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Original Research

The 41-gene classifier TRAR predicts response of HER2 positive breast cancer patients in the NeoALTTO study

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Abstract ***Background:*** Dual HER2-inhibition combined with neoadjuvant chemotherapy allows increased pathological complete response (pCR) rate. However, with the addition of new agents, there is a growing need to select patients to minimise overtreatment. Herein, we evaluated the 41-gene classifier TRAR to predict pCR to anti-HER2 therapies in the NeoALTTO trial.
Patients and methods: Gene expression data were obtained using RNA from 226 pretreatment tumour biopsies. Logistic regression analysis and the area under the receiver operating characteristic (ROC) curve (AUC) were used to evaluate TRAR predictive and discriminatory capabilities.

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Predictive biomarker

Results: TRAR levels were associated with pCR (odds ratio, OR: 0.25, 95% confidence interval, CI: 0.15–0.42). The ROC analysis showed AUC values of 0.73 (95% CI: 0.67–0.80) overall; 0.70 (0.59–0.81) and 0.71 (0.62–0.80) for positive and negative oestrogen receptor cases and 0.74 (0.60–0.88), 0.76 (0.65–0.87) and 0.71 (0.59–0.83) for trastuzumab, lapatinib and combined treatment arms, respectively. TRAR provided reliable predictive information beyond established clinico-pathological variables (OR: 0.26, 95% CI: 0.14–0.47). Furthermore, addition of TRAR to these variables provided greater predictive capability than the addition of PAM50: AUC 0.78 (0.72–0.84) versus 0.74 (0.67–0.81), p = 0.04.

Conclusion: TRAR represents a promising tool to refine the ability to identify patients sensitive to anti-HER2 (including trastuzumab-only)-based therapy and eligible for de-escalated treatment strategies.

1. Introduction

The HER2 gene is amplified and/or overexpressed in approximately 20% of breast cancer (BC) cases [1] and has been associated with aggressive disease and poor prognosis [2]. The introduction of dual anti-HER2 combinations, i.e. trastuzumab (T) plus (+) lapatinib (L) or T + pertuzumab (P), improved the clinical outcome of patients in this subgroup. T + L in the NeoALTTO trial showed higher pathological complete response (pCR) compared with either T or L with paclitaxel (51.3% versus 29.5% or 24.7%, respectively; $p < 0.01$ for both) [3]. Again, in the neoadjuvant setting, the Neosphere study reported increased pCR by adding P to T with docetaxel (45.8% versus 29.0%, $p = 0.014$) [4]. The development of strategies with dual anti-HER2 combinations was paralleled by the growing need to select patients for optimal treatment. When the results of the ALTTO study were published [5], it came out as a proof of concept that the simple combination of potentially active agents, i.e. L to standard adjuvant T-based therapy, does not necessarily yield survival gain. Furthermore, undoubtedly effective dual anti-HER2 combination may be unnecessary in patients who already benefit from a single agent [6]. Hence, finding positive predictive marker(s) of response could have huge impact towards de-escalating strategies in terms of single agent versus dual blockade and/or duration of chemotherapy. Different studies have failed to demonstrate that a single biomarker can identify patients who differentially respond to T [7], possibly because of the multifaceted drug mechanism of action [8]. Moreover, the recently reported interplay between primary tumour HER2-dependence and immune system [9] supports the concept that HER2 overexpression *per se* is not sufficient to define anti-HER2 responders.

At Fondazione IRCCS Istituto Nazionale dei Tumori—Milano (INT), we developed the 41-gene classifier TRAR, which is able to identify HER2-positive BC patients with differential risk of relapse upon treatment with adjuvant T [10] and provides reliable predictive information over established clinical factors in the neo-adjuvant setting [10,11]. The discriminatory capability of TRAR stands on its unique feature of including both HER2- and oestrogen receptor (ER)-related genes and to split tumours according to their immune infiltration characteristics [10]. Herein, we took advantage of the unique opportunity to profile RNA from pretreatment fresh tumour biopsies obtained from NeoALTTO patients, to assess whether TRAR is associated with pCR to single or dual HER2-targeted therapies.

2. Methods

2.1. Patients

The results of the multicentre randomised phase III NeoALTTO trial (NCT00553358) have already been published [3]. Briefly, patients with HER2-positive BC were randomised to preoperative L, T or their combination for 6 weeks followed by the addition of paclitaxel for further 12 weeks. After surgery, patients continued with three cycles of fluorouracil, epirubicin and cyclophosphamide followed by the same HER2-targeted therapy administered in the preoperative setting to complete 1 year of treatment. The primary end-point of the study, which was conducted in accordance with the Declaration of Helsinki, was pCR in the breast. Written informed consent was obtained from all patients at study entry, which also covered future biomarker research.

2.2. Gene expression profiling analysis

RNA was obtained from snap frozen core biopsies of primary tumours before the initiation of neoadjuvant therapy as already reported [12]. RNA samples stored at the central biobank of Vall d'Hebron University Hospital, Barcelona, were shipped to INT. RNA

concentration was determined by ND-1000 spectrophotometer (NanoDrop),and RNA quality was checked using TapeStation 2200 (Agilent) and the RNA integrity number. Gene expression data were generated using HumanHT12_v4 beadchips (Illumina, San Diego, CA), as per protocol and detailed in the Supplementary methods.

The 41-gene classifier TRAR (Supplementary Table S1) was computed as previously described [10]. Besides, gene expression–based biomarkers including *ERBB2* and *ESR1*, the research-based PAM50 subtype predictor [13], immune metagenes [14] and proliferation signatures [15] were also evaluated (Supplementary methods).

Gene set enrichment analysis (GSEA) was performed using 'fgsea' function from R package. The enrichment score values were calculated according to the gene list ordered on the bases of their fold change. The predefined gene sets from Gene Ontology (GO) annotation (http://bioinf.wehi.edu.au/software/MSigDB/index.html), including categories of molecular function, cellular component or biological process and KEGG pathways, were analysed. Then, the collapse pathways function was used to select only independent pathways from the list of statistically significant enriched pathways (i.e. false discovery rate [FDR], p-value<0.05).

2.3. Statistical analysis

The association of TRAR levels, measured on a continuous scale, with pCR as well as with other categorical clinicopathological variables was evaluated by resorting to the non-parametric Kruskal–Wallis test. The strength of the association was assessed by the Spearman correlation coefficient (rs) and its 95% confidence interval (95% CI) [16]. Univariate logistic regression analysis was implemented for each variable of interest to estimate the odds ratio (OR) and its 95% CI [17]. The relationship between continuous variables and pCR probability was investigated by resorting to a regression model based on restricted cubic splines [18]. The predictive performance of TRAR with respect to pCR was further evaluated by resorting to a multivariate logistic regression model by taking into account treatment arm (T-arm as reference), age (continuous), tumour size (≤5 cm versus >5 cm), nodal (N0/1 versus other) and ER status (negative versus positive). The discriminatory capability of each model was evaluated in terms of area under the receiver operating characteristic (ROC) curve (AUC) and the corresponding 95% CI [19]. The non-parametric approach of DeLong and Clarke-Pearson [20] was used to compare the discriminatory capability of TRAR with respect to PAM50. Finally, TRAR was dichotomised according to the cut-off value identified by maximising the Youden index from the univariate ROC curve. Within each of the obtained subgroups, i.e. TRAR-low and TRAR-high, a list of differentially expressed genes between patients achieving or not pCR was identified by resorting to Kruskal–Wallis test. All statistical analyses were carried out with the SAS (version 9.2.; SAS Institute, Inc., Cary, NC) and R software by adopting a significance level of $\alpha = 0.05$.

3. Results

3.1. Patient characteristics

Overall, 455 patients were enrolled in the NeoALTTO trial; from 226 of them (49.6%), gene expression data were obtained for the purpose of the present analysis (Fig. 1).

Specifically, RNA of enough quality and quantity was obtained for 232 patients and processed for microarray hybridisation. Six samples out of 232 did not pass the quality-control procedures. Baseline characteristics of patients evaluated for the present study, hereinafter called TRAR cohort, were superimposable with those of the whole NeoALTTO trial (Supplementary Table S2).

3.2. Evaluation of TRAR predictive capability

TRAR resulted significantly associated with ER status ($p < 0.001$) and tumour size ($p = 0.005$) (Supplementary Fig. S1). No statistically significant association was observed between TRAR and age, nodal status and treatment arm (Supplementary Fig. S1). Of the 226 evaluable cases, 80 (35%) achieved a pCR, specifically following L (n = 21, 27%), T (n = 19, 28%) and their combination (n = 40, 51%) plus paclitaxel.

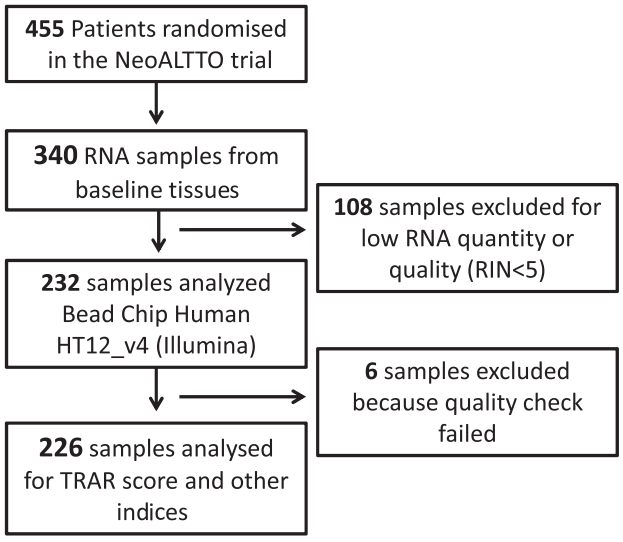

Fig. 1. **Flow diagram of patients and samples included in the analysis**.

TRAR significantly differed among patients with and without pCR ($p < 0.001$). Specifically, the median levels of TRAR were lower in patients achieving pCR (Fig. 2A). Univariate logistic regression analysis (Table 1) showed that TRAR levels were associated with pCR (OR: 0.25, 95% CI: 0.15–0.42) with an AUC of 0.73 (95% CI: 0.67–0.80) (Fig. 2B). The predictive value of TRAR was confirmed in both ER positive and negative cases, AUC 0.70 (95% CI: 0.59–0.81) and AUC 0.71 (95% CI: 0.62–0.80), respectively, and in all treatment arms: AUC 0.76 (95% CI: 0.65–0.87), AUC 0.74 (95% CI: 0.60–0.88) and AUC 0.71 (95% CI: 0.59–0.83) in L, T and their combination, respectively (Supplementary Fig. S2).

3.3. Multivariate analyses

In the TRAR cohort, the main clinicopathological drivers of pCR were ER status and treatment arm; TRAR retained its predictive role when considered together with these variables (OR: 0.26, 95% CI: 0.14–0.47) (Table 1). Gene expression data including *ERBB2*, *ESR1*, PAM50 and the MSK proliferation molecular score were significantly associated with pCR, in both univariate and multivariate analysis (Supplementary Table S3). Conversely, the immune-cell signatures, i.e. haematopoietic cell kinase (HCK), interferon (IFN), lymphocyte-specific kinase (LCK), MHC-II and STAT1 metagenes [14], were found not associated with pCR (Supplementary Table S4). As shown in Supplementary Fig. S3, TRAR correlated with *ERBB2* (r_s:- 0.70, 95% CI: −0.76 to −0.63), *ESR1* (r_s: 0.76, 95% CI: 0.71–0.81) and PAM50 ($p < 0.001$), even though TRAR seems to better explain the distribution of pCR in the study population with respect to the other tested gene expression–based indices (Fig. 3). Of note, a subset of TRAR-low tumours was not classified as HER2-enriched (HER2-E) (Supplementary Fig. S3c). Finally, TRAR did not correlate ($r_s = -0.19$, 95% CI: −0.32 to −0.05) but rather provided additional predictive capability in combination with the proliferation score MSK (Fig. 3).

TRAR retained its significant association with pCR in a multivariate regression model containing age, treatment arm, ER status, tumour size and nodal status (OR 0.25, 95% CI 0.13–0.46, $p < 0.0001$). Furthermore, the addition of these variables to TRAR provided significantly higher predictive capability as compared with their addition to PAM50 (dichotomised as HER2-

Table 1
Association of TRAR and clinicopathological variables with pathological complete response (pCR): Univariate and multivariate logistic regression model.

Variables	Univariate OR (95% CI)	Multivariate OR (95% CI)
TRAR score	**0.25 (0.15–0.42)**	**0.26 (0.14–0.47)**
Treatment		
L versus T	0.95 (0.46–1.97)	0.95 (0.43–2.09)
L+T versus T	**2.77 (1.39–5.52)**	**3.08 (1.45–6.58)**
ER status		
Neg versus Pos	**2.62 (1.46–4.69)**	1.25 (0.61–2.57)
Age	0.99 (0.96–1.01)	
Tumour size		
≤5 versus > 5	0.91 (0.52–1.59)	
Nodal status		
N0/1 versus ≥ N2	0.62 (0.31–1.26)	

The bold values correspond to statistically significant odds ratio
OR, odds ratio; CI, confidence interval; L, lapatinib; T, trastuzumab; ER, oestrogen receptor.

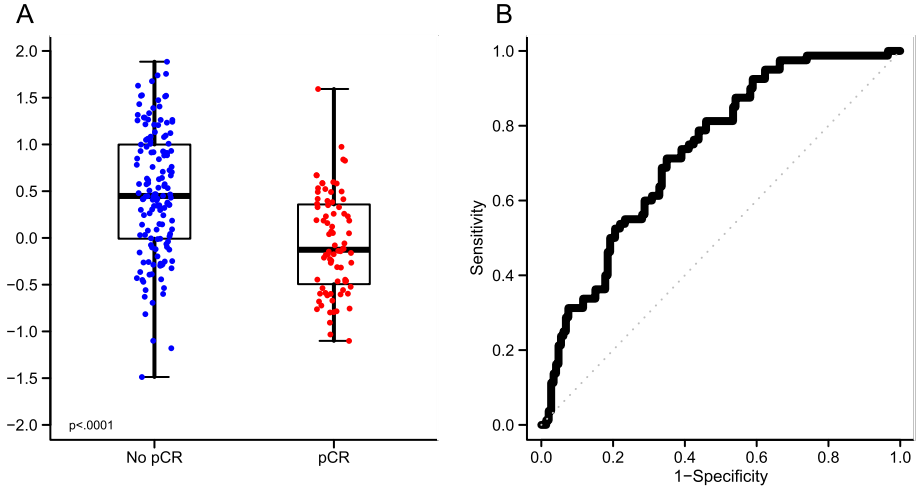

Fig. 2. **Distribution of TRAR score according to pathological complete response (pCR) and the corresponding receiver operating characteristic (ROC) curve in the TRAR cohort (n = 226).** (A) The box-plots show the distribution of TRAR score in patients with pCR and residual disease (No pCR) in the overall analysed cohort (n = 226). Shown are the 25th and the 75th percentiles of the distribution (box), the median (horizontal line) and the extreme values (whiskers). (B) ROC curve derived from the univariate logistic analysis of TRAR score modelling the probability of pCR. The reference line is in grey, a ROC curve lying on the reference line reflects that the performance of test is no better than chance.

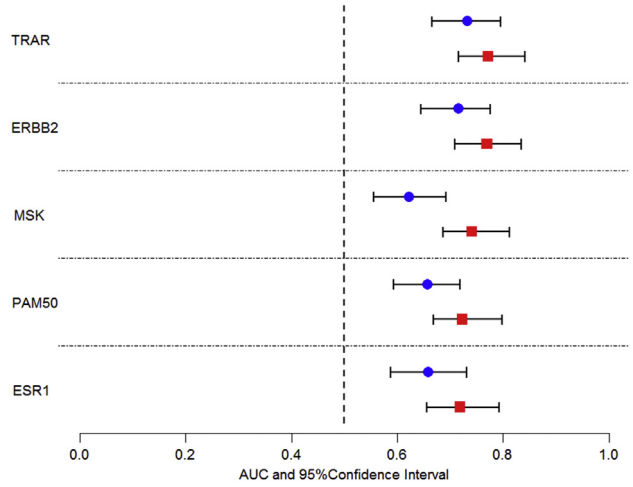

Fig. 3. **Predictive performance of TRAR and the other gene expression based indices in the TRAR cohort (n = 226)**. Blue circles and red squares indicate the area under the receiver operating characteristic (ROC) curves (AUC) obtained from the univariate (blue) and multivariate (red) model, respectively, that includes oestrogen receptor status and treatment arm. Horizontal bars indicate the 95% confidence interval of the AUC. Value of AUC is expected to be 0.5 in absence of predictive capability, whereas it tends to be 1.00 in the case of high predictive capacity. To aid the reader to interpret the value of this statistic, we suggest that values between 0.6 and 0.7 be considered as indicating a weak predictive capacity, values between 0.71 and 0.8 a satisfactory predictive capacity and values > 0.8 a good predictive capacity. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

E versus non-HER2-E), AUC 0.78 (0.72–0.84) versus 0.74 (0.67–0.81), p = 0.04 (Fig. 4).

3.4. Identification of genes associated with pCR in TRAR-low and TRAR-high BC

We next examined differences in gene expression in patients with and without pCR within the subgroups defined by the Youden cut-off as TRAR-low (n = 108, of whom 57 pCR) and TRAR-high (n = 118, of whom 23 pCR). By considering unadjusted p-value < 0.05, 1337 and 734 differentially expressed genes (DEGs) were identified in TRAR-low and TRAR-high subgroups, respectively. Supplementary Table S5 reports top DEGs (unadjusted p-value < 0.01 and fold change > 2 or < 0.5).

By GSEA, several gene sets were significantly (FDR < 0.05) and positively enriched (enrichment score, ES > 0) in cases achieving pCR, both in TRAR-low and TRAR-high cases, but none in cases with residual disease. To identify the pathways, which had the most significant involvement with the genes identified, DEGs were submitted into the GO and KEGG pathway analysis. The upregulated DEGs were enriched in GO terms associated with immune response, cell cycle and response to stimuli and metabolism (Supplementary Table S6). KEGG analysis identified no statistically significantly enriched pathways in TRAR-high subgroup; whereas, upregulated DEGs were significantly enriched in immune response and cell cycle pathways in TRAR-low subgroup (Supplementary Table S7). Therefore, we finally evaluated the predictive capability of the immune metagenes HCK, IFN, LCK, MHC-II and STAT1 and the proliferation molecular score MSK within each TRAR subgroup. HCK, STAT1 and MSK were statistically significant associated with pCR (Table 2) in the TRAR-low subgroup, whilst none of these signatures was associated with pCR in TRAR-high cases.

4. Discussion

Achievement of pCR after neoadjuvant therapy has emerged as a challenging goal, because pCR is associated with lower recurrence risk and higher overall survival in BC patients [21,22]. Though HER2 overexpression/amplification is widely accepted as a biomarker to assign anti-HER2 agents [23], it is not sufficient to recapitulate the heterogeneity of treatment response [24]. The establishment of predictive biomarkers for single or dual HER2-targeted therapy is therefore of utmost importance to personalise the 'right' amount of therapy, because proper patients' selection can avoid toxic/expensive drugs or, conversely, support the use of therapeutic escalation strategies.

Our results demonstrate that the TRAR classifier is able to identify HER2 positive BC patients likely to respond to either L, T or their combination plus paclitaxel. A significant association was found for TRAR and the levels of *ERBB2 and ESR1*. This is not surprising, as TRAR was constructed based on the expression of 41 genes, including *ERBB2* and *ESR1*. It is well known that ER may act as a direct regulator of *ERBB2* transcription [25] and that HER2 can in turn directly control ER genomic activity [26]. The predictive value of these latter genes has emerged in neoadjuvant trials using either T or its combination with L [12] or P [27,28]. However, we showed that TRAR has an added predictive value as compared with these single genes, probably resulting from the integration of *ERBB2* and *ESR1* with the other genes composing the signature. Remarkably, the predictive value of TRAR was independent of ER status, evaluated both at the protein level by immunohistochemistry, the standard measurement approach in clinical practice and at *ESR1* mRNA level (as ER testing was not centralised in the NeoALTTO trial). In addition, the predictive capability of TRAR was independent of the proliferation score MSK, which is consistently associated with pCR in patients treated

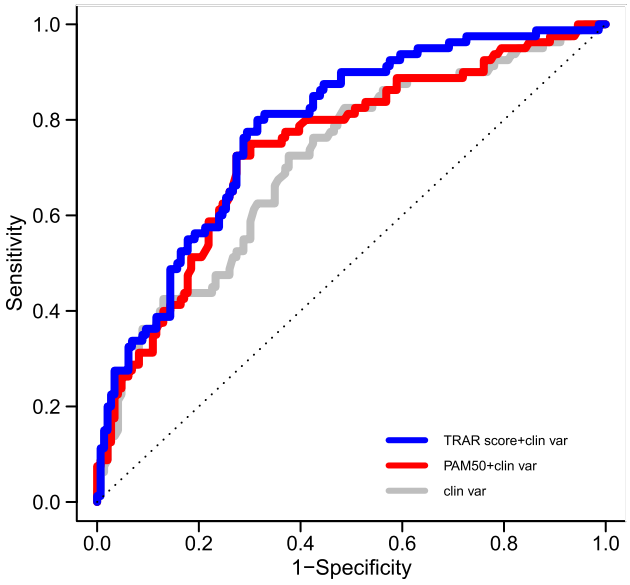

Fig. 4. **Comparisons of the discriminatory capability between TRAR and PAM50 classifier from the multivariate logistic regression model including clinical variables in the TRAR cohort (n = 226).** The receiver operating characteristic (ROC) curve derived from the multivariate logistic model including PAM50 and the clinical variables (i.e. treatment arm, oestrogen receptor status, age, tumour size and lymph node status) is reported in red. The ROC curve derived from the multivariate logistic model including TRAR and the clinical variables is reported in blue and the ROC curve derived from the multivariate logistic model including only clinical variables is reported in grey.

Table 2
Association of the immune metagenes and MSK score with pathological complete response within the two TRAR subgroups: univariate logistic regression model.

Variables	TRAR-low OR (95% CI)	TRAR-high OR (95% CI)
HCK	**2.65 (1.11;6.31)**	0.96 (0.42;2.19)
IFN	1.10 (0.68;1.77)	0.79 (0.45;1.39)
LCK	1.69 (0.90;3.17)	0.98 (0.55;1.78)
MHC-II	1.94 (1.00;3.78)	1.00 (0.54;1.87)
MSK	**3.15 (1.20;8.31)**	1.94 (0.58;6.49)
STAT1	**2.01 (1.13;3.60)**	1.31 (0.75;2.28)

The bold values correspond to statistically significant odds ratio. The Youden cut-off defines TRAR-low and TRAR-high cases.
OR, odds ratio; CI, confidence interval; HCK, haematopoietic cell kinase, IFN, interferon; LCK, lymphocyte-specific kinase.

with anti-HER2 therapies in combination with chemotherapy.

As expected, and consistently with our previous observations [10], most of TRAR-low tumours belong to the HER2-E subtype. Nevertheless, all subtypes could be further subdivided into TRAR-low and TRAR-high, indicating that TRAR may provide supplementary information to PAM50 subtypes. As a consequence, TRAR may discriminate among patients with differential response to anti-HER2 therapies similarly or even better than the molecular classifier PAM50.The predictive value of TRAR for pCR was shown to be independent of treatment arm. Despite the limitation of the small sample size, this evidence supports the hypothesis that TRAR might inform on the mechanisms of action shared by different anti-HER2 agents, including but not limited to inhibition of HER2 signalling and relevant in mediating tumour response, i.e. induction and/or promotion of ADCC, and tumour infiltration by T cells [9,29,30].

In our study, immune signatures did not significantly correlate with the likelihood of achieving a pCR after anti-HER2 treatments, in contrast with the results from others [31–33]. Differences between the trials, including the type of HER2-targeted agent used (the tyrosine kinase inhibitor L or the antibody P), the neoadjuvant chemotherapy backbone (anthracyclines + taxanes or taxanes alone) and the methodological approaches (platform used and immune signature tested) may have accounted for discrepancies. Another potential explanation is that the metagenes we tested probably provide only partial information on the complexity of the tumour-immune microenvironment and do not contain the immune genes associated with pCR in our data set. Nevertheless, in the TRAR-low subgroup, we found both an enrichment in immune processes and an association between STAT1 and HCK signatures in patients achieving pCR. Hence, our GO and KEGG findings support the concept that preexisting immune activation is a determinant of improved outcome in *ERBB2* high/*ESR1* low HER2 positive BC, as TRAR-low cases are. Indeed, our group has recently reported that infiltration of cells of the innate immune system in the tumour microenvironment of TRAR-low BC cases likely depends on the activity of HER2 receptor and the lack of ER inhibition on immune cell recruitment [34]. This regulation might also explain why in TRAR-high BC cases, which conversely are characterised by low *ERBB2*/high *ESR1* expression, immune signatures are not predictive of pCR at all.

Our analyses have some limitations, which have to be pointed out. The small patient population and the retrospective nature of the study do not allow us to draw any definitive conclusion about the effectiveness of the TRAR classifier. In addition, the small sample size of overall patient population and treatment arms hampered any significant analysis of treatment interaction and long-term outcome. However, this has not prevented us from showing TRAR as a compelling predictor of response to neoadjuvant anti-HER2-based therapy.

Incorporating genomic profile into a larger patient evaluation to better characterise not only primary tumour but also host-tumour interplay is a must if one is to overcome the limitations of predictive capability of molecular classifiers [6,35]. Although confirmatory studies on prospective independent case series are

needed to evaluate the reproducibility of TRAR and its possible integration with immune signatures or other biomarkers including those derived from liquid biopsy, the data presented support the development of TRAR as a promising tool to identify responders to T as monotherapy and to guide de-escalating therapy.

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Conflict of interest statement

SDC has received speaking fees from Novartis outside this work; TT, SP, LDC, LdP, MGD, SG, SC, VT, GA, PV and ET declare no competing interest. EdA received research grants, advisory boards, travel grants and honoraria from Roche/Genentech outside this work. DF's institution received support from GlaxoSmithKline until November 30, 2015 and from Novartis since then for the conduct of the NeoALTTO study. DF's institution receives support from AstraZeneca, Pfizer, Roche/Genentech, Servier and Tesaro outside the submitted work. LP has received consulting fees and honoraria from Astra Zeneca, Merck, Novartis, Genentech, Eisai, Pieris, Immunomedics, Seattle Genetics, Almac, Syndax and SynDevRx. NH received speaking and consulting fees from Novartis and Roche/Genentech outside this work. MI is currently employed at Novartis. JH received advisory boards and honoraria from Roche and research grants from Novartis. JB is Employee of AstraZeneca, serves on the Board of Directors of Foghorn and is a past board member of Varian Medical Systems, Bristol-Myers Squibb, Grail, Aura Biosciences and Infinity Pharmaceuticals. He performed consulting and/or advisory work for Grail; PMV Pharma, ApoGen, Juno, Lilly, Seragon, Novartis and Northern Biologics. He has stock or other ownership interests in PMV Pharma, Grail, Juno, Varian Medical Systems, Foghorn, Aura Biosciences, Infinity Pharmaceuticals, ApoGen, as well as Tango and Venthera for which is a co-founder. He previously received Honoraria or Travel Expenses from Roche, Novartis, and Lilly. MP received personal fees from Roche outside this work. FGdB received speakers bureau honoraria from Bristol-Myers Squibb, Eli Lilly, Roche, Amgen, AstraZeneca, Gentili, Fondazione Menarini, Novartis, MSD, Ignyta, Bayer, Noema S.r.l., ACCMED, Dephaforum S.r.l., Nadirex, Roche, Biotechspert Lts., PriME Oncology, Pfizer, and is a consultant/advisory board member for Bristol-Myers Squibb, Tiziana Life Sciences, Celgene, Novartis, Servier, Phanm Research Associated, Daiichi Sankyo, Ignyta, Amgen, Pfizer, Roche, Teogarms, and Pierre Fabre.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejca.2019.06.001.

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