference.

Validated datasets from our previously published RNAi screen were used for the derivation of the paclitaxel response metagene (webappendix pp 10–12). The RNAi screen focused on kinases and ceramide-pathway genes. In the kinase screen, we identified ten mitosis-associated genes that influenced sensitivity to paclitaxel in three different cell lines. In addition, we identified three genes from the ceramide pathway (COL4A3BP, GBA1, GBA3) that together with the published gene UGCG, encode proximal regulators of ceramide metabolism and influence paclitaxel sensitivity. Of these 14 genes (figure 1), six mitotic genes and three ceramide genes could be assayed on the Affymetrix HGU133A platform, and these were combined into the mitotic and the ceramide gene sets. The number of genes within each gene set was further reduced to the mitotic module (four genes) and the ceramide module (two genes) by only including genes that were significantly correlated with each other across four independent breast cancer datasets. The expression of the genes within each module was compressed by using the mean expression of the participating institutions. Further cohort-specific information is detailed in the webappendix pp 10–12.

Statistical analysis
A binomial test was used to test for enrichment in genes that predicted response to treatment with paclitaxel-containing neoadjuvant chemotherapy, among the genes that substantially affected paclitaxel sensitivity across three cell lines. The significance of association between module or metagene scores and pCR was estimated with AUC and logistic regression. Since the two modules were expected to predict sensitivity in the opposite direction (high mitotic expression=sensitivity, high ceramide expression=Resistance), the paclitaxel response metagene was defined as the mitotic module minus the ceramide module. As a comparison, 10,000 random combinations of nine-gene sets were subjected to the same correlative approach, across four clinical datasets, and the mean expression of significantly correlated genes were tested for their ability to predict pCR in T-FAC-treated cohorts. We also did a literature search and found 24 genes reported as affecting taxane sensitivity when either overexpressed or repressed (webappendix p 6). These genes were subjected to the same correlation analysis as the paclitaxel response metagene, and the reported direction of expression relating to paclitaxel sensitivity was included as weights (minus 1 if the gene induced sensitivity when repressed, 1 if the gene induced sensitivity when overexpressed). The genomic grade index and the stroma signatures were calculated as described previously.
treated cohorts to increase statistical power for univariate and multivariate logistic regression analysis. Univariate and multivariate logistic regression was done using iteratively reweighted least squares. All covariates were converted to binary, using the median as a threshold to determine paclitaxel response metagene status and Ki67 status. Analyses compared stage T3 and T4 versus T1 and T2; grade 3 versus grade 1 and 2; and N0 versus N1+. A p value of 0·05 or lower was considered significant for all statistical tests. All statistical analyses were done in R version 2.9.2 using the software packages affy, genefilter, beeswarm, gdata, ROCR, hgu133a.db, u133x3p.db, squash, boot, rmeta, and survplot (webappendix p 11).

**Role of the funding source**

The funding sources and sponsors of the trials had no role in the design of the study; collection, analysis, or interpretation of the data; or writing of this report. All authors had access to the raw data. The corresponding author had full access to all data and had the final responsibility to submit for publication.

**Results**

To develop an analytical framework based on experimentally established functional links instead of associative correlations, we revisited our previously published functional links instead of experimental evidence. Based on this screen and previously published data, we identified two gene modules of tightly correlated genes: a four-gene mitotic module where higher expression predicted sensitivity, and a two-gene ceramide module where higher expression predicted resistance. We then used the mean expression of the genes within each module as a single predictive value; an approach previously shown to improve reliability across many tumour samples.\(^1\)\(^2\) Finally, we combined the mitotic and ceramide modules into a single functionally derived paclitaxel-response metagene by subtracting the mean expression of the genes in the ceramide module from the mean expression of the genes in the mitotic module (figure 1). Therefore, this summary measure reflects the difference in the mean expression of the two modules and is predicted to correlate with paclitaxel sensitivity.

In the T-FAC treated triple-negative cohorts, the paclitaxel response metagene was highly predictive of pCR, with an AUC of 0·79 in MDA1 (p=0·0053; 95% CI 0·53–0·93; n=27), an AUC of 0·72 in MDA/MAQC (p=0·031; 0·48–0·90; n=30), and an AUC of 0·74 when the two cohorts were combined (p=0·0013; 0·58–0·86; n=57; table 2, figure 2 A and B, webappendix p 2).

Consistent with these data, the paclitaxel response metagene was also predictive of response across all patients in each cohort: for MDA1, AUC was 0·79 (p<0·0001; 0·69–0·87; n=133); for MDA/MAQC-II, AUC was 0·76 (p=0·00061; 0·61–0·87; n=100); and for the combined cohort, AUC was 0·77 (p<0·0001; 0·69–0·84; n=233; data not shown).

Measurements of gene expression in the cohorts treated with neoadjuvant paclitaxel were consistent with in-vitro functional observations; repression of genes in the mitotic module was associated with resistance to T-FAC therapy, and repression of genes in the ceramide module was associated with sensitivity to T-FAC. Moreover, genes whose suppression increased paclitaxel resistance across all three cancer cell lines of different tissue origin were enriched in a list of genes that are differentially expressed in patients who were sensitive or resistant to paclitaxel (a binomial test addressing the probability that four of six

| Mitotic module | Ceramide module | Paclitaxel response metagene | Paclitaxel response metagene including all module genes | Literature-based metagene |
|----------------|-----------------|-----------------------------|--------------------------------------------------------|---------------------------|
| **All patients** |                 |                             |                                                        |                           |
| MDA1           | 0·73; 0·63–0·82; <0·0001 | 0·74; 0·62–0·83; <0·0001 | 0·79; 0·69–0·87; <0·0001 | 0·79; 0·68–0·85; <0·0001 | 0·52; 0·39–0·62; 0·39 |
| MDA/MAQC-II    | 0·71; 0·52–0·82; 0·0059* | 0·73; 0·58–0·85; 0·0023*† | 0·76; 0·61–0·87; 0·0066*† | 0·75; 0·58–0·86; 0·00094* | 0·56; 0·35–0·72; 0·77† |
| TOP            | 0·49; 0·34–0·68; 0·57 | 0·50; 0·33–0·69; 0·51† | 0·50; 0·34–0·66; 0·52 | 0·50; 0·36–0·65; 0·49 | 0·56; 0·41–0·71; 0·801 |
| EORTCnr        | 0·57; 0·45–0·67; 0·11 | 0·59; 0·48–0·71; 0·061† | 0·62; 0·49–0·72; 0·025*† | 0·62; 0·51–0·72; 0·023* | 0·55; 0·43–0·66; 0·811 |
| EORTCir        | 0·53; 0·37–0·68; 0·64 | 0·54; 0·39–0·69; 0·71† | 0·53; 0·38–0·67; 0·65 | 0·52; 0·37–0·70; 0·61† | 0·55; 0·40–0·70; 0·761 |

| **Triple-negative** |                   |                             |                                                        |                           |
|---------------------|------------------|-----------------------------|--------------------------------------------------------|---------------------------|
| MDA1                | 0·70; 0·45–0·87; 0·028* | 0·65; 0·41–0·86; 0·093† | 0·79; 0·53–0·93; 0·0052* | 0·79; 0·51–0·9; 0·017* | 0·55; 0·29–0·79; 0·23 |
| MDA/MAQC-II         | 0·62; 0·36–0·85; 0·34 | 0·67; 0·38–0·86; 0·075† | 0·72; 0·48–0·9; 0·031* | 0·71; 0·43–0·88; 0·035* | 0·58; 0·31–0·78; 0·751 |
| TOP                 | 0·50; 0·33–0·72; 0·51 | 0·55; 0·4–0·74; 0·31† | 0·53; 0·31–0·77; 0·38 | 0·52; 0·3–0·71; 0·42 | 0·64; 0·38–0·83; 0·911 |
| EORTCnr             | 0·54; 0·36–0·72; 0·3 | 0·50; 0·33–0·68; 0·52† | 0·53; 0·36–0·71; 0·35 | 0·54; 0·36–0·69; 0·33 | 0·59; 0·41–0·74; 0·871 |
| EORTCir             | 0·63; 0·44–0·80; 0·921 | 0·64; 0·44–0·79; 0·93 | 0·64; 0·43–0·81; 0·93† | 0·61; 0·40–0·78; 0·89* | 0·52; 0·31–0·70; 0·591 |
| DFCI                | 0·57; 0·04–0·87; 0·33 | 0·56; 0·28–0·89; 0·35 | 0·59; 0·22–0·82; 0·28 | 0·56; 0·20–0·94; 0·35 | 0·70; 0·0–0·98; 0·921 |

Data are AUC; 95% CI; p value. Histopathological subtypes were based on pathology review by ER/PR/HER2 immunohistochemistry or HER2 FISH, supplemented by expression if histopathological data were unavailable. AUCarea under the curve. FEC=fluorouracil, epirubicin, and cyclophosphamide. TET=docetaxel followed by epirubicin and docetaxel. ER=oestrogen receptor. PR=progesterone receptor. *Significant association between module or metagene and treatment response. †These values predict resistance to paclitaxel, all others predict sensitivity.

Table 2: Association between gene sets and treatment response for all patients and for triple-negative patients for each cohort
mitotic genes from the RNAi screen were significantly predictive of pCR in the MDA1 cohort when compared with the background probability of 3802/26138 that a gene in the MDA1 cohort was significantly predictive of pCR (OR 11.40, 95% CI 2.088–62.29; p=0.0052) and that four of six mitotic genes from the RNAi screen were significantly predictive of pCR in the MDA/MAQC-II cohort compared with the background probability of 1986/26138 that a gene in the MDA1 cohort was significantly predictive of pCR (OR 23.55, 95% CI 4.31–128.69; p=0.00044), and that four of six mitotic genes from the RNAi screen were significantly predictive of pCR in both the MDA1 and the MDA/MAQC-II cohorts compared with the background probability of 3802/26138 that a gene in both cohorts were significantly predictive of pCR (OR 11.40, 95% CI 2.088–62.29; p=0.0052) and that four of six mitotic genes from the RNAi screen were significantly predictive of pCR in both the MDA1 and the MDA/MAQC-II cohorts compared with the background probability of 3802/26138 that a gene in both cohorts were significantly predictive of pCR (OR 11.40, 95% CI 2.088–62.29; p=0.0052) and that four of six mitotic genes from the RNAi screen were significantly predictive of pCR in both the MDA1 and the MDA/MAQC-II cohorts compared with the background probability of 3802/26138 that a gene in both cohorts were significantly predictive of pCR (OR 11.40, 95% CI 2.088–62.29; p=0.0052) and that four of six mitotic genes from the RNAi screen were significantly predictive of pCR in both the MDA1 and the MDA/MAQC-II cohorts compared with the background probability of 3802/26138 that a gene in both cohorts were significantly predictive of pCR (OR 11.40, 95% CI 2.088–62.29; p=0.0052) and that four of six mitotic genes from the RNAi screen were significantly predictive of pCR in both the MDA1 and the MDA/MAQC-II cohorts compared with the background probability of 3802/26138 that a gene in both cohorts were significantly predictive of pCR (OR 11.40, 95% CI 2.088–62.29; p=0.0052) and that four of six mitotic genes from the RNAi screen were significantly predictive of pCR in both the MDA1 and the MDA/MAQC-II cohorts compared with the background probability of 3802/26138 that a gene in both cohorts were significantly predictive of pCR (OR 11.40, 95% CI 2.088–62.29; p=0.0052). These data suggest that the concordance of the functional genomic and clinical trial genomics datasets is unlikely to result from a chance association.

For multivariate analysis, we combined the two T-FAC-treated MDA1 and MDA/MAQC-II cohorts to increase statistical power. We found that the paclitaxel response metagene was the covariate most significantly associated with pCR (p=0.0039; odds ratio 19.92; 95% CI 12.62–151.57) in T-FAC-treated patients with triple-negative breast cancer, more than nodal status, T stage, tumour grade, and Ki67 (table 3). The paclitaxel response metagene also did better than the genomic grade index and the stroma signature in both univariate and multivariate analysis of the combined T-FAC clinical trials, when all patients were considered and in the triple-negative cohorts (table 4). These data support the conclusion that the paclitaxel response metagene derived from an RNAi screen has predictive power in two paclitaxel clinical trial cohorts that had no role in the discovery of the genes included in the metagene. The response metagene was not significantly associated with recurrence-free survival in two untreated triple-negative breast cancer cohorts, indicating the predictive rather than prognostic power of the metagene (webappendix p 4).

To further validate the functional importance of the genes contained within the metagene and limit the possibility that the correlation step would artificially enrich for genes predictive of pCR, we tested the ability to predict for pCR of 10000 random nine-gene sets subjected to the same correlative approach. None of the 10000 combinations predicted for pCR better than the paclitaxel response metagene (p=0.0001). We addressed the relevance of the RNAi screening process to select genes for inclusion in the metagene by performing the same analysis with 24 genes reported to be associated with paclitaxel or docetaxel resistance. The literature metagene did not show any predictive value in the MDA1 or MDA/MAQC-II cohorts. In a further analysis, we eliminated the correlation step from the derivation of the paclitaxel response metagene. Although the nine-gene set (figure 1) selected before the expression correlation step was still predictive of pCR with T-FAC, this gene set did not do as well as the paclitaxel response metagene. These results suggest that unbiased selection of correlated genes with consistent phenotypes across an RNAi screen improves the performance of the predictive metagene (table 2).

To assess the paclitaxel specificity of the paclitaxel response metagene, we assessed its predictive power in four cohorts that did not receive paclitaxel (table 1, webappendix p 5): the EORTC 10994 FEC trial cohort, EORTC 10994 TET trial cohort, the TOP epirubicin trial cohort, and the triple-negative DFCI cisplatin-treated cohort. The paclitaxel response metagene did not predict response in the triple-negative subtype (p>0.05, figure 2 C–F). Notably, the paclitaxel response metagene did not predict a significant response to the TET regimen. The triple-negative tumours analysed were somewhat homogeneous (reflecting the neoadjuvant setting of these trials), with similar nodal status, T stage, and were higher grade tumours (webappendix p 5), indicating that tumour heterogeneity is unlikely to account for this result.

Figure 2: Receiver operating characteristic (ROC) curves for the gene modules and the paclitaxel response metagene

ROC curve in triple-negative patients in the T-FAC-treated cohorts MDA1 (A) and MDA/MAQC-II (B), the epirubicin-treated TOP cohort (C), the FEC-treated EORTC cohort (D), the TET-treated EORTC cohort (E), and in the cisplatin-treated DFCI cohort (F). AUC=area under the curve. FEC=fluorouracil, epirubicin, and cyclophosphamide. TET=docetaxel followed by epirubicin and docetaxel. *These values predict paclitaxel resistance, all others predict sensitivity.
Using a meta-analysis, we combined the odds ratios of the paclitaxel response metagene to pCR in the two paclitaxel-treated cohorts and in the four non-paclitaxel treated cohorts. We found that the summary odds ratio of the paclitaxel treated cohorts was 5·65 (95% CI 1·67–19·11; p=0·0053), whereas the summary odds ratio of the non-paclitaxel treated cohorts was 0·87 (95% CI 0·44 to 1·67; p=0·67), consistent with improved predictive power of the paclitaxel response metagene in paclitaxel treated cohorts (figure 3).

Finally, we combined paclitaxel-treated with non-paclitaxel-treated triple-negative cohorts and did logistic-regression analysis using a mixed effects model, considering paclitaxel treatment, binary metagene status, and their interaction. We observed a significant interaction term between paclitaxel treatment and binary metagene status (OR 5·9, 95% CI 1·61–23·18; p=0·0089), indicating that the paclitaxel response metagene has paclitaxel-specific predictive power.

**Discussion**

This study supports the use of high-throughput RNAi functional genomics screening to accelerate discovery of predictive biomarkers in cancer medicine. By filtering for common paclitaxel resistance pathways through RNAi screening across three cell lines of different tumour origin, and selecting genes which correlate across independent cohorts, we derived a paclitaxel response metagene that is predictive of T-FAC response in two clinical trial datasets, but not in cohorts treated without paclitaxel. Our results show the usefulness of this approach to identify drug-specific response predictors. Data reported here support a specific response predictors. Data reported here support a model whereby expression of genes that regulate mitotic spindle assembly checkpoint signalling, and genes that arrest and chromosomal stability, mediated through a model whereby expression of genes that regulate mitotic spindle assembly checkpoint signalling, and genes that arrest and chromosomal stability, mediated through a common paclitaxel resistance pathways through RNAi screen in a triple-negative breast-cancer cell line, we investigated the performance of the paclitaxel response metagene in vivo (figure 4). Since the metagene was derived from an RNAi screen in a triple-negative breast-cancer cell line, we investigated the performance of the paclitaxel response metagene compared with published gene-expression signatures in univariate and multivariate logistic regression (combined analysis).

**Table 3: Univariate and multivariate analysis for response to treatment (combined analysis)**

| All patients | Univariate analysis | Multivariate analysis |
|--------------|--------------------|----------------------|
| Stroma metagene | 0·45; 0·23–0·86; 0·016* | 0·55; 0·27–1·11; 0·095 |
| Genomic grade index | 4·11; 1·98–8·54; 0·00015* | 1·59; 0·66–3·80; 0·30 |
| Paclitaxel response metagene | 7·28; 3·23–16·41; <0·0001* | 5·37; 2·12–13·64; 0·00040* |
| Triple-negative | | |
| Stroma metagene | 0·28; 0·09–0·85; 0·025* | 0·27; 0·07–1·03; 0·055 |
| Genomic grade index | 2·35; 0·65–8·52; 0·19 | 0·41; 0·06–2·68; 0·35 |
| Paclitaxel response metagene | 4·51; 1·41–14·43; 0·011* | 5·47; 1·21–24·76; 0·027* |

Data are odds ratios; 95% CI; p value. All covariates are binary. The binary metagene and binary Ki67 is defined as the upper 50th percentile. T stage is T3–4 vs T2–0. Grade is G3 vs G1 and G2. Node is N+ versus N0. *Significant values.

**Table 4: Performance of the paclitaxel response metagene compared with published gene-expression signatures in univariate and multivariate logistic regression (combined analysis)**

| All patients | Univariate analysis | Multivariate analysis |
|--------------|--------------------|----------------------|
| MDA1 | | |
| MDA/MAQC-II | | |
| TOP | | |
| EORTC | | |
| EORTC | | |
| DFCI | | |

**Figure 3: Odds ratio for pathological complete response in patients with high paclitaxel response metagene score, compared with a low score.**

FEC=fluorouracil, epirubicin, and cyclophosphamide. TET=docetaxel followed by epirubicin and docetaxel. 13 (8%) would be possible by sacrificing specificity, resulting in the sparing of suboptimal therapy in seven of 14 patients with resistant disease. A false negative proportion of 8% would compare with the false negative proportion of ER analysis in breast cancer (Sotiriou C, unpublished data). We plan to assess whether the...
performance of the paclitaxel response metagene approach might be improved through gene selection from genome-wide RNAi screening approaches targeting more than 21000 genes across multiple cell lines, by contrast with the 829 genes assessed in this study.

Sources of random and systematic error should be considered when interpreting these data. Notably, the triple-negative breast-cancer cohorts are likely to be molecularly heterogeneous. Although no patients enrolled in the T-FAC trials were known to have germline BRCA mutations, the same DNA repair-pathway mechanisms may be disrupted in sporadic breast cancers that have also been implicated in taxane resistance in vitro.22,23 Also, it should be noted that the T-FAC clinical trial datasets were acquired from fine needle aspirations whereas the non-paclitaxel trials were acquired from core biopsies. RNA yield and expression profiling are similar using both techniques,24 but we cannot exclude the possibility that the enrichment of stromal elements in the core biopsy datasets contribute to the lack of predictive power of the paclitaxel response metagene. There is heterogeneity between the clinical studies including the timing and measurement of pCR by different pathological centres. The five trials examined here varied in chemotherapy exposure from 12 to 24 weeks, and two trials used monotherapy schedules that might affect the proportion of patients achieving a pCR.

A metagene derived from reports of genes implicated in taxane resistance did not show T-FAC predictive power. Furthermore, none of 10000 random nine-gene sets performed better than the metagene. While these results support the RNAi approach to biomarker discovery and argue against the role of chance in these findings, the modest cohort sizes and the heterogeneity of the non-paclitaxel trials require replication in larger prospective studies to confirm the relevance of this method with a clinically applicable gene-expression assay. Experience with the Oncotype DX assay has shown that RNA-based expression measurements (real-time PCR) from paraffin-fixed tumour material can inform clinical decision making. A similar assay to assess the expression of the paclitaxel response metagene could be developed that, we estimate, would cost less than €30 per patient. With this assay, exact thresholds of mitotic and ceramide module expression should be defined retrospectively with tumours from the T-FAC trial before testing the defined threshold in a prospective trial. A randomised clinical trial comparing a paclitaxel with a non-paclitaxel regimen will be required to formally support the paclitaxel-specificity of the metagene and the relevance of RNAi to the biomarker discovery process. The usefulness of this approach in routine clinical practice should then be assessed, since patients enrolled in clinical trials may not accurately reflect the demographics and clinical stage of patients diagnosed with primary breast cancer.

Notably, the paclitaxel response metagene was not predictive of pCR in the EORTC-10 cohort (docetaxel then epirubicin and docetaxel), which may be explained by the non-overlapping pathways of drug resistance to the two taxanes. The metagene was derived from a screen to identify mediators of paclitaxel not docetaxel resistance across three cell lines, and preclinical data has shown that docetaxel binds to β-tubulin with greater affinity than paclitaxel and has increased interphase (G1/S/G2) cell-cycle activity, mediating cell death through the induction of BCL2 phosphorylation.25–28 Finally, 19–31% of patients respond to paclitaxel having progressed on or after docetaxel,22–24 supporting the divergent drug-resistance mechanisms of these two taxanes.

We cannot be certain that the paclitaxel response metagene is paclitaxel specific, despite the test for interaction, since silencing of COL4A3BP and UGCG has been shown to promote doxorubicin sensitisation.29 Consistent with this hypothesis, we note that the paclitaxel response metagene is weakly predictive of pCR in the EORTC database across all patients (AUC 0·62; 95% CI 0·49–0·72; p=0·025). The ceramide module is likely the main contributor, since COL4A3BP expression alone predicts for pCR in all patients (0·59; 0·48–0·71; p=0·061); this would support the role of the ceramide pathway in the regulation of multidrug sensitivity in vivo.

In summary, we used in-vitro functional genomics analyses to guide the development of a metagene to predict paclitaxel response in patients with breast cancer. Although we cannot conclude that our approach is better than associative predictive strategies, the latter strategy...
requires training of two large cohorts and validation of such signatures. The functional genomics approach used in this study could be an efficient method to accelerate biomarker development for experimental therapies in single-cohort early phase clinical trials, where stratification of response according to tumour expression of a functional metagene could be considered.

Contributors
NJ, ZS, and CSw designed the study and the research concept. NJ, ACE, BH-K, and QI did the statistical and bioinformatics analysis. CSw, LP, VV, EA, FJE, CD, BH-K, and WFS provided datasets and supervised the clinical trials. NJ, RAB, MG, ACE, ZS, and CSw wrote the paper. All authors reviewed the manuscript.

Conflicts of interest
The authors declared no conflicts of interest.

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