ARTICLE

Molecular Diagnostics

The immunologic constant of rejection classification refines the prognostic value of conventional prognostic signatures in breast cancer

François Bertucci1-2,3, Pascal Finetti1, Ines Simeone4, Wouter Hendrickx5, Ena Wang5, Francesco M. Marincola5-8, Patrice Viens, Emilie Mamessier, Michele Ceccarelli6,7, Daniel Birnbaum and Davide Bedognetti

BACKGROUND: The immunologic constant of rejection (ICR) is a broad phenomenon of Th-1 immunity-mediated, tissue-specific destruction.

METHODS: We tested the prognostic value of a 20-gene ICR expression signature in 8766 early breast cancers.

RESULTS: Thirty-three percent of tumours were ICR1, 29% ICR2, 23% ICR3, and 15% ICR4. In univariate analysis, ICR4 was associated with a 36% reduction in risk of metastatic relapse when compared with ICR1-3 (p = 2.30E-03). In multivariate analysis including notably the three major prognostic signatures (Recurrence score, 70-gene signature, ROR-P), ICR was the strongest predictive variable (p = 9.80E-04). ICR showed no prognostic value in the HR+/HER2− subtype, but prognostic value in the HER2+ and TN subtypes. Furthermore, in each molecular subtype and among the tumours defined as high risk by the three prognostic signatures, ICR4 patients had a 41–75% reduction in risk of relapse as compared with ICR1-3 patients. ICR added significant prognostic information to that provided by the clinico-genomic models in the overall population and in each molecular subtype. ICR4 was independently associated with achievement of pathological complete response to neoadjuvant chemotherapy (p = 2.97E-04).

CONCLUSION: ICR signature adds prognostic information to that of current proliferation-based signatures, with which it could be integrated to improve patients' stratification and guide adjuvant treatment.

British Journal of Cancer (2018) 119:1383–1391; https://doi.org/10.1038/s41416-018-0309-1

BACKGROUND

Despite recent progresses, ~15% of patients with breast cancer still develop metastases and die. During the last decades, genomic analysis revealed the extent of the molecular heterogeneity of disease. Based on gene expression profiling, a new molecular classification was defined, confirming that breast cancer is a group of molecularly distinct subtypes associated with different clinical outcome and prognostic features. In parallel, multigene signatures prognostic and/or predictive for response to chemotherapy were developed. Several commercially available prognostic classifiers have been cleared by the Food and Drug Administration for clinical use or endorsed by American Society of Clinical Oncology (ASCO), National Comprehensive Cancer Network (NCCN), and Saint-Gallen guidelines to assist clinicians in making decisions about adjuvant chemotherapy, in particular for patients with HR+/HER2− tumour. Indeed, those signatures, mainly based on genes involved in cell proliferation, provide modest prognostic information for patients with classically proliferative HER2+ or triple-negative (TN) tumours. The role of immunity in countering tumour progression is clearly recognised.4,5 Classically, breast cancer is considered less immunogenic than melanoma or renal cell carcinoma. Nonetheless, the role of immunity has emerged with the demonstration of a favourable predictive impact of the presence of tumour-infiltrating lymphocytes (TILs) and of gene expression signatures of immune response (IR), notably for TN and HER2+ tumours.6,7 Given the recent therapeutic success of immune checkpoint inhibitors in several types of cancers,8,9 these drugs were tested in breast cancer:10-12 no or very low activity was observed in HR+ tumours, whereas higher activity was reported in small subsets of heavily pre-treated TN tumours preselected with an increased PD-L1 expression with respective 18.5 and 24% objective response rates with pembrolizumab (n = 27) and atezolizumab (n = 21),13 and remarkably durable responses. Recent data suggest that not only the composition of tumour-infiltrating immune cells, but also their functional orientation might serve as a prognostic/predictive marker to select systemic therapies.5 The functional orientation towards cytotoxic response is observed in tumours undergoing regression following immunotherapy and, in melanoma, has been associated with responsiveness to interleukin-2, adoptive therapy, vaccines, and checkpoint inhibitors.14-19 Although prognostic immune signatures defined in breast cancer differ in term of gene composition,
most of them include transcripts underlying a cytotoxic response.\textsuperscript{20–22} The corresponding pathways are also activated during other forms of immunity-mediated tissue-specific destruction, such as allograft rejection,\textsuperscript{23} graft-versus-host disease,\textsuperscript{24} and flares of autoimmunity.\textsuperscript{25} We defined them as the immunologic constant of rejection (ICR).\textsuperscript{5,18} More specifically, the ICR consists in a signature including genes involved in Th-1 signalling interferon (IFNG, TBX21, CD8A/B, IL12B, STAT1, and IRF1), Th-1 chemotraction (such as the CXCR3 and CCR5 ligands, respectively, CXCL9 and CXCL10, and CCL5), and cytotoxic functions (GNLY, PRF, GZMA, GZMB, and GZMH). Interestingly, the expression of these pro-cytotoxic transcripts in tumours is associated with the counter activation of suppressive mechanisms, such as the expression of IDO1, CTLA4, CD274 (PD-L1), PDCD1 (PD-1), and FOXP3.\textsuperscript{26} In a study\textsuperscript{27} centred on the TCGA data set, we found that breast cancers can be classified in four classes according to the ICR signature. In such classification, the level of immune antitumour response progressively decreased from ICR4 to ICR1. The ICR4 tumours, characterised by the coordinate activation of the ICR pathways, displayed a prolonged survival as compared with ICR1-3 tumours in univariate analysis.

Here, to further assay its clino-biological value, we expanded the ICR classification to a set of 8766 non-metastatic, invasive primary breast cancers. We searched for correlations with clinico-biological data, including metastasis-free survival (MFS) and pathological complete response (pCR) to neoadjuvant chemotherapy.

**MATERIALS AND METHODS**

Breast cancer samples and gene expression profiling

Our institutional series included 352 tumour samples from pre-treatment invasive primary mammary carcinomas either surgically removed or biopsied.\textsuperscript{28} The study was approved by our institutional review board. Each patient had given a written informed consent for research use. Samples had been profiled using Affymetrix U133 Plus 2.0 human microarrays (Santa Clara, CA, USA). We pooled them with 34 public breast cancer data sets comprising both gene expression profiles generated using DNA microarrays and RNA-Seq and clinico-pathological annotations. These sets were collected from the National Center for Biotechnology Information (NCBI)/Genbank GEO and ArrayExpress databases, and authors’ website (Supplementary Table 1). The final pooled data set included 8766 non-redundant non-metastatic, non-inflammatory, primary, invasive breast cancers.

**Gene expression data analysis**

Before analysis, several steps of data processing were applied. The first step was the normalisation of each set separately. It was done in R using Bioconductor and associated packages; we used quantile normalisation for the available processed data from non-Affymetrix-based sets (Agilent, SwiGene, and Illumina), and Robust Multichip Average (RMA) with the non-parametric quantile normalisation for the available processed data from non-Affymetrix-based sets (Agilent, SweGene, and Illumina). Before analysis, several steps of data processing were applied. The first step was the normalisation of each set separately. It was done in R using Bioconductor and associated packages; we used quantile normalisation for the available processed data from non-Affymetrix-based sets (Agilent, SweGene, and Illumina), and Robust Multichip Average (RMA) with the non-parametric quantile normalisation for the available processed data from non-Affymetrix-based sets (Agilent, SweGene, and Illumina). In a study\textsuperscript{27} centred on the TCGA data set, we found that breast cancers can be classified in four classes according to the ICR signature. In such classification, the level of immune antitumour response progressively decreased from ICR4 to ICR1. The ICR4 tumours, characterised by the coordinate activation of the ICR pathways, displayed a prolonged survival as compared with ICR1-3 tumours in univariate analysis.

Here, to further assay its clino-biological value, we expanded the ICR classification to a set of 8766 non-metastatic, invasive primary breast cancers. We searched for correlations with clinico-biological data, including metastasis-free survival (MFS) and pathological complete response (pCR) to neoadjuvant chemotherapy.

We applied in each data set separately several multigene expression-based classification algorithms. First, the ICR classifier based on consensus clustering (CC) analysis of the expression levels of 20 representative immune genes (namely, CCL5, CD274, CD8A, CD8B, CTLA4, CXCL9, CXCL10, FOXP3, GNLY, GZMA, GZMB, GZMH, IDO1, IFNG, IL12B, IRF1, PDCD1, PRF1, STAT1, and TBX21) as previously described.\textsuperscript{29} Briefly, the CC analysis was performed in R using the Bioconductor package “ConsensusClusterPlus\textsuperscript{30}” setting as input parameters 5000 repetitions, 80% item resampling (pitem), a number of groups (k) fixed to 4 (in order to have all data sets stratified with the same number of classes, 4 being the optimal number of groups for the TCGA cohort),\textsuperscript{27} and the use of an agglomerative hierarchical clustering with ward criterion (Ward.D2) inner and complete outer linkage. We also applied the three major prognostic multigene classifiers of breast cancer: Recurrence score,\textsuperscript{31} 70-gene signature,\textsuperscript{32} and Risk of Relapse score based on PAM50 subtype and proliferation Risk of Relapse (ROR-P).\textsuperscript{2} Other signatures included the metagenes associated with immune cell populations such as T cells, CD8+ T cells and B cells defined by Palmer et al.,\textsuperscript{33} the transcriptional signatures of 24 different innate and adaptive immune cell subpopulations defined by Bindea et al.,\textsuperscript{34} the cytolytic activity score,\textsuperscript{35} the activation score of IFNa, IFNγ, and tumor necrosis factor (TNFα) immune-related and TP53 biological pathways,\textsuperscript{36} and a chromosomal instability signature.\textsuperscript{31} We also applied to each data set separately three immune gene signatures reported as prognostic in specific molecular subtypes of breast cancer: the IR signature\textsuperscript{37} and the lymphocyte-specific kinase (LCK) signature\textsuperscript{20} in ER− breast cancers, the Immune 28-kinase signature\textsuperscript{31} in basal/TN breast cancers, and the LCK signature\textsuperscript{20} in HER2+ breast cancers. Finally, we calculated the mitogen-activated protein kinase (MAPK) mut score using MAPK genes upregulated in MAPK2K4/MAP3K1 mutated vs. wild-type tumours, as listed elsewhere.\textsuperscript{27}

Statistical analysis

Correlations between tumour classes and clinicopathological variables were analysed using the one-way analysis of variance (ANOVA) or the Fisher’s exact test when appropriate. MFS was calculated from the date of diagnosis until the date of distant relapse. Follow-up was measured from the date of diagnosis to the date of last news for event-free patients. Survivals were calculated using the Kaplan–Meier method and curves were compared with the log-rank test. Uni- and multivariate prognostic analyses for MFS were done using Cox regression analysis (Wald test). The variables submitted to univariate analyses included patients’ age at diagnosis (≤ 50 years vs. > 50), pathological type (lobular vs. ductal vs. other), pathological axillary lymph node status (pN: negative vs. positive), pathological tumour size (pT1 vs. pT2 vs. pT3), pathological grade (1 vs. 2 vs. 3), molecular subtypes (HR−/HER2− vs. HER2+ vs. HER2+ vs. TN), and classifications based on ICR and prognostic multigene signatures. The likelihood ratio (LR) tests were used to assess the prognostic information provided beyond that of a clinical model and other signatures, assuming a χ² distribution. Changes in the LR values (LR Δχ²) measured quantitatively the relative amount of information of one model compared with another. We also analysed the pCR after neoadjuvant chemotherapy, defined as absence of invasive cancer in both breast and axillary lymph nodes. Uni- and multivariate analyses for pCR were done using logistic regression. Variables with a p-value < 0.05 in univariate analyses were tested in multivariate analyses. All statistical tests were two sided at the 5% level of significance. Statistical analysis was done using the survival package (version 2.30) in the R software (version 2.9.1; http://www.cran.r-project.org/). We followed the reporting recommendations for tumour MARKer prognostic studies (REMARK criteria).\textsuperscript{39}
RESULTS

Breast cancer population and ICR classification

We applied the ICR classification to a series of 8766 pre-treatment cancer samples. Most of the patients were >50 years old and most of the tumours were ductal type, pT1−pT2, pN−, grade 2−3, ER+, HER2− (Supplementary Table 2). Sixty-six percent were HR+/HER2−, 12% were HER2+, and 22% were TN. ICR classification defined 2874 tumours (33%) as ICR1, 2516 (29%) as ICR2, 2061 (23%) as ICR3, and 1315 (15%) as ICR4, with progressive decrease of the percentage of high-risk samples according to the ICR classes (Fig. 1), and the cytolytic activity score.36 This immune pattern was confirmed and refined using the 24 Bindea signatures for immune cell subsets,35 showing a strong enrichment from ICR1 to ICR4 for T cells, cytotoxic T cells, CD8+ T cells, T-helper cells, and Th6 cells, activated NK CD56dim cells and neutrophils (p < 1.00E−100; Supplementary Figure 2). Among T-helper cells, the Th-1/Th-2 ratio increased from ICR1 to ICR4, whereas Th-17 enrichment, often associated with unfavourable prognosis,36,40 decreased. This antitumour activation was also correlated to subsets involved in antigen presentation, such as activated dendritic cells (aDCs), DC, B cells, and macrophages. Mast cells and eosinophils decreased from ICR1 to ICR4. Finally, the percentage of high-risk samples increased from ICR1 to ICR4 (p < 1.00E−06) for the 70-gene signature,25 the Recurrence score,32 and the ROR-P score6 (Fig. 1).

The activation score of TP53 pathway37 decreased from ICR1 to ICR4, whereas the percentage of samples with chromosomal instability, as defined by the Carter signature,38 increased (Fig. 1). The MAPK-mut score, reflection of the degree of MAPK deregulation, decreased from ICR1 to ICR4 in the whole series and in each molecular subtype separately (Supplementary Figure 3, as previously reported.27 Here too, for all tested signatures, a continuum was present between the four classes. Of note, all correlations with the ICR classes remained significant after adjustment on the molecular subtype (Supplementary Table 5).

ICR classification and MFS in the whole population

We assessed the prognostic value of the ICR classification in term of MFS in the 3046 informative patients non-metastatic at diagnosis, operated and without neoadjuvant systemic therapy: 2415
In order to further assess the complementarity of ICR classification and late (> 5 years) metastatic relapses (HR 0.20 with other signatures, we repeated the same analysis in each of the four ICR classes.

The immunologic constant of rejection classification was associated with both early (0–5 years) metastatic relapses (HR = 0.67, 95% CI, 0.49–0.91, p = 1.16E–02, Wald test) and late (> 5 years) metastatic relapses (HR = 0.43, 95% CI, 0.20–0.91, p = 2.85E–02, Wald test).

ICR classification and MFS in each molecular subtype

In order to further assess the complementarity of ICR classification with other signatures, we repeated the same analysis in each molecular subtype separately (Supplementary Table 6, Figs. 2c–e). In the HER2+ subtype (n = 352), the ICR classification and the LCK signature were associated with MFS in univariate analysis, with a HR for MFS event equal to 0.56 (95% CI, 0.38–0.81) in ICR1-3 compared with ICR4. In multivariate analysis, only the ICR classification remained significant. In the TN subtype (n = 563), ICR classification displayed strong prognostic value with a HR for MFS event equal to 0.44 (95% CI, 0.28–0.69; p = 3.42E–04, Wald test) in ICR4 when compared with ICR1-3. The other immune signatures (IR, LCK, and 28-kinase) were also significant in univariate analysis, but in multivariate analysis, only the ICR signature kept its prognostic value (p = 1.57E–02, Wald test). Finally, in the HR+/HER2− subtype (n = 2131), the ICR classification was not associated with MFS, whereas most of the clinico-pathological variables and all classical prognostic signatures were.

These three commercial gene signatures are mainly used to decide whether HR+/HER2− patients need adjuvant chemotherapy (high risk) or not (low risk). We investigated whether the ICR signature could identify prognostic classes within the different risk groups defined by those signatures in the 2131 HR+/HER2− patients. A prognostic value existed in the high-risk groups, but not in the intermediate-risk groups, and even less in the low-risk groups (Fig. 3). In the high-risk group defined by the 70-gene signature (n = 1414), the HR for MFS event was 0.56 (95% CI, 0.35–0.90; p = 1.76E–02, Wald test) in ICR4 (9% of samples) compared with ICR1-3. In the Recurrence score-defined high-risk group (n = 583), the corresponding HR was 0.59 (95% CI, 0.33–1.05; p = 7.47E–02, Wald test) with 13.5% of samples in ICR4, and in the ROR-P score-defined high-risk group (n = 871), it was 0.50 (95% CI, 0.30–0.84; p = 8.33E–03, Wald test) with 10.5% of samples in ICR4. Interestingly, for each signature, the MFS curves
were not significantly different between the high-risk/ICR4 class and the low-risk or low/intermediate-risk group, but significantly differed from those of the high-risk/ICR1-3 class. For example regarding the Recurrence score, the 10-year MFS were 76% (95% CI, 73–79) in the low/intermediate-risk group, 77% (95% CI, 65–72) in the high-risk/ICR4 class, and 66% (95% CI, 60–72) in the high-risk/ICR1-3 class. For the ROR-P score, the corresponding 10-year MFS were 79% (95% CI, 76–82), 79% (95% CI, 70–90), and 62% (95% CI, 58–67).

The same analysis gave similar results in the HER2+ and TN subtypes. In these subtypes, the low-risk and intermediate-risk groups were the minority and were pooled for analysis. A prognostic value existed in the high-risk groups, but not in the low or low/intermediate-risk groups. In the 352 HER2+ patients, for the 70-gene signature, the 10-year MFS were 85% (95% CI, 75–96) in the high-risk/ICR4 class, and 61% (95% CI, 54–68) in the high-risk/ICR1-3 class. For the 70-gene signature, risk, the pathological response to anthracycline-based neoadjuvant chemotherapy. Among them, 283 (23%) displayed pCR, whereas 946 did not. In univariate analysis (Table 3), ICR classification was associated with pCR (43% pCR in ICR3 class vs 20% in the ICR1-3 class; p = 2.88E–10), with an odds ratio (OR) for pCR equal to 2.99 (95%CI 2.24–3.97). The other significant variables were high grade, and HER2+ and TN subtypes. In multivariate analysis, all variables remained significant, including the ICR classification (p = 2.97E–04, logit function). Here too, a continuum existed in term of pCR rate between the four ICR classes, from 14% (ICR1) to 20% (ICR2), 28% (ICR3), and 43% (ICR4). Such correlation between ICR classes and pCR rate was maintained in each molecular subtype separately (Supplementary Table 7).

Based on these results and the MFS results, we postulated that the prognostic value of ICR classification could be mediated, at least in part, by its association with response to chemotherapy. Thus, we analysed its prognostic value in our MFS data set according to the delivery or not of adjuvant chemotherapy, which was informed for 2355 patients, including 1653 HR+ patients and no interaction; by contrast, interaction was significant (p = 4.71E–02) in the TN patients, with strong prognostic value in the chemotherapy-treated group (p = 1.80E–03, log-rank test), but not in the chemotherapy-naive group (p = 0.18); however, interaction was not significant (p = 0.14). Analysis per molecular subtype revealed no prognostic value for ICR classification in both groups in the HR+/HER2− patients and no interaction; by contrast, interaction was significant (p = 4.71E–02) in the TN patients, with strong prognostic value in the chemotherapy-treated group (p = 1.80E–03, log-rank test) and no prognostic value in the chemotherapy-naive group (p = 0.47); in HER2+ patients, there was no significant interaction, with strong prognostic value in the chemotherapy-naive group (p = 2.84E–02, log-rank test)

### Table 1. Univariate and multivariate Cox regression analyses for MFS in breast cancer

| Characteristics          | Univariate | Multivariate |
|--------------------------|------------|--------------|
|                          | Hazard ratio [95% CI] | p-Value | Hazard ratio [95% CI] | p-Value |
| Age (years)              |            |              |                        |          |
| >50 vs ≤50               | 0.89 [0.74–1.08] | 0.250        | 1.29 [1.02–1.63] | 0.030    |
| Pathological type        |            |              |                        |          |
| Lobular vs. ductal       | 0.83 [0.52–1.33] | 0.892        | 1.28 [1.00–1.65] | 0.001    |
| Mixed vs. ductal         | 0.97 [0.48–1.97] |            |                        |          |
| Other vs. ductal         | 0.93 [0.54–1.61] |            |                        |          |
| Pathological tumour size (pT) |        |              |                        |          |
| pT2 vs. pT1              | 1.47 [1.19–1.81] | 0.001        | 1.29 [1.02–1.63] | 0.030    |
| pT3 vs. pT1              | 1.57 [1.07–2.31] |            |                        |          |
| Pathological axillary node status (pN) |        |              |                        |          |
| Positive vs. negative    | 1.18 [1–1.4] | 0.053        | 1.29 [1.02–1.63] | 0.030    |
| Pathological grade       |            |              |                        |          |
| 2 vs. 1                  | 2.04 [1.43–2.91] | <1.00E–06 | 1.29 [1.02–1.63] | 0.030    |
| 3 vs. 1                  | 3.98 [2.83–5.60] |            |                        |          |
| Molecular subtype        |            |              |                        |          |
| HER2+ vs. HR+/HER2−      | 1.64 [1.32–2.05] | 0.000        | 1.29 [1.02–1.63] | 0.030    |
| TN vs. HR+/HER2−         | 1.74 [1.43–2.10] |            |                        |          |
| 70-gene signature, risk  |            |              |                        |          |
| Poor vs. good            | 2.45 [1.95–3.08] | 0.000        | 1.29 [1.02–1.63] | 0.030    |
| Recurrence score, risk   |            |              |                        |          |
| Poor vs. good            | 2.21 [1.83–2.67] | 0.000        | 1.29 [1.02–1.63] | 0.030    |
| Intermediate vs. good    | 1.78 [1.41–2.25] |            |                        |          |
| Risk of relapse, ROR-P   |            |              |                        |          |
| Poor vs. good            | 2.58 [2.12–3.14] | <1.00E–06 | 1.29 [1.02–1.63] | 0.030    |
| Intermediate vs. good    | 1.94 [1.50–2.52] |            |                        |          |
| ICR classification       |            |              |                        |          |
| ICR4 vs. ICR1-3          | 0.64 [0.47–0.85] | 0.002        | 1.29 [1.02–1.63] | 0.030    |

The immunologic constant of rejection classification refines the...F Bertucci et al.
no prognostic value in the chemotherapy-treated group despite strong difference in MFS between the two ICR classes \((p = 0.21)\). Thus, these data confirm that, in breast cancer, the ICR4 class is associated with higher response to chemotherapy, particularly in the TN subtype.

**DISCUSSION**

Here, we show that the transcriptional ICR signature, reflecting an immune antitumour response, defines a continuum of clinically and biologically relevant classes of breast cancers. The signature is associated with classical prognostic features and immunity-related parameters, and with MFS, where it refines the prognostic value of classical prognostic signatures, and with pathological response to chemotherapy.

Our approach tested the prognostic and predictive value for our signature in an independent series of samples, thus avoiding the problem of overfitting. We analysed a retrospective pooled set of 8766 pre-therapeutic samples of non-metastatic and invasive primary breast cancers, including 3046 cases informative for MFS and 1229 for pathological response to chemotherapy. Such figures allowed testing our hypothesis in uni- and multivariate analyses in the whole population, but also in each molecular subtype separately. Moreover, the whole-genome transcriptional data allowed testing several other gene signatures and modules relevant to breast cancer.

An immunological continuum was observed with increasing enrichment, from ICR1 to ICR4, of scores reflecting the presence of an antitumour IR, such as lymphocyte infiltrate, expression signatures of immune cell types including T cells, cytotoxic T cells, Th-1 cells, CD8+ T cells, T-helper cells, Tγδ cells, and antigen-presenting cells, and scores of IFNγ pathway activation and of cytolytic activity. Although the molecular subtype is classically associated with immunologic infiltrate, such correlations persisted in multivariate analysis including the molecular subtypes. The level of immune activation captured by the ICR classification positively correlated with classical negative prognostic features of breast cancer, as the scores of standard prognostic signatures (70-gene signature, Recurrence score, and ROR-P score). Here too, a continuum was observed from ICR1 to ICR4, the latter being associated with the poorer-prognosis features. The activation score of TP53 pathway decreased from ICR1 to ICR4, in agreement with the higher rate of inactivating TP53 mutations reported in ICR4, whereas chromosomal instability increased.

Importantly, although associated with poor-prognosis features (including the TN subtype and high-risk defined by classical prognostic signatures), the ICR4 class displayed longer MFS than the three other classes, which showed similar MFS and were pooled. In the whole population, the 5-year MFS was 84% in ICR4 and 78% in pooled ICR1-3, with a HR for relapse equal to 0.64. Multivariate analysis showed that such prognostic value was independent from that of classical prognostic variables and of the three major prognostic signatures of breast cancer, clearly suggesting that IR (reflected by our classification) and tumour cell proliferation (reflected by the three other signatures) provide complementary prognostic information. Of note, the lymphocyte infiltration, relatively simple measure of IR, which was available in 929 TCGA samples, including 929 with available follow-up (88 HER2+, 180 TN, and 661 HR+/HER2–), was not associated with MFS in univariate analysis, whereas our ICR classification was (data not shown). In fact, the prognostic value of
our ICR classification was dependent upon the molecular subtype of samples and complementary to that of other signatures: it was present in the TN and HER2+ subtypes, classically highly proliferative, but absent in the HR+/HER2− subtype, classically less proliferative. The opposite was observed for the three proliferation-based signatures currently used in clinical practice in patients with HR+/HER2− disease. Nevertheless, in HR+/HER2− patients, ICR stratified into prognostic classes the high-risk patients defined by these three signatures. Between 9 and 14% of high-risk patients (according to the signatures tested) were classified ICR4, and such patients had a 41–50% reduction of risk of distant relapse as compared with ICR1–3 high-risk patients. Interestingly, high-risk ICR4 patients exhibited high 10-year MFS (between 77 and 81%), similar to the 10-year MFS of low-risk or low/intermediate-risk patients. Regarding the TN and HER2+ subtypes, no prognostic signature is marketed to date. However, interestingly, in these subtypes also, ICR stratified into prognostic values the...

The immunologic constant of rejection classification refines the...
F Bertucci et al.

Table 2. Comparison of prognostic information for MFS

| Patient group               | All    | TN     | HER2+  | HR+/HER2− |
|-----------------------------|--------|--------|--------|-----------|
| N+ patients                 | 3046   | 563    | 352    | 2131      |
| N+ distant Evt              | 631    | 143    | 97     | 391       |
| Clinical model              | LR-X²  | 75.00  | ---    | 45.45     |
| p-Value                     | 3.84E-14| ---    | ---    | 3.21E-09  |
| ICR classification          | LR-X²  | 10.57  | 15.38  | 12.19     |
| p-Value                     | 1.15E-03| 8.79E-05| 4.81E-04| 0.198     |
| 70-gene signature           | LR-X²  | 72.09  | 0.11   | 1.08      |
| p-Value                     | <2.00E-16| 0.735  | 0.298  | 1.15E-13  |
| Clinical + 70-gene + ICR4   | LR-X²  | 90.2   | 15.41  | 12.66     |
| p-Value                     | <2.00E-16| 4.51E-04| 1.78E-03| 2.81E-11  |
| Clinical + 70-gene + ICR4 vs. Clinical + 70-gene | LR-ΔX² | 10.31 | 15.30 | 11.57 |
| p-Value                     | 1.33E-03| 9.19E-05| 6.69E-04| 0.052     |
| Recurrence score (RS)       | LR-X²  | 73.76  | 0.53   | 2.35      |
| p-Value                     | 1.11E-16| 0.769  | 0.309  | 4.49E-09  |
| Clinical + RS + ICR4        | LR-X²  | 110.90 | 16.20  | 15.06     |
| p-Value                     | <2.00E-16| 1.03E-03| 1.76E-03| 1.05E-13  |
| Clinical + RS + ICR4 vs. Clinical + RS | LR-ΔX² | 10.39 | 15.68 | 12.72 |
| p-Value                     | 1.27E-03| 7.52E-05| 3.63E-04| 3.46E-02  |
| Risk of relapse (ROR-P)     | LR-X²  | 103.20 | 0.65   | 3.02      |
| p-Value                     | <2.00E-16| 0.723  | 0.221  | <2.00E-16 |
| Clinical + ROR-P + ICR4     | LR-X²  | 106.5  | 16.12  | 16.11     |
| p-Value                     | <2.00E-16| 1.07E-03| 1.08E-03| 2.10E-13  |
| Clinical + ROR-P + ICR4 vs. Clinical + ROR-P | LR-ΔX² | 12.41 | 15.47 | 13.09 |
| p-Value                     | 4.27E-04| 8.38E-05| 2.97E-04| 1.63E-02  |

* Clinical model variables

pT, grade & HR mRNA none none pT & grade

N° distant Evt 631 143 97 391
N° patients 3046 563 352 2131

*the clinical variables mentioned correspond to the variables significant in multivariate analysis and integrated in the Clinical model.

Table 3. Univariate and multivariate analyses for pathological complete response to neoadjuvant chemotherapy in breast cancer

| Characteristics            | Univariate | Multivariate |
|----------------------------|------------|--------------|
|                            | N         | Odds ratio [95% CI] | p-Value | N        | Odds ratio [95% CI] | p-Value |
| Age (years)                | >50 vs. ≤50| 1227 0.84 [0.67-1.05] | 0.210 |          |            |            |
| Pathological type          | Lobular vs. ductal | 526 1.69 [0.62-4.31] | 0.368 |          |            |            |
|                           | Mixed vs. ductal | 526 0.74 [0.36-1.42] | 0.476 |          |            |            |
|                           | Other vs. ductal | 526 0.82 [0.44-1.46] | 0.593 |          |            |            |
| Pathological grade         | 2 vs. 1    | 1118 3.27 [1.15-14.3] | 0.108 | 1118 2.42 [0.84-10.6] | 0.235 |
|                           | 3 vs. 1    | 1118 11.5 [4.16-49.5] | 7.58E-04 | 1118 5.86 [2.07-25.5] | 1.61E-02 |
| Molecular subtype          | HER2+ vs. HR+/HER2− | 1229 3.98 [2.83-5.60] | 2.46E-11 | 1118 3.12 [2.14-4.54] | 6.09E-07 |
|                           | TN vs. HR+/HER2− | 1229 3.50 [2.70-4.57] | 5.76E-15 | 1118 2.56 [1.90-3.47] | 2.99E-07 |
| ICR classification         | ICR4 vs. ICR1-3 | 1229 2.99 [2.24-3.97] | 2.88E-10 | 1118 1.99 [1.45-2.72] | 2.97E-04 |
classes the high-risk patients defined by the three proliferation-based commercial signatures. In the HER2+ subtype, 17–18% of high-risk patients (according to the signatures tested) were classified ICR4, and such patients had a 68–75% reduction of risk of distant relapse as compared with ICR1-3 high-risk patients. Similarly in the TN subtype, 27–30% of high-risk patients (according to the signatures tested) were classified ICR4, with a 56–60% reduction of risk of distant relapse as compared with ICR1-3 high-risk patients. Clearly, our ICR signature added substantial prognostic information beyond that provided by the combination of clinical model and each major prognostic multi-gene signature.

Finally, the ICR classification was also independently associated with pathological response to anthracycline-based chemotherapy, with 43% pCR rate in ICR4 vs. 20% in ICR1-3, and an OR close to 3. Here too, there was a continuum between ICR1 and ICR4 in term of pCR rate, further linking the degree of antitumour response to the degree of chemosensitivity of breast cancer.11,42 Such correlation was observed in each molecular subtype. Unfortunately, no expression data are currently available in the literature for testing the eventual value of our signature as predictor for response to checkpoint inhibitors.

In conclusion, our 20-gene ICR signature displays robust predictive values for MFS and for pathological response to anthracycline-based chemotherapy in breast cancer. Among aggressive tumours, those with a coordinated antitumour response (ICR4) display better prognosis and better respond to chemotherapy than those without, further reinforcing the fact that immune reaction is an important component of breast cancer and complementary to cell proliferation in prognostic term. Our study displays several strengths: (i) the large size of the series, which represents to our knowledge one of the largest series reported so far analysing the prognostic/predictive value of gene signatures in breast cancer; (ii) the analysis per molecular subtype, demonstrating that the prognostic value is absent in the whole population of HR+/HER2− tumours, but major in the TN tumours; (iii) the persistence of prognostic and predictive values in multivariate analysis including classical prognostic signatures; (iv) the analysis per relapse risk in each molecular subtype, demonstrating that the prognostic value is present in high-risk tumours only; (v) the added prognostic value beyond that provided by the clinical model and each major prognostic signature; (vi) the biological relevance of the signature, which reveals a gradient of antitumour IR in breast cancer and suggests the potential therapeutic interest of stimulating a pro-IR response; (vii) the small number of genes in the signature, which should facilitate its clinical application by using other tests applicable to formaldehyde-fixed paraffin-embedded samples such as quantitative reverse transcriptase-PCR. The main limitation is the retrospective nature of our series and associated biases.

The perspectives are therapeutic. Indirectly, the integration of ICR classification with classical prognostic signatures can improve prognostication of breast cancer. For example, identification of poor or poor-prognosis cases within operated TN breast cancers should help tailor the systemic treatment: although the 5-year MFS of ICR4 class remains insufficient (83%) and cannot preclude the use of adjuvant chemotherapy, the strong MFS difference suggests that the ICR1-3 patients should need a more aggressive treatment than ICR4 patients. The same is true in HR+/HER2− patients defined as high risk according to the classical prognostic signatures. Such hypothesis should be tested prospectively to identify additional women that might be spared from unnecessary chemotherapy, or perhaps, which can be treated with adjuvant immune-modulatory approaches. More directly, since the antitumour IR seems to play a pivotal role regarding the clinical outcome, the manipulation of genes and/or pathways interfering with its development should provide new therapeutic weapons for treating these poor-prognosis tumours.

ACKNOWLEDGEMENTS
Our work is supported by the the Ligue Nationale Contre le Cancer (label DB) and SIRIC (INCA-DGOS-Inserm 6038 grant).

AUTHOR CONTRIBUTIONS
F.B. was involved in the conception and design of study, analysis and interpretation of data, and draft of the manuscript. P.F. was involved in the acquisition, analysis, and interpretation of all data. I.S., W.H., E.W., F.M.M., P.V., E.M., M.C., D. Birnbaum, and D. Bedognetti were involved in the analysis and interpretation of all data. All authors read critically and approved the final manuscript.

DATA AVAILABILITY
All data were collected from and are available in public databases listed in Supplementary Table 1.

ADDITIONAL INFORMATION
Supplementary Information is available for this paper at https://doi.org/10.1038/s41416-018-0309-1.

Competing interests: The authors declare no competing interests.

Consent to participate: Our study is based upon public data from published studies in which the consent to participate was already obtained by authors.

Ethics approval: Our study is based upon public data from published studies in which ethics approval was already obtained by authors.

Note: This work is published under the standard license to publish agreement. After 12 months the work will become freely available and the license terms will switch to a Creative Commons Attribution 4.0 International (CC BY 4.0)

REFERENCES
1. Sotiriou, C. & Pusztai, L. Gene-expression signatures in breast cancer. N. Engl. J. Med. 360, 790–800 (2009).
2. Parker, J. S. et al. Supervised risk predictor of breast cancer based on intrinsic subtypes. J. Clin. Oncol. 27, 1160–1167 (2009).
3. Sabatier, R., Goncalves, A. & Bertucci, F. Personalized medicine: present and future of breast cancer management. Crit. Rev. Oncol. Hematol. 91, 223–233 (2014).
4. Zitvogel, L., Tesniere, A. & Kroemer, G. Cancer despite immunosurveillance: immunoselection and immunosubversion. Nat. Rev. Immunol. 6, 715–727 (2006).
5. Galon, J., Angell, H. K., Bedognetti, D. & Marincola, F. M. The continuum of cancer immunosurveillance: prognostic, predictive, and mechanistic signatures. Immunology 39, 11–26 (2013).
6. Salgado, R. et al. The evaluation of tumor-infiltrating lymphocytes (TILs) in breast cancer: recommendations by an International TILs Working Group 2014. Ann. Oncol. 26, 259–271 (2015).
7. Bedognetti, D., Hendrickx, W., Marincola, F. M. & Miller, L. D. Prognostic and predictive immune gene signatures in breast cancer. Curr. Opin. Oncol. 27, 433–444 (2015).
8. Miller, L. D. et al. Immunogenic subtypes of breast cancer delineated by gene classifiers of immune responsiveness. Cancer Immunol. Res. 4, 600–610 (2016).
9. Zitvogel, L. & Kroemer, G. Targeting PD-1/PD-L1 interactions for cancer immunotherapy. Oncoimmunology 1, 1223–1225 (2012).
10. Sharma, P. & Allison, J. P. The future of immune checkpoint therapy. Science 348, 56–61 (2015).
11. Bertucci, F. & Goncalves, A. Immunotherapy in breast cancer: the emerging role of PD-1 and PD-L1. Curr. Oncol. Rep. 19, 64 (2017).
12. Nanda, R. et al. Pembrolizumab in patients with advanced triple-negative breast cancer: phase Ib KEYNOTE-012 study. J. Clin. Oncol. 34, 2460–2467 (2016).
13. Emens, L. A. et al. Inhibition of PD-L1 by MPDL3280A leads to clinical activity in patients with metastatic triple-negative breast cancer (TNBC). Cancer Res. 75, Abstract PD1–PD6 (2015).
14. Bedognetti, D. et al. CXCR3/CCR5 pathways in metastatic melanoma patients treated with adoptive therapy and interleukin-2. Br. J. Cancer 109, 2412–2423 (2013).
15. Ji, R. R. et al. An immune-active tumor microenvironment favors clinical response to ipilimumab. Cancer Immunol. Immunother. 61, 1019–1031 (2012).
16. Uloa-Montoya, F. et al. Predictive gene signature in MAGE-A3 antigen-specific cancer immunotherapy. J. Clin. Oncol. 31, 2388–2395 (2013).
Van Allen, E. M. et al. Genomic correlates of response to CTLA-4 blockade in metastatic melanoma. Science 350, 207–211 (2015).

Wang, E., Bedognetti, D. & Marincola, F. M. Prediction of response to anticancer immunotherapy using gene signatures. J. Clin. Oncol. 31, 2369–2371 (2013).

Weiss, G. R. et al. Molecular insights on the peripheral and intratumoral effects of systemic high-dose rIL-2 (aldesleukin) administration for the treatment of metastatic melanoma. Clin. Cancer Res. 17, 7440–7450 (2011).

Rody, A. et al. T-cell metagene predicts a favorable prognosis in estrogen receptor-negative and HER2-positive breast cancers. Breast Cancer Res. 11, R15 (2009).

Sabatier, R. et al. Kinome expression profiling and prognosis of basal breast cancers. Mol. Cancer 10, 86 (2011).

Teschendorff, A. E., Miremadi, A., Pinder, S. E., Ellis, I. O. & Caldas, C. An immune response gene expression module identifies a good prognosis subtype in estrogen receptor negative breast cancer. Genome Biol. 8, R157 (2007).

Spivey, T. L. et al. Gene expression profiling in acute allograft rejection: challenging the immunologic constant of rejection hypothesis. J. Transl. Med. 9, 174 (2011).

Imanguli, M. M. et al. Increased T-bet + cytotoxic effectors and type I interferon-mediated processes in chronic graft-versus-host disease of the oral mucosa. Blood 113, 3620–3630 (2009).

Yoshida, S. et al. Gene expression analysis of rheumatoid arthritis synovial lining regions by cDNA microarray combined with laser microdissection: up-regulation of inflammation-associated STAT1, IRF1, CXCL9, CXCL10, and CCL5. Scand. J. Rheumatol. 41, 170–179 (2012).

Bedognetti, D., Hendrickx, W., Ceccarelli, M., Miller, L. D. & Seliger, B. Disentangling the relationship between tumor genetic programs and immune responsiveness. Curr. Opin. Immunol. 39, 150–158 (2016).

Hendrickx, W. et al. Identification of genetic determinants of breast cancer immune phenotypes by integrative genome-scale analysis. Oncomineology 6, e1253654 (2017).

Sabatier, R. et al. Down-regulation of ECRG4, a candidate tumor suppressor gene, in human breast cancer. PLoS ONE 6, e27656 (2011).

Bertucci, F., Finetti, P., Viens, P. & Birnbaum, D. EndoPredict predicts for the response to neoadjuvant chemotherapy in ER-positive, HER2-negative breast cancer. Cancer Lett. 355, 70–75 (2014).

Lehmann, B. D. et al. Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies. J. Clin. Invest. 121, 2750–2767 (2011).

Wilkerson, M. D. & Hayes, D. N. ConsensusClusterPlus: a class discovery tool with confidence assessments and item tracking. Bioinformatics 26, 1572–1573 (2010).

Paik, S. et al. A multigene assay to predict recurrence of tamoxifen-treated, node-negative breast cancer. N. Engl. J. Med. 351, 2817–2826 (2004).

van de Vijver, M. J. et al. A gene-expression signature as a predictor of survival in breast cancer. N. Engl. J. Med. 347, 1999–2009 (2002).

Palmer, C., Diehn, M., Alizadeh, A. A. & Brown, P. O. Cell-type specific gene expression profiles of leukocytes in human peripheral blood. BMC Genom. 7, 115 (2006).

Bindea, G. et al. Spatiotemporal dynamics of intratumoral immune cells reveal the immune landscape in human cancer. Immunity 39, 782–795 (2013).

Rooney, M. S., Shukla, S. A., Wu, C. J., Getz, G. & Hacohen, N. Molecular and genetic properties of tumors associated with local immune cytolytic activity. Cell 160, 48–61 (2015).

Gatza, M. L. et al. A pathway-based classification of human breast cancer. Proc. Natl. Acad. Sci. USA 107, 6994–6999 (2010).

Carter, S. L., Eklund, A. C., Kohane, I. S., Harris, L. N. & Szallasi, Z. A signature of chromosomal instability inferred from gene expression profiles predicts clinical outcome in multiple human cancers. Nat. Genet. 38, 1043–1048 (2006).

McShane, L. M. et al. REPorting recommendations for tumour MARKer prognostic studies (REMARK). Br. J. Cancer 93, 387–391 (2005).

Spivey, T. L. et al. The stable traits of melanoma genetics: an alternate approach to target discovery. BMC Genom. 13, 156 (2012).

Stoll, G. et al. Immune-related gene signatures predict the outcome of neoadjuvant chemotherapy. Oncoimmunology 3, e27884 (2014).

Pruner, G., Vingiani, A. & Denkert, C. Tumor infiltrating lymphocytes in early breast cancer. Breast 37, 207–214 (2018).

Disis, M. L. & Park, K. H. Immunomodulation of breast cancer via tumor antigen specific Th-1. Cancer Res. Treat. 41, 117–121 (2009).