

## ARTICLE OPEN

A multifactorial ‘Consensus Signature’ by *in silico* analysis to predict response to neoadjuvant anthracycline-based chemotherapy in triple-negative breast cancerNatalie Turner<sup>1,4</sup>, Mattia Forcato<sup>2,4</sup>, Simona Nuzzo<sup>2</sup>, Luca Malorni<sup>1,3</sup>, Silvio Bicciato<sup>2,5</sup> and Angelo Di Leo<sup>1,5</sup>

**BACKGROUND:** Owing to the complex processes required for anthracycline-induced cytotoxicity, a prospectively defined multifactorial Consensus Signature (*ConSig*) might improve prediction of anthracycline response in triple-negative breast cancer (TNBC) patients, whose only standard systemic treatment option is chemotherapy.

**AIMS:** We aimed to construct and evaluate a multifactorial signature, comprising measures of each function required for anthracycline sensitivity in TNBC.

**METHODS:** *ConSigs* were constructed based on five steps required for anthracycline function: drug penetration, nuclear topoisomerase IIa (topolla) protein location, increased topolla messenger RNA (mRNA) expression, apoptosis induction, and immune activation measured by, respectively, HIF1 $\alpha$  or SHARP1 signature, LAPTM4B mRNA, topolla mRNA, Minimal Gene signature or YWHAZ mRNA, and STAT1 signature. TNBC patients treated with neoadjuvant anthracycline-based chemotherapy without taxane were identified from publicly available gene expression data derived with Affymetrix HG-U133 arrays (training set). *In silico* analyses of correlation between gene expression data and pathological complete response (pCR) were performed using receiver-operating characteristic curves. To determine anthracycline specificity, *ConSigs* were assessed in patients treated with anthracycline plus taxane. Specificity, sensitivity, positive and negative predictive value, and odds ratio (OR) were calculated for *ConSigs*. Analyses were repeated in two validation gene expression data sets derived using different microarray platforms.

**RESULTS:** In the training set, 29 of 147 patients had pCR after anthracycline-based chemotherapy. Various combinations of components were evaluated, with the most powerful anthracycline response predictors being *ConSig1*: (STAT1+topolla mRNA +LAPTM4B) and *ConSig2*: (STAT1+topolla mRNA+HIF1 $\alpha$ ). *ConSig1* demonstrated high negative predictive value (85%) and high OR for no pCR (3.18) and outperformed *ConSig2* in validation sets for anthracycline specificity.

**CONCLUSIONS:** With further validation, *ConSig1* may help refine selection of TNBC patients for anthracycline chemotherapy.

*npj Breast Cancer* (2015) 1, 15003; doi:10.1038/npjbcancer.2015.3; published online 2 June 2015

## INTRODUCTION

Preferred breast cancer (neo)adjuvant chemotherapy regimens are generally anthracycline based, given the improved outcomes compared with cyclophosphamide/methotrexate/fluorouracil.<sup>1</sup> However, across all anthracycline-treated patients, only a small percentage actually receives benefit while these agents are associated with significant toxicities. Breast cancer is well recognized as a heterogeneous disease and therefore treating all breast cancers with the same chemotherapeutic agents could be considered illogical. Of considerable use would be a predictive marker of response to distinguish patients likely to receive benefit from those who are not, sparing predicted ‘poor responders’ from associated toxicities. Unfortunately, suitable predictive biomarkers for chemotherapeutic agents have remained elusive to date.

Topoisomerase IIa gene (*TOP2A*) is a putative marker of anthracycline sensitivity, with its gene product being the direct target of anthracyclines. *TOP2A* amplification has been shown to predict increased sensitivity to anthracyclines in several studies,<sup>2–7</sup> although this finding has not been entirely consistent.<sup>8,9</sup> Indeed, a

single biomarker may not be sufficient to predict anthracycline response, and a multifactorial approach using gene signatures might be required.<sup>7,10</sup> In the TOP trial<sup>7</sup> of 149 patients with estrogen receptor (ER)-negative early or locally advanced breast cancer treated with a single agent epirubicin, *TOP2A* amplification was significantly associated with pathological complete response (pCR). In addition, a multifactorial anthracycline sensitivity score (the A-score), comprised of three gene signatures, was evaluated, demonstrating a very high negative predictive value (NPV), although a much lower positive predictive value (PPV).<sup>7</sup>

Gene signatures are often defined through retrospective analyses of tumor tissue gene expression patterns correlated with patient outcomes. Although concordance in outcome prediction between signatures has been demonstrated,<sup>11</sup> signatures derived in this manner often contain few genes in common as well as numerous genes of unknown function, making their clinical relevance less certain. It might be possible to improve clinical relevance of a signature by identifying the molecular processes required for a specific cellular function, such as anthracycline-

<sup>1</sup>Sandro Pitigliani Medical Oncology Department, Prato Hospital, Istituto Toscano Tumori, Prato, Italy; <sup>2</sup>Department of Biomedical Sciences, Centre for Genome Research, University of Modena and Reggio Emilia, Modena, Italy and <sup>3</sup>Translational Research Unit, Prato Hospital, Istituto Toscano Tumori, Prato, Italy.

Correspondence: A Di Leo (adileo@usl4.toscana.it)

<sup>4</sup>These authors contributed equally to this work.

<sup>5</sup>These authors contributed equally to this work.

Received 17 March 2015; accepted 20 March 2015

induced cytotoxicity, and construct a signature containing measures of each of these functions. On the basis of this hypothesis, we aimed to construct and evaluate a multifactorial Consensus Signature for predicting anthracycline sensitivity in triple-negative breast cancer (TNBC). The term Consensus Signature was chosen to reflect the concept that, by each selected component acting as a surrogate marker of the different steps required for anthracycline cytotoxicity, included components (the genes and gene signatures) would work synergistically to provide an overall measure of effective anthracycline function.

We focused specifically on TNBC for the following reasons. First, there has been substantial work performed already evaluating the predictive role of *TOP2A* in HER2<sup>+</sup> breast cancer, due to the known relationship between *TOP2A* amplification and HER2 amplification. Anthracyclines are commonly used in TNBC and appear to have activity. We wanted to assess this without the confounding factor of HER2 overexpression. Second, with treatment options in TNBC limited to chemotherapy, more effective use of chemotherapy would be of considerable benefit. Finally, with growing understanding of breast cancer biological diversity, evaluation of a predictive biomarker within a specific subtype might be preferable, as positive results could otherwise be masked if evaluated across a heterogeneous combined cohort.

## MATERIALS AND METHODS

### Data set

The construction and evaluation of consensus signatures (*ConSigs*) were carried out using a retrospective cohort study design, with *in silico* analyses of previously collected genetic data, clinical characteristics, and responses. The data set comprised gene expression profiles of patients who had received neoadjuvant anthracycline-based chemotherapy without a taxane. As the *ConSigs* were designed to be specific for anthracyclines, taxane use was considered a confounding factor.

Data derived from Affymetrix (Santa Clara, CA, USA) gene expression arrays based on build 133 of UniGene database (HG-U133) were combined and evaluated as a single group, designated the 'breast compendium' (details on the construction and composition of the breast compendium are reported in Supplementary Table 1). The subset of samples treated with anthracycline-based neoadjuvant chemotherapy was used as training set to derive the *ConSigs*, whereas the cohort of patients treated with anthracycline plus taxane-based neoadjuvant chemotherapy served as a control group to assess *ConSig* specificity for anthracyclines. In addition, two cohorts of patients treated with anthracycline-based neoadjuvant chemotherapy with gene expression data derived from different microarray platforms served as validation sets. The European Organisation for Research and Treatment of Cancer (EORTC)/BIG00-01 data set included gene expression data obtained with the Affymetrix X3P array from patients with locally advanced, inflammatory, or large operable breast cancers treated with either fluorouracil/epirubicin/cyclophosphamide or docetaxel followed by docetaxel/epirubicin under the auspices of the EORTC 10994 trial.<sup>12</sup> The data set was available in the Gene Expression Omnibus repository under accession number GSE6861. The Netherlands Cancer Institute (NKI) data set (accessible under accession number GSE34138) used the Illumina (San Diego, CA, USA) HumanWG 6 v3.0 expression beadchip for gene expression profiling and included patients with intermediate or high-risk ER-breast cancer treated with neoadjuvant dose-dense doxorubicin/cyclophosphamide.<sup>13</sup>

For this study, only TNBC patients in the training and control subsets of the compendium and in the validation sets were considered. Further details on the construction of the breast compendium and on the definition of TNBCs are reported in the Supplementary Methods.

### Design of the consensus signature

In order for anthracyclines to be effective, we postulated that the following steps must occur: (1) penetration of the drug into the tumor bed, (2) location of the target (topolla protein) within the nucleus, (3) increased topolla messenger RNA (mRNA) expression above that related to proliferation alone, (4) induction of apoptosis, and (5) active immune/stromal function.

For each step, a representative gene or gene signature was selected, with gene signatures chosen over single genes where possible. In some cases, more than one potential genes/gene signatures was evaluated for each step, to compare their relative utility. Details of the marker(s) evaluated, their association with anthracycline response, and rationale for their selection are listed in Table 1.

### Quantification of genes and gene signatures

SHARP1 signature,<sup>14</sup> HIF1 $\alpha$  hypoxia signature (HIF),<sup>14</sup> and the Minimal Gene signature<sup>15</sup> were quantified as previously described. Briefly, each signature was calculated by summarizing the standardized expression levels of the genes in the signature into a combined score with zero mean. AURKA, STAT1, and PLAU signatures were computed as previously described<sup>16</sup> using *genefu* R package. Briefly, for each sample, the signature was quantified as:  $s = \sum \omega_i \xi_i / \sum |\omega_i|$  where  $\xi_i$  is the expression of a gene  $i$  included in the set of genes of interest and  $\omega_i$  is either +1 or -1 depending on the sign of the association under study. Gene expression lists for each signature are included in Supplementary Table 2.

LAPTM4B, AURKA, YWHAZ, and topolla mRNA expression levels were calculated using the corresponding probe sets or the median expression if multiple probe sets were available for each gene. For the NKI data set, probes were filtered on the basis of their quality, keeping only probes classified as 'perfect' and 'good' in the Bioconductor illumina Human v3.db annotation package.

### Quantification of the Consensus Signature

The Consensus Signature score was calculated in a continuous form, consisting of a linear combination of the various components/signatures. Prior to combination, each component/signature was scaled to have the interquartile range equal to 1 and the median equal to 0. On the basis of the association with pCR (Table 1), we hypothesized that a high Consensus Signature score would predict for increased pCR rate, whereas a low score should predict anthracycline resistance.

### Statistical analysis

All statistical analyses were performed in R version 2.15.1.<sup>17</sup> Odds ratios (ORs) were used to compare pCR rates between groups defined by different clinical and molecular characteristics (*stats* R package). The area under the curve (AUC) was used to assess the prediction performance of any signature score (*ROCR* R package). AUC was estimated through the concordance index (*survcomp* R package) under the alternative hypothesis that AUC was greater than 0.5, as each signature score was designed to have positive AUC. Its significance and confidence interval were estimated assuming asymptotic normality. Because of the differences in array design and technology between the training and the two validation sets, the threshold *ConSig* score for each cohort was calculated using the score value that corresponded to the 75th percentile of the score distribution for that cohort.  $P$  values of  $< 0.05$  were considered significant.

## RESULTS

### Data set

The training set was derived from a breast cancer data set originally consisting of 4,600 samples collected in 27 different studies. After exclusion of duplicate samples ( $n=939$ ) and adjusting for batch effect using ComBat,<sup>17</sup> 1,069 samples (29%) were classified as TNBC by the SCMOD2 subtype clustering classifier (subtype clustering model)<sup>18</sup> contained in *genefu* R package.<sup>19</sup> Among these samples, 491 had information about neoadjuvant chemotherapy. Eleven samples were from patients treated with taxane but not anthracycline and were excluded, whereas 147 and 333 samples were from patients treated with anthracycline-based therapy without taxane and anthracycline-based therapy with taxane, respectively (Figure 1).

Clinical and tumor characteristics for the patients in the training set ( $n=147$ ) treated with anthracycline-based chemotherapy are listed in Table 2. The samples were originally contained in four different data sets,<sup>7,20-22</sup> and included 29 (19.7%) pCRs and 118 (80.3%) samples with residual disease. pCR was defined as ypT0/is, ypN0 in all included studies.

**Table 1.** Consensus Signature components based on putative steps required for effective anthracycline-induced cytotoxicity

Step	Surrogate marker	Association with pCR	Rationale
Penetration of drug into the tumor bed	SHARP1 signature Hypoxia signature (HIF)	Negative Negative	Hypoxia, promoted by HIFs, is a well-known contributor to decreased drug penetration, and chemoresistance. <sup>1</sup> Montagner <i>et al.</i> recently described a hypoxia signature of 22 genes, with increased expression correlated with increased HIF activity. <sup>2</sup> A direct interaction between SHARP1 (a downstream target of the tumor suppression gene p63) and HIF1 $\alpha$ and HIF2 $\alpha$ was demonstrated, with a signature of low SHARP1 activity in TNBC conferring increased HIF function and increased hypoxia. <sup>2</sup> With the SHARP1 signature measuring low SHARP activity and thus increased HIF function, it has a negative association with pCR.
Location of topoll $\alpha$ protein within the nucleus	LAPTM4B	Negative	In order to work effectively, the target of anthracyclines, topoll $\alpha$ protein, must have access to nuclear DNA; thus, it must be located in the nucleus. Nuclear export of topoll $\alpha$ protein may contribute to anthracycline resistance. <sup>3,4</sup> topoll $\alpha$ protein nuclear location might be inferred using the expression level of LAPTM4B. <sup>5</sup> <i>LAPTM4B</i> gene resides on chromosome 8q22, with overexpression shown to increase sequestration of anthracyclines in the cytoplasm. Increased levels of LAPTM4B mRNA have been correlated with increased anthracycline resistance, whereas selective depletion of LAPTM4B significantly increased sensitivity to anthracycline, but not cisplatin or taxane, chemotherapy. <sup>5</sup>
Increased expression of topoll $\alpha$ mRNA, independent of proliferation	topoll $\alpha$ mRNA topoll $\alpha$ mRNA: AURKA topoll $\alpha$ mRNA: AURKA signature	Positive Positive Positive	<i>TOP2A</i> transcription can be enhanced by proliferation signals independently of gene aberrations and topoll $\alpha$ protein is strongly influenced by proliferation. <sup>6</sup> Increased expression of topoll $\alpha$ protein therefore may be seen in the setting of highly proliferating tumors, without correlating with an increased likelihood of response specifically to anthracyclines. By determining the ratio of expression of topoll $\alpha$ mRNA relative to that of a known proliferation marker (Aurora kinase A gene, AURKA, <sup>7,8</sup> or AURKA gene signature), tumors with increased topoll $\alpha$ mRNA independent of proliferation might be determined.
Induction of apoptosis	YWHAZ Minimal gene signature (MS)	Negative Positive	The anti-apoptotic gene YWHAZ (coding for 14-3-3 $\zeta$ ) resides on chromosome 8q22 close to <i>LAPTM4B</i> gene and may promote <i>de novo</i> anthracycline resistance. <sup>5</sup> Increased expression has been associated with increased doxorubicin resistance in breast cancer cell lines, and early relapses after anthracycline chemotherapy. siRNA knockdown of YWHAZ in breast cancer cell lines significantly increased doxorubicin-induced apoptosis. <sup>5</sup> An alternate marker of apoptosis is the MS, <sup>9</sup> comprising two genes, <i>SHARP1</i> and <i>CCNG2</i> . As with SHARP1, CCNG2 is a downstream target of p63. As p63 is inhibited by mutant p53, lack of MS expression implies dysfunction in the p53 pathway, the major apoptotic pathway in the presence of oncogenic stress, and may be a suitable surrogate for lack of apoptosis.
Active immune function	Immune function signature (STAT1) Stromal signature (PLAU)	Positive Negative	Both innate and adaptive immune responses are important in anthracycline toxicity. <sup>10-13</sup> Anthracyclines trigger immunogenic cell death by eliciting tumor-specific IFN $\gamma$ CD8 <sup>+</sup> cytotoxic T lymphocytes, thus an anthracycline-induced anticancer immune response can help eradicate residual cancer cells, or maintain residual cells in state of dormancy. Moreover, immune module scores <sup>14,15</sup> have been associated with higher probability of achieving pCR after anthracycline $\pm$ taxane chemotherapy among all breast cancer subtypes when defined by immunohistochemistry. <sup>16</sup> Closely related to immune function, stromal signatures may also be useful in predicting anthracycline sensitivity or resistance. <sup>15,17</sup>

Abbreviations: HIF, hypoxia-inducible factor; IFN $\gamma$ , interferon gamma; LAPTM4B, lysosomal-associated protein transmembrane 4B gene; mRNA, messenger RNA; MS, minimal gene signature; pCR, pathological complete response; siRNA, small interfering RNA; TNBC, triple-negative breast cancer. References for this table are listed in Supplementary Materials.

#### Clinical characteristics

All clinical variables were tested for their ability to predict pCR, with no significant association between any clinical characteristics and pCR found (data not shown).

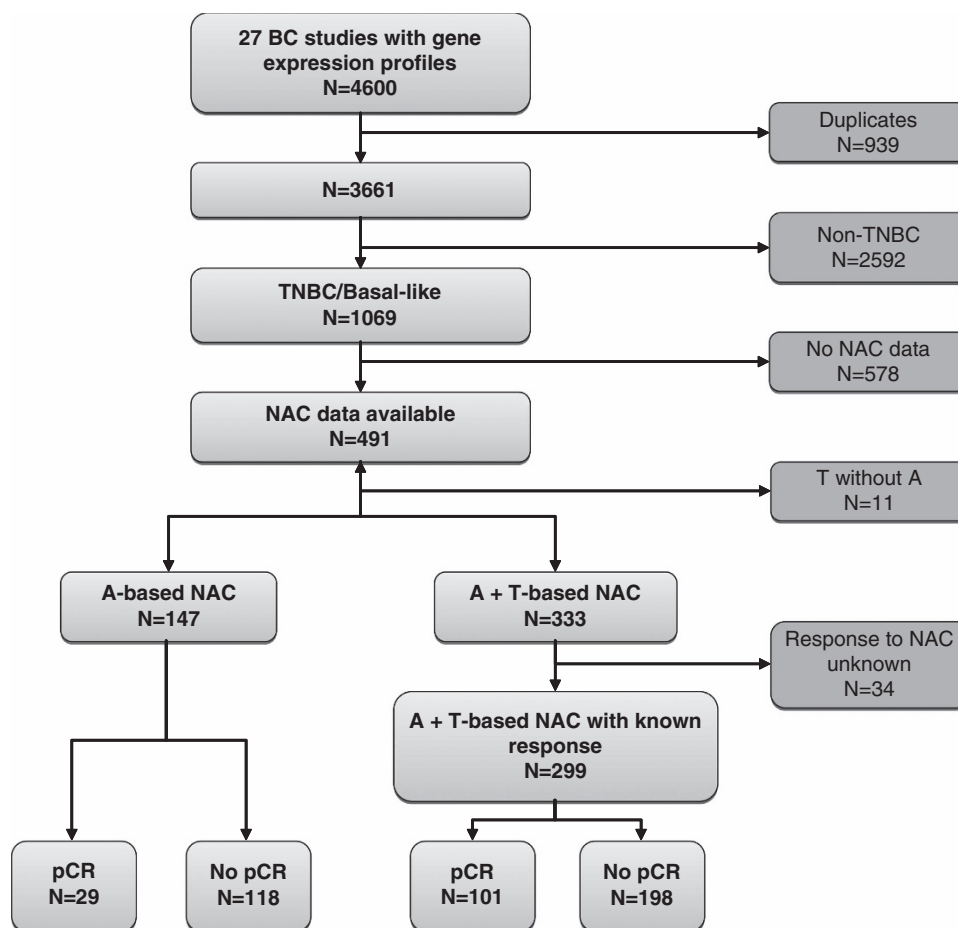
#### Predictive power of single gene or gene signatures

Using receiver-operating characteristic curves, the ability of any single component (gene or gene signature) to discriminate patients with pCR from patients with residual disease in the training set was assessed. STAT1 was significantly associated with

pCR status (Supplementary Table 3; Supplementary Figure 1a). All other components, when considered individually, were not significantly correlated with pCR. Topoll $\alpha$  mRNA corrected for proliferation with either AURKA mRNA or AURKA signature showed no increased correlation with pCR compared with topoll $\alpha$  mRNA alone (data not shown).

#### Predictive power of *ConSigs*

*ConSigs* was constructed using various combinations of components, with the starting point being components shown to have significant or near-significant predictive capability when



**Figure 1.** Consort diagram for selection of samples in the training set. A, anthracycline-based chemotherapy; BC, breast cancer; NAC, neoadjuvant chemotherapy; pCR, pathological complete response; T, taxane; TNBC, triple-negative breast cancer.

used alone, that is, STAT1, topolla, HIF, and LAPTM4B. Using a continuous score to quantify *ConSig* expression level, all combinations of core components demonstrated a significant correlation with pCR in patients in the training set treated with anthracycline-based chemotherapy without taxane. The two most predictive combinations were designated *ConSig1*: (STAT1+topolla+LAPTM4B) with AUC 0.70 (Supplementary Figure 1b),  $P=3.9 \times 10^{-5}$ , and *ConSig2*: (STAT1+topolla+HIF) with AUC 0.71,  $P=4.2 \times 10^{-6}$ . High correlation with pCR was maintained with the addition of further component genes/gene signatures to either *ConSig1* or *ConSig2*, but overall predictive power was not better than with three components (Table 3). The combination of STAT1+PLAU, the components for TNBC of another multifactorial scoring signature, the *A*-score,<sup>7</sup> was correlated with pCR, although less strongly than other combinations. Substituting topolla mRNA with topolla mRNA corrected for proliferation did not improve the performance of any of the *ConSigs*.

To assess specificity of *ConSigs* for anthracycline response compared with other chemotherapy regimens, we analyzed their respective performances in a control group of patients who received taxane in addition to anthracycline ( $n=333$ ), 299 of whom had information about response and 101 with pCR. For the *ConSigs* with the highest predictive power in the training set, i.e., *ConSig1* and *ConSig2*, neither was correlated with pCR in this control group (Supplementary Figure 1c and d). Although (STAT1+PLAU) had predicted response to anthracycline-based chemotherapy, it did not appear to be anthracycline specific, performing similarly in anthracycline+taxane-treated patients (Table 3).

#### Classification performance of *ConSigs*

A threshold score that could be used to classify a patient in the training set as a putative responder or as resistant was determined for each *ConSig* by selecting the score value corresponding to the 75th percentile of the score distribution. PPV and NPVs, sensitivity, specificity, and OR were then calculated. For *ConSig1* NPV was high (85%) as was OR for lack of pCR (OR=3.18,  $P=0.008$ ) (Table 4). PPV, however, was modest (PPV=35%). *ConSig2* performed similarly, with high NPV and OR, but modest PPV. In the control group of 299 patients treated with anthracycline plus taxane, NPVs for *ConSig1* and *ConSig2* were lower, at 66 and 67%, respectively, and ORs for lack of pCR were no longer statistically significant for either *ConSig* (Table 4), further supporting the specificity of these *ConSigs* for anthracycline response.

#### Evaluation of *ConSig1* and *ConSig2* in two independent 'validation' data sets

Two data sets, NKI and EORTC/BIG00-01, were selected as validation sets. The EORTC/BIG00-01 data set<sup>12</sup> comprised 161 samples, 85 of which were TNBC, with 46 patients treated with anthracycline without taxane (18 pCRs) and 39 treated with anthracycline plus taxane (18 pCRs). The NKI data set<sup>13</sup> included 178 ER-negative breast cancer samples, 52 of which were TNBC. Of these 52 patients, 24 had pCR. All patients received anthracycline-based neoadjuvant chemotherapy without taxane.

In the NKI data set, both *ConSig1* and *ConSig2* were significantly correlated with pCR for patients receiving anthra-

cycline without taxane (Table 5). In the EORTC/BIG00-01 data set, *ConSig1* remained specific for anthracyclines in the data set, whereas *ConSig2* was correlated with pCR for both anthracycline-based and anthracycline+taxane-based neoadjuvant chemotherapy.

**Table 2.** Clinical and tumor characteristics of TNBC patients in the training set treated with anthracycline-based neoadjuvant chemotherapy without taxane ( $n = 147$ )

Characteristic	No. of patients (%)
<b>Age (years)</b>	
< 40	12 (8)
40–60	40 (27)
> 60	7 (5)
NA	88 (60)
<b>Tumor size</b>	
T1	15 (10)
T2	90 (61)
T3	22 (15)
T4	19 (13)
NA	1 (1)
<b>Nodal status</b>	
N0	14 (9)
N1	13 (9)
N2	6 (4)
NA	114 (78)
<b>Histologic grade</b>	
G1	2 (1)
G2	25 (17)
G3	112 (76)
NA	8 (6)
<b>pCR</b>	
Yes	29 (20)
No	118 (80)

Abbreviations: NA, not available; pCR, pathological complete response; TNBC, triple-negative breast cancer.

## DISCUSSION

Overall, the best performing combination of components was *ConSig1* (STAT1+topolla+LAPTM4B). Our results suggest that *ConSig1* has excellent ability to predict anthracycline resistance within a cohort of anthracycline-treated TNBC patients. This is clinically relevant, as, if further validated, *ConSig1* could be used to identify TNBC patients for whom the addition of anthracycline is likely to add toxicity without benefit, and thus for whom an alternate chemotherapy regimen might be selected. When evaluated in TNBC patients who received anthracycline and taxane, the predictive ability of *ConSig1* was lost. Although not conclusive, a lack of predictive utility in patients who also received taxanes supports the anthracycline specificity of *ConSig1*. *ConSig2* was most strongly correlated with pCR in the training set; however, in the validation set *ConSig2* did not show discrimination in performance between patients treated with anthracycline-based and anthracycline plus taxane-based neoadjuvant chemotherapy.

Although we considered that five main processes should take place for anthracyclines to cause cell death, markers for two of these processes, that is, induction of apoptosis and hypoxia/drug penetration, did not appear to contribute significantly to the predictive power of *ConSig1*. Given that immune function (STAT1) was a powerful contributor to *ConSig1* and *ConSig2*, we postulated that in the setting of a highly active immune response, intact apoptotic pathways as measured by Minimal Gene signature<sup>15</sup> or

**Table 4.** Performance of *ConSig1* and *ConSig2* in the training set for predicting pathological complete response

<i>ConSig</i>	NAC	NPV (%)	PPV (%)	OR	95% CI	P value
<i>ConSig1</i>	A	85	35	3.18	1.34–7.54	$8.2 \times 10^{-3}$
	A+T	66	35	1.05	0.60–1.82	0.85
<i>ConSig2</i>	A	85	32	2.63	1.10–6.20	$2.8 \times 10^{-2}$
	A+T	67	37	1.23	0.71–2.12	0.45

Abbreviations: A, anthracycline-based chemotherapy; A+T, anthracycline +taxane chemotherapy; CI, confidence interval; *ConSig*, Consensus Signature; NAC, neoadjuvant chemotherapy; NPV, negative predictive value; OR, odds ratio for lack of pathological complete response; PPV, positive predictive value.

**Table 3.** Correlation with pCR for various combinations of *ConSig* components in the training set

<i>ConSig</i> combination	Type of NAC	AUC	95% CI	P value
STAT1+PLAU	A	0.63	0.52–0.73	$9.0 \times 10^{-3}$
	A+T	0.56	0.50–0.63	$3.1 \times 10^{-2}$
STAT1+topoll	A	0.68	0.57–0.78	$7.6 \times 10^{-4}$
	A+T	0.60	0.54–0.67	$1.2 \times 10^{-3}$
STAT1+topolla+LAPTM4B ( <i>ConSig1</i> )	A	0.70	0.60–0.80	$3.9 \times 10^{-5}$
	A+T	0.55	0.48–0.61	0.08
STAT1+topolla+HIF1 ( <i>ConSig2</i> )	A	0.71	0.62–0.80	$4.2 \times 10^{-6}$
	A+T	0.52	0.45–0.58	0.33
STAT1+topolla+YWHAZ	A	0.66	0.56–0.76	$6.8 \times 10^{-4}$
	A+T	0.54	0.48–0.61	0.10
STAT1+topolla+LAPTM4B+YWHAZ	A	0.66	0.56–0.76	$8.0 \times 10^{-4}$
	A+T	0.51	0.45–0.58	0.34
STAT1+topolla+LAPTM4B+MS+PLAU	A	0.62	0.51–0.72	$1.3 \times 10^{-2}$
	A+T	0.56	0.49–0.62	$4.2 \times 10^{-2}$

Abbreviations: AUC, area under the ROC curve; CI, confidence interval; *ConSig*, Consensus Signature; HIF, hypoxia-inducible factor; LAPTM4B, lysosomal-associated protein transmembrane 4B gene; MS, minimal gene signature; NAC, neoadjuvant chemotherapy; pCR, pathological complete response; ROC, receiver-operating characteristic.

For A-based chemotherapy,  $n = 147$  with 29 pCRs. For A+T chemotherapy,  $n = 299$  with 101 pCRs.

A: anthracycline-based neoadjuvant chemotherapy without taxane; A+T: anthracycline plus taxane neoadjuvant chemotherapy.

**Table 5.** Performance of *ConSig1* and *ConSig2* in the validation sets for predicting pathological complete response

Validation set	<i>ConSig</i>	NAC	AUC	95% CI	P value	NPV (%)	PPV (%)	OR	95% CI	P value
EORTC/BIG00-01	<i>ConSig1</i>	A	0.70	0.55–0.85	$5.2 \times 10^{-3}$	74	75	8.33	2.00–44.44	$6.0 \times 10^{-3}$
		A+T	0.60	0.43–0.77	0.12	62	70	3.81	0.87–20.75	0.09
	<i>ConSig2</i>	A	0.65	0.50–0.80	$2.4 \times 10^{-2}$	62	42	1.15	0.29–4.40	0.83
		A+T	0.65	0.49–0.81	$3.6 \times 10^{-2}$	59	60	2.13	0.50–9.92	0.31
NKI	<i>ConSig1</i>	A	0.74	0.61–0.87	$1.3 \times 10^{-4}$	64	77	5.95	1.53–29.99	$1.6 \times 10^{-2}$
	<i>ConSig2</i>	A	0.64	0.49–0.78	$3.1 \times 10^{-2}$	59	62	2.30	0.65–8.86	0.20

Abbreviations: A, anthracycline-based chemotherapy; A+T, anthracycline+taxane chemotherapy; AUC, area under the ROC curve; CI, confidence interval; *ConSig*, Consensus Signature; EORTC, European Organisation for Research and Treatment of Cancer; NAC, neoadjuvant chemotherapy; NKI, Netherlands Cancer Institute; NPV, negative predictive value; OR, odds ratio for lack of pathological complete response; PPV, positive predictive value; ROC, receiver-operating characteristic.

YWHAZ<sup>23</sup> might be less important. However, predictive power was not improved using either Minimal Gene signature or YWHAZ in absence of the immune/stromal components (STAT1 and PLAU) (data not shown). Although the inclusion of a component marker of hypoxia had the highest correlation with pCR in the training set (*ConSig2*), this combination did not show consistent anthracycline specificity, with similar performance in anthracycline- and anthracycline plus taxane-treated patients in the EORTC validation set. This might be because hypoxia is a critical factor in the function of other chemotherapy agents, not only anthracyclines. Interestingly, although topolla protein expression is known to relate to proliferation,<sup>24</sup> correcting topolla mRNA expression level for proliferation made no difference to the predictive utility of the *ConSigs*. The lack of discriminating ability of the proliferative markers, AURKA or AURKA signature, might relate to the fact that the majority of tumors in our data set (76%) were grade 3 with most of the rest being grade 2, and thus were all moderately to highly proliferating. With no comparator group of low-proliferating tumors, the influence of a proliferation marker cannot easily be evaluated. Furthermore, when considering a cohort of TNBC, proliferation is characteristically high, making the ability to measure variability of topolla protein expression due to low versus high proliferation arguably less critical. Although the influence of proliferation itself in the results is uncertain, the specificity of *ConSig1* for anthracycline response over and above that of proliferation is supported by the fact that it is not predictive in patients treated with anthracyclines plus taxanes, a situation where proliferation is still important.

Differently from the A-score, which evaluated ER-/HER2<sup>+</sup> and ER-/HER2<sup>-</sup> tumors, we focused specifically on TNBC, a group where treatment is limited to chemotherapy, and thus where optimization of chemotherapy regimen would be of considerable utility. Interestingly, the predictive ability of *ConSig1* for TNBC patients treated with anthracycline-based chemotherapy without taxane appeared to be better than the combination of STAT1 +PLAU, the component gene signatures in the A-score for TNBC.

By constructing a biologically relevant multifactorial *ConSig*, we hypothesized that this should predict anthracycline response, as well as resistance; however, PPV of all *ConSigs* in the training set were lower than anticipated, and only around 35% for *ConSig1*. Conversely, *ConSig1* predicts likelihood of lack of response (NPV), which is still a finding of considerable clinical utility if further validated. In general, high PPV for biomarkers has previously been shown to be hard to achieve. Considering two well-established biomarkers and the only two routinely used in breast cancer management, ER and HER2, positivity predicts treatment response in only around 50% of patients for endocrine therapy<sup>25</sup> and less than 40% for trastuzumab as a single agent.<sup>26</sup> However, it is worth noting that the small number of pCRs in the training set (29/147; 19.7%) might have contributed to less than stable results for

PPV. Indeed, in the validation sets, where pCR rates are 39% (EORTC/BIG00-01) and 46% (NKI), PPV is higher than 75% (Table 5).

Finally, our sample size was relatively limited. The training set used contained all publicly available data on the same platform for TNBC treated with anthracycline-based chemotherapy. However, this incorporated only 147 TNBC patients. Thus, further validation of *ConSig1* in independent TNBC cohorts is necessary.

In conclusion, this project demonstrated the feasibility of defining a multifactorial Consensus Signature for predicting anthracycline response and, when applied in a TNBC patient cohort, *ConSig1* was highly predictive for anthracycline resistance. With further validation, this signature may provide clinicians with a useful tool for improved selection of TNBC patients for anthracyclines, potentially leading to better treatment tolerance and more effective therapy.

## ACKNOWLEDGMENTS

We acknowledge the generous support of the Italian Association for Cancer Research (AIRC) that provided funding for this project under the auspices of the AIRC Special Program Molecular Clinical Oncology '5 per mille' research initiative.

## CONTRIBUTIONS

All the authors contributed to the conception and design of the manuscript and to the data analysis and interpretation. NT, MF, and SN contributed also to the collection and assembly of the data. All authors have written and approved the manuscript. ADL and SB are the manuscript's guarantors.

## COMPETING INTERESTS

The authors declare no conflict of interest.

## FUNDING

The authors declare that no funding was received.

## REFERENCES

- Peto R, Davies C, Godwin J, Gray R, Pan HC, Clarke M *et al*. Comparisons between different polychemotherapy regimens for early breast cancer: meta-analyses of long-term outcome among 100,000 women in 123 randomised trials. *Lancet* 2012; **379**: 432–444.
- Di Leo A, Desmedt C, Bartlett JM, Piette F, Ejlertsen B, Pritchard KI *et al*. HER2 and TOP2A as predictive markers for anthracycline-containing chemotherapy regimens as adjuvant treatment of breast cancer: a meta-analysis of individual patient data. *Lancet Oncol* 2011; **12**: 1134–1142.
- Di Leo A, Gancberg D, Larsimont D, Tanner M, Jarvinen T, Rouas G *et al*. HER-2 amplification and topoisomerase IIalpha gene aberrations as predictive markers in node-positive breast cancer patients randomly treated either with an anthracycline-based therapy or with cyclophosphamide, methotrexate, and 5-fluorouracil. *Clin Cancer Res* 2002; **8**: 1107–1116.
- Press MF, Sauter G, Buyse M, Bernstein L, Guzman R, Santiago A *et al*. Alteration of topoisomerase II-alpha gene in human breast cancer: association with responsiveness to anthracycline-based chemotherapy. *J Clin Oncol* 2011; **29**: 859–867.

- 5 Slamon D, Eiermann W, Robert N, Pienkowski T, Martin M, Press M *et al*. Adjuvant trastuzumab in HER2-positive breast cancer. *N Engl J Med* 2011; **365**: 1273–1283.
- 6 Arriola E, Rodriguez-Pinilla SM, Lambros MB, Jones RL, James M, Savage K *et al*. Topoisomerase II alpha amplification may predict benefit from adjuvant anthracyclines in HER2 positive early breast cancer. *Breast Cancer Res Treat* 2007; **106**: 181–189.
- 7 Desmedt C, Di Leo A, de Azambuja E, Larsimont D, Haibe-Kains B, Selleslags J *et al*. Multifactorial approach to predicting resistance to anthracyclines. *J Clin Oncol* 2011; **29**: 1578–1586.
- 8 Bartlett JM, Munro A, Cameron DA, Thomas J, Prescott R, Twelves CJ. Type 1 receptor tyrosine kinase profiles identify patients with enhanced benefit from anthracyclines in the BR9601 adjuvant breast cancer chemotherapy trial. *J Clin Oncol* 2008; **26**: 5027–5035.
- 9 Martin M, Romero A, Cheang MC, López García-Asenjo JA, García-Saenz JA, Oliva B *et al*. Genomic predictors of response to doxorubicin versus docetaxel in primary breast cancer. *Breast Cancer Res Treat* 2011; **128**: 127–136.
- 10 Di Leo A, Moretti E, Oakman C, Biganzoli L, Santaripa L. Predictive molecular markers of anthracycline effectiveness in early breast cancer. *Eur J Cancer* 2011; **Supp 9**: 16–21.
- 11 Fan C, Oh DS, Wessels L, Weigelt B, Nuyten DS, Nobel AB *et al*. Concordance among gene-expression-based predictors for breast cancer. *N Engl J Med* 2006; **355**: 560–569.
- 12 Farmer P, Bonnefoi H, Anderle P, Cameron D, Wirapati P, Becette V *et al*. A stroma-related gene signature predicts resistance to neoadjuvant chemotherapy in breast cancer. *Nat Med* 2009; **15**: 68–74.
- 13 deRonde JJ, Lips EH, Mulder L, Vincent AD, Wesseling J, Nieuwland M *et al*. SERPINA6, BEX1, AGTR1, SLC26A3, and LAPTM4B are markers of resistance to neoadjuvant chemotherapy in HER2-negative breast cancer. *Breast Cancer Res Treat* 2013; **137**: 213–223.
- 14 Montagner M, Enzo E, Forcato M, Zancanato F, Parenti A, Rampazzo E *et al*. SHARP1 suppresses breast cancer metastasis by promoting degradation of hypoxia-inducible factors. *Nature* 2012; **487**: 380–384.
- 15 Adorno M, Cordenonsi M, Montagner M, Dupont S, Wong C, Hann B *et al*. A Mutant-p53/Smad complex opposes p63 to empower TGFbeta-induced metastasis. *Cell* 2009; **137**: 87–98.
- 16 Desmedt C, Haibe-Kains B, Wirapati P, Buyse M, Larsimont D, Bontempi G *et al*. Biological processes associated with breast cancer clinical outcome depend on the molecular subtypes. *Clin Cancer Res* 2008; **14**: 5158–5165.
- 17 Johnson WE, Li C, Rabinovic A. Adjusting batch effects in microarray expression data using empirical Bayes methods. *Biostatistics* 2007; **8**: 118–127.
- 18 Wirapati P, Sotiriou C, Kunkel S, Farmer P, Pradervand S, Haibe-Kains B *et al*. Meta-analysis of gene expression profiles in breast cancer: toward a unified understanding of breast cancer subtyping and prognosis signatures. *Breast Cancer Res* 2008; **10**: R65.
- 19 Haibe-Kains B, Schroeder M, Bontempi G, Sotiriou C, J Quackenbush. *genefu*: Relevant function for gene expression analysis, especially in breast cancer. R package version 1.6.1; <http://www.bioconductor.org/packages/release/bioc/vignettes/genefu/inst/doc/genefu.pdf> (Accessed on 31st October 2013).
- 20 Hatzis C, Pusztai L, Valero V, Booser DJ, Esserman L, Lluch A *et al*. A genomic predictor of response and survival following taxane-anthracycline chemotherapy for invasive breast cancer. *JAMA* 2011; **305**: 1873–1881.
- 21 Tabchy A, Valero V, Vidaurre T, Lluch A, Gomez H, Martin M *et al*. Evaluation of a 30-gene paclitaxel, fluorouracil, doxorubicin, and cyclophosphamide chemotherapy response predictor in a multicenter randomized trial in breast cancer. *Clin Cancer Res* 2010; **16**: 5351–5361.
- 22 Iwamoto T, Bianchini G, Booser D, Qi Y, Coutant C, Shiang CY *et al*. Gene pathways associated with prognosis and chemotherapy sensitivity in molecular subtypes of breast cancer. *J Natl Cancer Inst* 2011; **103**: 264–272.
- 23 Li Y, Zou L, Li Q, Haibe-Kains B, Tian R, Li Y *et al*. Amplification of LAPTM4B and YWHAZ contributes to chemotherapy resistance and recurrence of breast cancer. *Nat Med* 2010; **16**: 214–218.
- 24 Oakman C, Moretti E, Di Leo A. Re-searching anthracycline therapy. *Breast Cancer Res Treat* 2010; **123**: 171–175.
- 25 Schiff R, Massarweh S, Shou J, Osborne CK. Breast cancer endocrine resistance: how growth factor signaling and estrogen receptor coregulators modulate response. *Clin Cancer Res* 2003; **9**: 447S–454S.
- 26 Nahta R, Esteva FJ. HER2 therapy: molecular mechanisms of trastuzumab resistance. *Breast Cancer Res* 2006; **8**: 215.



This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International License. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit <http://creativecommons.org/licenses/by-nc-nd/4.0/>

Supplementary Information accompanies the paper on the *npj Breast Cancer* website (<http://www.nature.com/npjbcancer>)