



HAL
open science

Identification of Factors Predicting Sensitivity or Resistance to Neoadjuvant Chemotherapy in Breast Cancer

Anne-Sophie Hamy

► **To cite this version:**

Anne-Sophie Hamy. Identification of Factors Predicting Sensitivity or Resistance to Neoadjuvant Chemotherapy in Breast Cancer. Cancer. Université Paris Saclay (COMUE), 2019. English. NNT : 2019SACLS129 . tel-02281751

HAL Id: tel-02281751

<https://theses.hal.science/tel-02281751>

Submitted on 9 Sep 2019

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Identification of factors predicting sensitivity or resistance to neoadjuvant chemotherapy in breast cancer

Thèse de doctorat de l'Université Paris-Saclay
préparée à l'institut Curie – laboratoire RT2

École doctorale n°582 Cancérologie : biologie - médecine – santé
Spécialité de doctorat: Science de la vie et de la santé

Thèse présentée et soutenue à Paris, le 24 juin 2019, par

Anne-Sophie HAMY-PETIT

Composition du Jury :

Frédérique Penault Llorca PU-PH, Directrice du Centre Jean Perrin (Clermont-Ferrand))	Présidente
Emmanuelle Charaffe-Jauffret PU-PH, Centre de recherche en Cancérologie de Marseille Equipe Epithelial stem cells and cancer	Rapportrice
Christos Sotiriou Professeur, Institut Jules Bordet (Belgium) Laboratoire de recherche translationnelle en cancérologie mammaire	Rapporteur
Pierre Fumoleau PU-PH, Directeur de l'Institut Curie	Examineur
Alain Puisieux PU-PH Directeur du Centre de Recherche en Cancérologie de Lyon UMR INSERM 1052 / CNRS 5286 Mixte CLB, UCBL, ISPB	Examineur
Sylvie Giacchetti Praticien hospitalier, hôpital Saint Louis Equipe Optimisation de la Chronothérapie des Cancers et de la Fonction Hépatique Post-opératoire	Examinatrice
Fabien Reyat PU-PH, Institut Curie Chef d'équipe « Résidu tumoral & réponse au traitement (RT2Lab)	Directeur de thèse
Joshua Waterfall Chef équipe, Insitut Curie « Génomique fonctionnelle intégrative du cancer (GFIC) »	Co-Directeur de thèse
Marc Espié MCU-PH, Directeur du Sénopôle, hôpital Saint Louis	Invité

Remerciements

Un immense merci à mes 2 superviseurs de thèse : @Fabien Reyal, pour m’avoir embarqué dans cette aventure, pour la qualité de son mentorat, pour la liberté qu’il me laisse, pour son niveau d’exigence qui me pousse toujours plus haut; @Joshua Waterfall, pour avoir accepté en cours de route de m’encadrer, pour son temps, pour la qualité et la finesse de ses remarques, pour sa patience, sa présence et sa constance malgré ma dispersion.

Merci à Sci-hub, qui promeut la libération et le partage de la connaissance, et qui contribue à accélérer la recherche.

Merci au Pr Frédérique Penault-Llorca, Présidente du Jury, aux Professeur.e.s. Emmanuelle Charaffe Jauffret et Christos Sotiriou, rapporteurs, et à tous les autres membres du jury pour le temps qu’ils consacrent à évaluer mon travail. Merci au Pr Pierre Fumoleau pour son soutien concret et constant.

Merci à tous ceux, qui, rencontrés par hasard, ont permis de co-écrire cette histoire: Maël Priour, Julien Guérin, Thomas Balezeau, Marick Laé, Sandrine Tury, Brice Aouchiche, Elise Dumas, Lisa Derosa. Un immense merci au Pr Laurence Zitvogel et au Pr Guido Kroemer de m’avoir fait l’honneur de collaborer avec eux sur le projet des comédications. Merci à Sebastian Amigorena d’avoir intégré RT2Lab à son environnement d’excellence, tout en ayant réussi à conserver une ambiance familiale.

Merci à toute l’équipe de chirurgie pour son accueil chaleureux, particulièrement à Enora Laas, Virginie Fourchette, et Jean-Guillaume Féron. Qu’ils trouvent ici la marque de mon profond respect pour le travail titanesque qu’ils réalisent, avec un professionnalisme et une humilité exemplaires et pour le service qu’ils rendent aux patients. J’ai une chance inouïe de travailler à leurs côtés.

Merci à l’organisme ITMO-Cancer-INSERM d’avoir financé ce doctorat que j’ai réalisé dans des conditions matérielles idéales.

Merci à Monoprix, et particulièrement à Lilian Rosas et Régis Schultz pour leur implication . Mon travail sera à la hauteur de leur engagement.

Merci à Marc Espié et Sylvie Giacchetti, premiers et éternels Maîtres, à qui je dois ma vocation et bien plus encore. Merci à Bernard Asselain, recours ultime de chacun de mes travaux, pour sa disponibilité, sa gentillesse et sa compétence irremplaçables. Merci à Laurence Bozec et à tous mes collègues du département d’oncologie médicale de Curie St Cloud, qui m’ont permis de reprendre une activité clinique passionnante dans une ambiance d’entraide et de bienveillance.

Merci à mes amis de l’équipe RT2 pour ces années de fun, trop courtes mais inoubliables.

Merci à tous les internes, étudiants, et autres collaborateurs qui m'ont épaulée dans ces nombreux travaux pour leur motivation, leur aide et leur sérieux.

Merci à Jenny, guide qui m'éclaire et m'accompagne depuis tant d'années.

Merci à Papa, qui m'a transmis l'amour du travail et à maman, mon plus précieux soutien. Merci à Jacques, et à la fabuleuse Lilia, anges gardiens de mes 2 têtes blondes, pour leur soutien affectif et logistique inestimable.

Last but not least, le plus grand des mercis est pour Vincent, pour sa patience, sa hauteur, sa sagesse et son amour. Merci à Léonard et Joseph, qui me font reprioriser l'essentiel.

Thèse dédiée à Aurore S.

Contents

0.1	Remerciements	3
0.2	Outline of the thesis	6
1	Neoadjuvant treatment	37
1.1	Article n°1 : Reyal, F. ESMO Open 3, e000371 (2018)	37
1.2	Article n°2 : Brandao, ESMO Open (2019)	41
1.3	Article n°3 : Giacchetti, S. et al. Eur. J. Cancer 75, 323–332 (2017)	46
1.4	Article n°4 : Hamy, A. S. et al. Breast Cancer Res. Treat. 159, 499–511 (2016)	58
1.5	Article n°5 : Bonsang-Kitzis, H. et al. PloS One 10, e0144359 (2015)	80
1.6	Article n°6 : Hamy-Petit, A.-S. et al. Br. J. Cancer 114, 44–52 (2016)	97
1.7	Article n°7 : Morel et al, in revision in PlosOne	119
1.8	Article n°8 : Labrosse, J. et al. The Breast 42, 61–67 (2018)	145
1.9	Article n°9 : Hamy, A.-S. et al. Breast Cancer Res. Treat. (2018)	161
1.10	Validation study of the Residual Cancer Burden score (RCB)	227
2	Neoadjuvant and Immunity	241
2.1	Article n°10 : Sadacca, B. et al. Sci. Rep. 7, 15126 (2017)	241
2.2	Article n°11 : Bonsang-Kitzis, H. et al. Oncoimmunology 5, e1061176 (2016)	255
2.3	Article n°12 : Hamy, A.-S. et al. PloS One 11, e0167397 (2016)	350
2.4	Article n°13 : Hamy, A.-S. et al. Ann. Oncol. Off. J. Eur. Soc. Med. Oncol. 28, 2233–2240 (2017)	392
2.5	Article n°14 : Hamy, A.-S. et al., CCR Apr 2019, in press	422
3	Comedication, neoadjuvant and Immunity	461
3.1	Article n°15 : Cremoux, P. D. et al. Anticancer Res. 38, 1485–1490 (2018)	461
3.2	Article n°16 : Hamy, A.-S. et al. J. Clin. Oncol. JCO.18.00636 (2019)	469
3.3	Article n°17 : Hamy, A.-S. et al., submitted to Oncoimmunology	505
3.4	Synthèse	541

Neoadjuvant treatment setting (chemotherapy before surgery) is widely being used in poor prognosis breast carcinoma. Beyond clinical benefits, it serves as a test of *in vivo* chemosensitivity, and represent a major research opportunity to understand resistance or sensitivity mechanisms to chemotherapy. The core of the thesis is to identify factors predicting sensitivity or resistance to treatment, while studying clinical, pathological, genomic, transcriptomic data from BC patients.

The outline of the thesis is summarized as follows :

- ***In the first chapter***, we will present the neoadjuvant model as a valuable, underexplored research tool. We will describe how the analysis of clinical trials or real-life data can represent an opportunity for raising new research hypotheses.

- ***In the second chapter***, we will focus on the relationships between immunity and breast cancer. Immunity has emerged as the top cancer research field in the last decade, but little is known about the evolution during neoadjuvant chemotherapy. We will describe and analyse the respective part of immunity in every breast cancer subtype, and their evolution before and after neoadjuvant chemotherapy.

- ***In the third chapter***, we will describe two examples of associations between comedication use and oncologic outcomes. We will adress the full potential of systematically analysing non anticancerous drugs in cancer patients, with the objective of discovering unsuspected associations with immune infiltration, prognosis or response to treatment. Such discoveries could lead to either (i) warnings to patients and cancer practitioners in case of associations worsening outcomes; (ii) drug repurposing clinical trials for associations improving patients outcome.

The articles published during the PhD are highlighted in red.

Chapter 1 : Neoadjuvant treatment is an optimal framework for translational research.

Breast cancer (BC) is the most commonly diagnosed cancer and remains the leading cause of cancer-related death in women. In 2008, an estimated 1.38 millions new cases have been diagnosed worldwide and the incidences rate in Western Europe was 89.7 per 100.000 women (www.iacr.org).

Neoadjuvant setting (*i.e* treatment before surgery) is currently being used in patients with advanced disease. The clinical benefits are supported by: a) an increase rate of breast conserving surgery, b) a similar prognosis of breast cancer patients receiving a neoadjuvant versus an adjuvant therapy regimen, c) an accumulation of evidences showing a strong correlation between achieving a pathological complete response (pCR) after neoadjuvant chemotherapy and a good prognosis in specific subgroups (triple negative, *HER2* positive). Additional benefits include access to oncogenetic screening, and avoidance of delays to systemic treatments due to surgical complications. Beyond the direct clinical benefit, the neoadjuvant setting represents an opportunity for patients to have a more rapid access to innovation. Finally, from the research and development point of view, NAC enables studying and monitoring “in vivo” treatment-sensitivity of the tumor and evaluating selection or resistance acquisition processes, and represents an unique strategic opportunity for translational research. **Article N°1, Reyal et al., 2018, ESMO OPEN, p. 37, Article N°2 Brandao et al., 2019, ESMO OPEN, p. 41.**

The Residual Tumor and Response to Treatment team (Dr Fabien REYAL, RT2Lab) has started a translational research program on “identification of factors predicting sensitivity or resistance to therapies for breast cancer” through various modalities, from clinical data to transcriptomics and genomics.

Clinical trials in the neoadjuvant setting evaluate whether a drug or a strategy may modify response to treatment (mainly evaluated at surgery by pathological complete response rate) or long-term prognosis (mainly evaluated by disease-free survival).

The **REMAGUS 02** was a multicenter randomized phase II trial including 340 patients with locally advanced BC. Patients were randomly assigned to receive neoadjuvant sequential chemotherapy [NAC: (1) epirubicin/cyclophosphamide, followed by: (2) docetaxel alone or docetaxel plus celecoxib (400 mg twice daily, orally) for *HER2*-negative tumors (n=220, stratum A); or docetaxel alone or docetaxel plus trastuzumab for *HER2*-positive tumors (n=120, stratum B)]. In the stratum A, the trial previously published found no benefit of celecoxib, in terms of pathological complete response 21 (primary objective) ; conversely, in the stratum B, patients who were randomized to receive trastuzumab during docetaxel had pCR rates modestly increased when compared to patients not receiving trastuzumab (26% versus 19%, p=0.05). After a median follow-up of 94 months, no survival benefit was evidenced in any of the two experimental arms of each stratum (stratum A : celecoxib / stratum B : trastuzumab) **Article N°3, Giacchetti et al. 2017, European Journal of Cancer, p. 49**. In *HER2*-negative BC patients, only classical factors such as initial clinical tumor size, PR negativity, and pathological complete response status were prognostic for DFS ; whereas the only prognostic factor was axillar pCR in the *HER2*-positive BC population.

In an effort to increase the accuracy of predictive and prognostic models for response to treatment and prognosis, we evaluated if transcriptomic data could help predicting patients outcome **Article n°4 Hamy et al, 2016, Breast cancer research and treatment, p.61**. We highlighted that only genes related to Estrogen pathways (TTF1) or proliferation (MYBL2) increased the performance of the model to predict pCR. Similarly, in the population of

patients failing to reach pCR, only BIRC5- a gene related to proliferation – was associated with an impaired prognosis.

=> Alltogether, these results suggest that well-known pathways such as estrogen and proliferation remain cornerstones of the prediction of response to treatment and prognosis in breast cancer.

Real life data represent an unprecedented amount of information, which is to date underexploited for research purposes. RT2Lab handles a unique cohort of primary BC including 1200 patients treated by NAC in Institut Curie between 2002 and 2011 (NEOREP cohort), with fully annotated clinical database, pathologic assessment of response to treatment as well as survival data. Investigating clinical and pathological parameters from this database separately in BC subtypes enabled us to identify the different predictive and prognostic value of several factors.

In the first study, we identified that the population of TNBC patients could be separated into 5 different subgroups with very different prognoses, based on axillary involvement, menopausal status, and BMI **Article n°5, Bonsang-Kitzis et al., 2015, Plos One, p. 76.**

In a second study on 287 patients with *HER2*-positive tumors, we highlighted that the dramatic pCR and survival gains were achieved in real-life data since the routine use of trastuzumab era **Article n°6 Hamy et al., 2016, British Journal of Cancer, p. 93.**

We further investigated if other baseline factors – such as the presence of a pre-NAC DCIS component – **Article n°7 Morel. C et al., 2019, in revision in Plos One, p. 104,** or the occurrence of a breast cancer the year following pregnancy [Pregnancy-associated breast cancer (PABC)] - **Article n°8, Labrosse et al., 2018, The Breast, p. 129-** were associated

with response to NAC and/or prognosis, and we found that these factors were not predictive of response to treatment.

Beyond the binary criteria of pathological response to treatment, we next investigated if further prognostic information could be retrieved while studying residual specimen. We evaluated the prognostic value of lymphovascular invasion (LVI) on residual specimen **Article n°9 Hamy et al, 2018, Breast cancer research and treatment, p.138**. We found that LVI was a very strong, independent prognostic marker of poor disease-free, metastasis-free, and overall survival after NAC. Finally, we identified a strong interaction between BC subtype and LVI, meaning that the prognostic value of LVI at NAC completion was worse in *HER2*-positive patients than in luminal or TNBCs.

In the same vein, we identified a strong interaction between BC subtype and the prognostic value of axillary nodal involvement following NAC. In luminal BC patients, intermediate nodal involvement (1 to 3 axillary nodes involved) was not associated with decreased DFS or OS compared to patients with no nodal involvement, whereas an increased number of nodes involved was associated with an impaired prognosis.

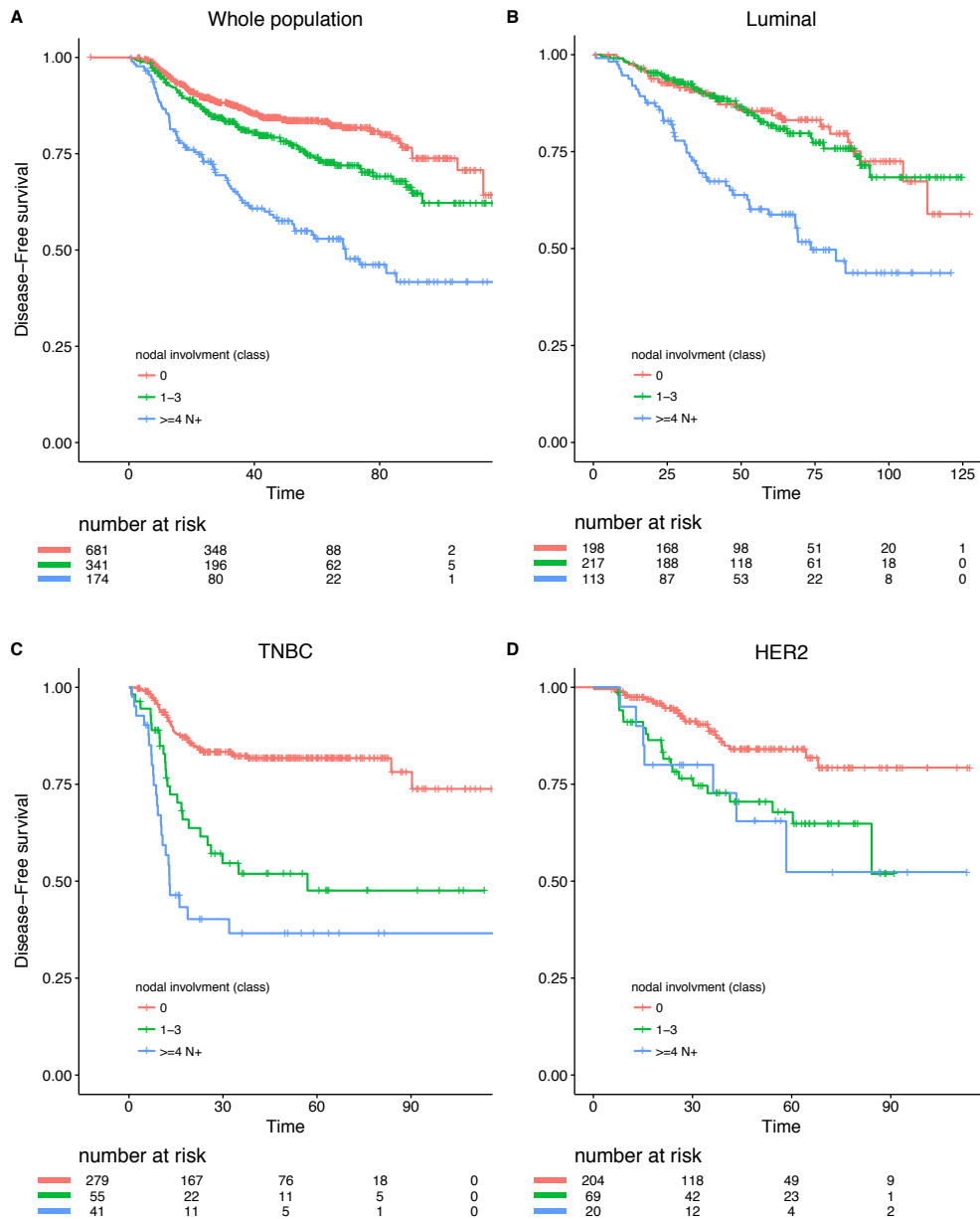


Figure 1: Kaplan-Meier curves of the association between nodal involvement and disease-free survival in the cohort from Institut Curie treated with neoadjuvant treatment (NEOREP cohort). The interaction between BC subtypes and nodal involvement is statistically significant ($P_{interaction}=0.006$)

In addition, we validated the residual cancer burden score developed by Fraser Symmans and colleagues, and demonstrated a high prognostic accuracy of this score. **Validation study of the residual cancer burden score, unpublished, p.183.**

⇒ *Alltogether, these findings support the fact that datasets contain a very large amount of information, and that several associations remain to discover. As a perspective for future research, we hypothesized than rather investigating each factor in a supervised manner, tools for systematic exploration and exploratory analyses could be applied to unravel hidden associations.*

In the last decade, health data have become increasingly available, and the wide opening of data sets represent a wealth of new information for research purposes. Due to their heterogeneity, their complexity and to the volume of data, the need for visualization tools has risen, and **data vizualisation** is progressively emerging as a new field in research. No solely has it been demonstrated that more richly illustrated journal articles have higher citation counts, but clear figures make associated concepts more memorable. Visualizations can be used for two main purposes when analyzing data (i) *exploratory analysis*, looking for relationships in the data and enabling discoveries, thus leveraging the full potential of database; (ii) *explanatory analysis*, aiming at graphically highlighting insights from a work and being an important support for communication among scientists.

The research team of institut Curie headed by Herve Isambert (U830) developped an online innovative tool to provide data-visualization together with causal relationships. MIIC algorithm is a network learning method combining constraint-based and information-theoretic frameworks to reconstruct causal, non-causal or mixed networks from non-perturbative data. Starting from a fully connected network, the algorithm first removes dispensable edges by iteratively subtracting the most significant information contributions from indirect paths between each pair of variables, and the remaining edges are then filtered based on their confidence assessment or oriented based on the signature of causality in observational data.

MIIC online server was first developed for biological and genomic data, and its application was secondarily extended to mixed heterogeneous both categorical and continuous data.

We tested the relevance of the MIIC network on the in-house dataset of patients treated with neoadjuvant chemotherapy in Institut Curie (NEOREP cohort) (Figure 2).

The MIIC algorithm successfully enabled: (i) Clusterizing items into clinically relevant categories (demographics ; outcome ; disease presentation); (ii) Performing quality controls ; (iii) Identifying intra and inter-modality correlations (clinical / radiological / pathological data) ; (iv) Highlighting clinical practice habits including center specificities ; (v) Redrawing the natural history of the disease ; (vi) Weighting the relative effects of factors ; (vii) Pointing out unsuspected and hidden associations ; (viii) Enabling new discoveries.

Such visualisation tools offer major perspective for quick and efficient exploratory analyses.

MIIC is planned to be released as open access tool to the medical and scientific community in a near future.

Chapter 2 : Immunity and neoadjuvant treatment for breast cancer

With the rise of high throughput technologies in the late 90's, microarrays gene expression profiling has identified a first robust stratification level of invasive breast cancer defined by three main tumor categories with very specific features [Luminal, Basal or Triple negative breast cancer (TNBCs), HER2]. Curtis et al published a gene-expression profile analysis of 2000 early breast cancer showing the high level of heterogeneity of this disease and identified up to ten independent molecular subgroups with different profile and prognosis. These subtypes have also been shown to differ in terms of clinical presentation, and sensitivity to systemic treatment [1][2], supporting the view that breast cancer is a disease composed of very different and independent molecular subgroups.

We first investigated the heterogeneity of cancer by studying gene expression from cell lines from CCLE and CGP public datasets **Article n°10, Sadacca et Hamy *et al.*, Scientific reports 2018, p.197**. We developed a robust classification grouping cell lines into clusters displaying greater homogeneity of drug sensitivity than when grouped based on tissue of origin. Notably, 15 TNBC cell lines were split in 7 different clusters with different drug sensitivity.

These findings are important, as (i) they point out the need for further development of basket trials (ii) they highlight the major heterogeneity of cancers.

To further **decipher tumor heterogeneity within the three BC subtypes** [Luminal, Basal, HER2], we analyzed 3,247 primary human breast cancer bulk tumors samples from 21 publicly available datasets, using a five-step method: (1) selection of BC simplified subtypes

using bimodal filtering on ER-HER2 and PR, (2) normalization of the selected samples, (3) selection of the most variant genes, (4) identification of gene clusters and biological gene selection within gene clusters on the basis of String© database connections and gene-expression correlations, (5) summarization of each gene cluster in a metagene. We then assessed the ability of these metagenes to predict response to treatment and prognosis on external public datasets.

In TNBCs, **Article n°11 Bonsang et al. 2015, Oncoimmunology,p.211**, we identified a six-metagene signature (167 genes) in which the metagenes were enriched in different gene ontologies (Immunity1, Immunity2, Proliferation/DNA damage, AR-like, Matrix/Invasion1 and Matrix2). Only the Immunity2 metagene had a strong prognostic value.

Secondly, *HER2*-positive BCs could be split into a six-metagene signature (138 genes) (Immunity, Tumor suppressors/proliferation, Interferon, Signal transduction, Hormone/survival and Matrix clusters) **Article n°12 Hamy et al. 2016, Plos One, p.278** Similarly, the immunity” metagene was associated with higher pathological complete response rates after NAC and with a better prognosis in *HER2*-positive/ER- negative breast cancers.

Finally, luminal BCs (**Hamy AS., unpublished**) could be split into a six metagene signature (104 genes, Immunity, Interferon, Breast_metabolism, DNA_replication, Matrix_1, Matrix_2).

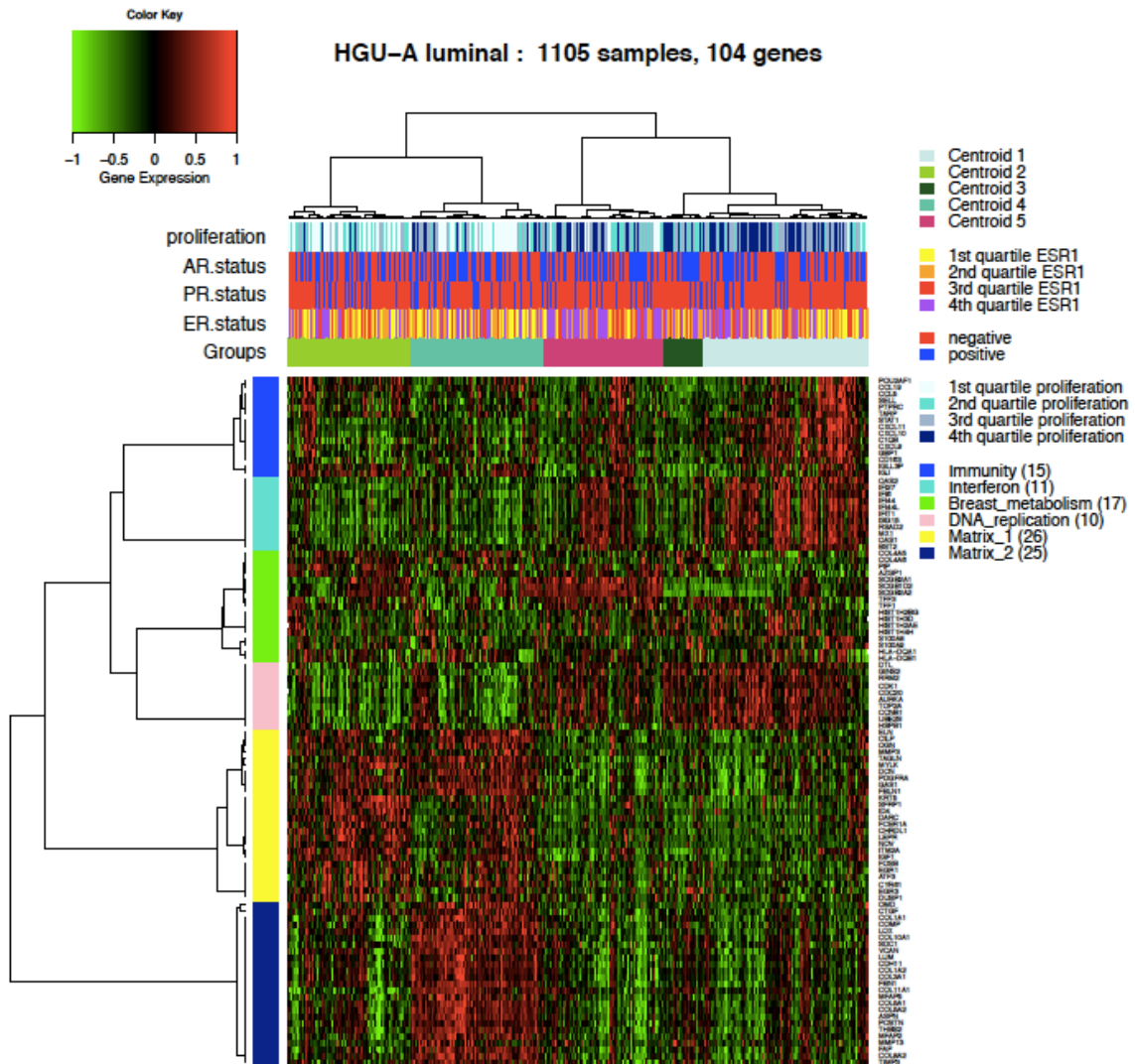


Figure 3: Heatmap showing the 105 most variable genes in the 1105 luminal BC samples (training set).

Combined with the proliferation gene expression, the Immunity metagene was associated with increasing response rates to chemotherapy.

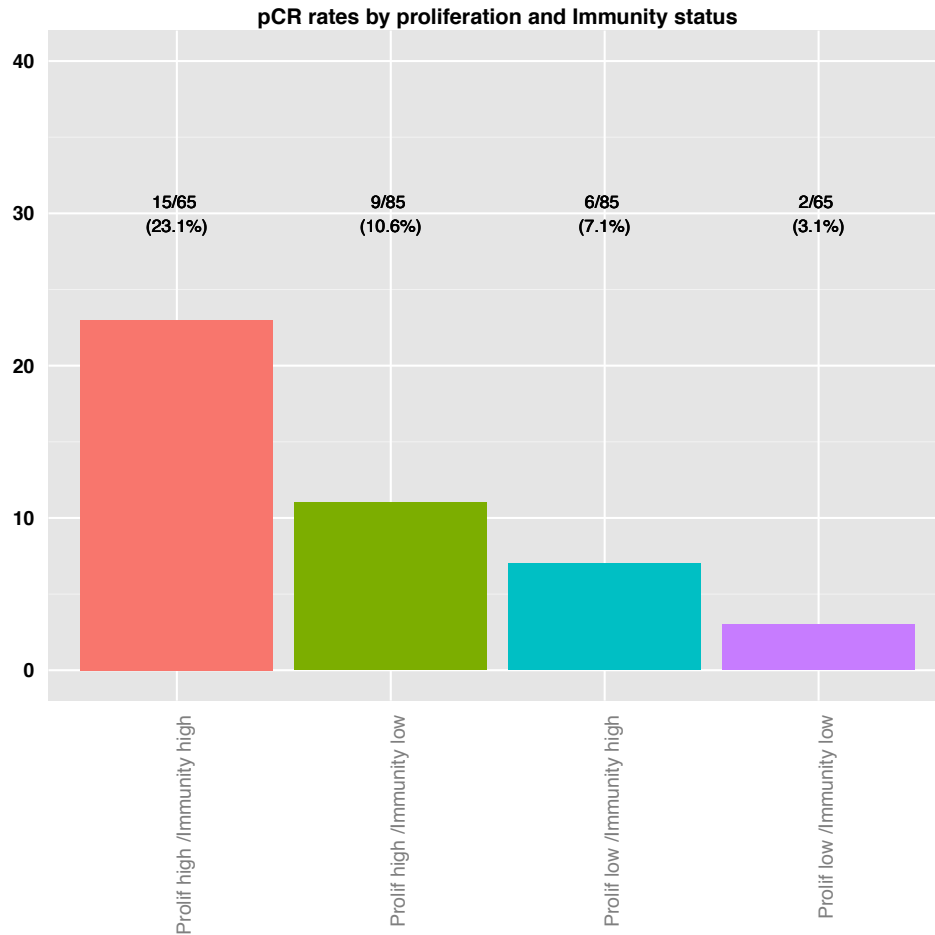


Figure 4: Pathological complete response rates according to the Immunity and the proliferation metagene status, in the luminal BCs from the Ignatiadis dataset.

Additionally, we identified an inverse correlation between the expression of immune genes and ESR1 expression, both in terms of morphological lymphocytic infiltration (Fig.5a) and in terms of immune gene expression (Fig.5b) in 6 independent BC public datasets.

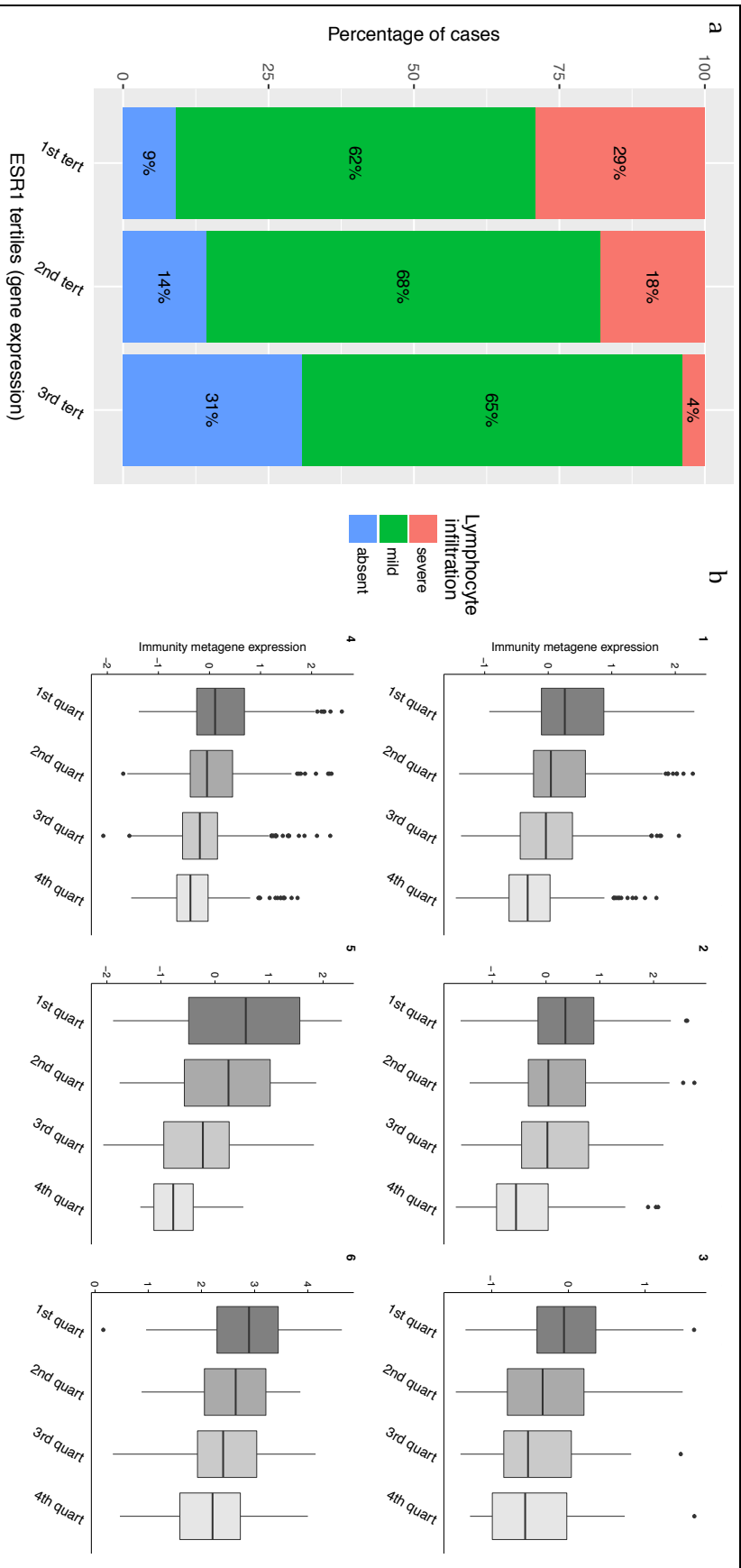


Figure 5a: Repartition of morphological lymphocyte infiltration by tertiles of ESR1 gene expression in luminal breast cancer (METABRIC dataset). **Figure 5b:** Immunity metagene expression by quartiles of ESR1 expression in 6 independent previously published datasets of luminal breast cancers with different gene expression technologies (1:Lehmann, Affymetrix chip type A, n=1105; 2: Lehmann, Affymetrix chip type P2, n=446 ; 3: Ignatiadis, Affymetrix chip type A, n=300, 4:METABRIC, Illumina chip, n=1307; 5: REMAGUS02 trial, chip P2, n=96 ; 6: TCGA, Agilent chip, n=308)

=> Altogether, these results suggest that a subset of patients display tumors enriched in immune genes, that may play an important role in breast cancer response to treatment or prognosis.

Beyond their important predictive and prognostic role in breast cancer, we focused on unraveling the **evolution of TILs before and after NAC**. In this aim, we evaluated the immune infiltration in paired matched breast cancer samples before and after neoadjuvant chemotherapy in a large cohort of 716 patients treated with NAC. Pre and post-treatment BC samples were reviewed for: (i) quantitative lymphocyte infiltration evaluation; (ii) response to treatment assessed both by the occurrence of a pathological complete response (pCR) and by the RCB score (Residual Cancer Burden).

Several results emerged from this project: (i) Pre-NAC TIL levels were associated to pathological complete response (pCR) in a non- linear manner in triple negative BC and were not associated with pCR in *HER2*-positive BC. (ii) TIL levels decreased after chemotherapy completion and this decrease was strongly associated with pCR. (iii) High post-NAC TIL levels were associated with impaired survival in *HER2*- positive BC but not in the other subtypes. **Article n°13 Hamy et al, 2017, Annals of Oncology, p.320, Article n°14 Hamy et al., 2019, Clinical Cancer Research, p.347.**

⇒ *Altogether, those findings provide a strong rationale for a differential composition of the TILs according to the subtype of BC and pre- versus post-NAC. TILs subsetting seems critical to further identify the different immune subpopulations in residual specimen and understand if their localization, their quantity or their state of activation is associated with the non-linear predictive impact and/or their different prognostic value before and after NAC among BC subtypes.*

As perspective, we are currently leading a research program (*Neocheck*) aiming at characterizing extensively the different immune subpopulations in 42 specimen of tumor resistant to treatment (RCB-III) from various subtypes TNBC (n=15)/ luminal (n=15) / and *HER2*-positive BC (n=12)). We are using multispectral Vectra imaging system (PerkinElmer) and Inform* software to distinguish and localize the different cell populations (stromal/tumor) and the expression of immune checkpoint / check point ligand in residual specimen of the 3 BC subtypes. Deciphering the nature of the immune infiltration in post-neoadjuvant residual tumor burden is the biological core of this project.

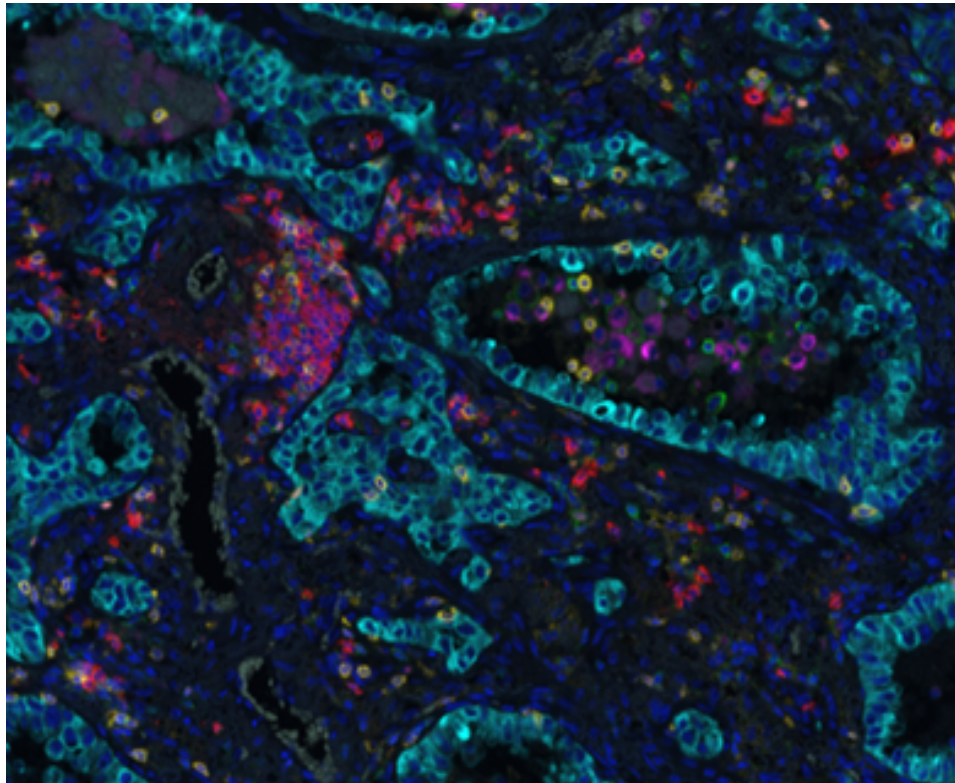


Figure 6: Multispectral Vectra imaging system on a breast cancer specimen.

Chapter 3 : Comedications, immunity and response to neoadjuvant treatment in breast cancer

The incidence of **breast cancer** (BC) increases with age, as does the incidence of many other chronic diseases, such as diabetes, hypertension, and cardiovascular disease. **Comorbidity** is defined as the “coexistence of disorders in addition to a primary disease of interest”. Comorbidities have been shown to influence BC history, cancer treatment decisions as well as short- and long-term survival.

In parallel, there is growing interest in **comedications** – *i.e.* chronically used medications - that may influence the risk for, as well as the progression of cancer [3],[4]. Hence, the chemoprevention approach is the matter of growing interest in the cancer field, and several recent conferences addressed drug repurposing as a potential approach for cancer prevention[5]. Epidemiological evidence has reported associations between **some medications** such as aspirin or non-steroidal anti-inflammatory drugs (NSAID) and a **decrease in BC risk** [6]. Others, as statins [7], NSAIDs [8] beta blockers (BB) [9] and metformin were found to be associated with a **decrease in BC recurrence or to an improved survival** after BC [10], [11].

Concomittant drugs taken during anticancer treatment may modify its pharmacodynamics or pharmacokinetics. However, interactions between comedications and chemotherapy have been so far little investigated. They were evaluated essentially from the toxicity point of view, *i.e.* regarding adverse events in patients with polypharmacy [12], or interactions with oral antineoplastic agents[13]. Regarding response to treatment, Jiralerspong *et al.* reported increased response rates to NAC in 68 diabetic BC patients treated by metformin (24%) compared with 87 patients without metformin (8.0%, $p=0.007$), and *versus* 2374 nondiabetic patients (16%, $p=0.04$) [14].

The **REMAGUS02** trial was a multicenter randomized phase II trial that included 340 patients with locally advanced breast cancer. In the *HER2*-negative stratum of the trial (n=220), patients were randomly assigned to receive celecoxib (400 mg twice daily, orally) in addition to neoadjuvant sequential chemotherapy (NAC, epirubicin/cyclophosphamide followed by docetaxel) versus standard treatment. The trial failed to demonstrate a benefit in the experimental arm with celecoxib, both for pathological complete response [15] (primary objective) or disease-free survival (DFS, secondary objective)[16].

We performed a secondary analysis of the randomized clinical trial REMAGUS02 (neoadjuvant chemotherapy (NAC) for breast cancer +/- celecoxib) **according to COX activation status** evaluated by *PTGS2* expression.

We identified a significant interaction between *PTGS2* expression and response to treatment assessed by pathological complete response **Article n°15 Cremoux et al., 2018, Anticancer research, 2018, p.387.**

In addition, we found a significant interaction between *PTGS2* expression and celecoxib on prognosis; and we evidenced an unexpected paradoxical effect: patients in the *PTGS2*-low group from the celecoxib arm had **impaired EFS** and **impaired OS** compared with the standard arm, whereas no association was seen in the *PTGS2*-high group **Article n°16 Hamy et al, 2019, Journal of Clinical Oncology, p.395.**

⇒ *These results suggest that strong, complex interactions exist between gene expression, response to treatment, prognosis and neoadjuvant chemotherapy, and that those associations might be masked from basic statistical analyses. We hypothesize that several interactions between comedications and chemotherapy remain to discover in datasets from clinical trials.*

On the other hand, comedications may enhance response to treatment. In a cohort of 1023 breast cancer patients treated with neoadjuvant chemotherapy (NAC), we systematically analyzed the concurrent comedications (n=1178) on the density of tumor infiltrating lymphocytes (TILs) and pathological complete responses (pCR). Pre-NAC TIL density was increased by medications targeting nervous system in triple negative BC (TNBC), and psycholeptics use was independently associated with pathological complete response. These experiments were reproduced in BC bearing mice, where psycholeptics reduced tumor growth and increased the anti-cancer activity of cyclophosphamide in a T cell-dependent manner.

Article n°17, Hamy et al, submitted to Oncoimmunology, p.431

This prompted us to launch a confirmatory research program, aiming at analyzing the relationships between co-morbidities, comedications, immune infiltration, response to treatment and toxicities, as well as breast cancer outcomes in a very large dataset of breast cancer patients (**COMBIMMUNO** project). Several independent cohorts from international trials (EORTC 10994/BIG 1-00 n=1856, GeparSexto, GeparSepto (n≈2000)) or national trials (PACS-08, PACS-09, n≈1000), or real life cohorts (cohorte CANTO n≈11 000, data Institut Curie n≈20000) are currently being aggregated. A robust statistical methodology will be developed to take into account both confounding factors and the aggregation of heterogeneous data. A large integrative analysis will be analyzed on a pooled analysis of nearly 50000 BC patients.

These analyses will identify drugs or drug combinations modulating immune infiltration, increasing or decreasing response to treatment, improving or harming prognosis. The ultimate goal of such analyses is to help designing drug-repositioning clinical trials aiming at prospectively validate whether the addition of comedications could modify the natural course of breast cancer.

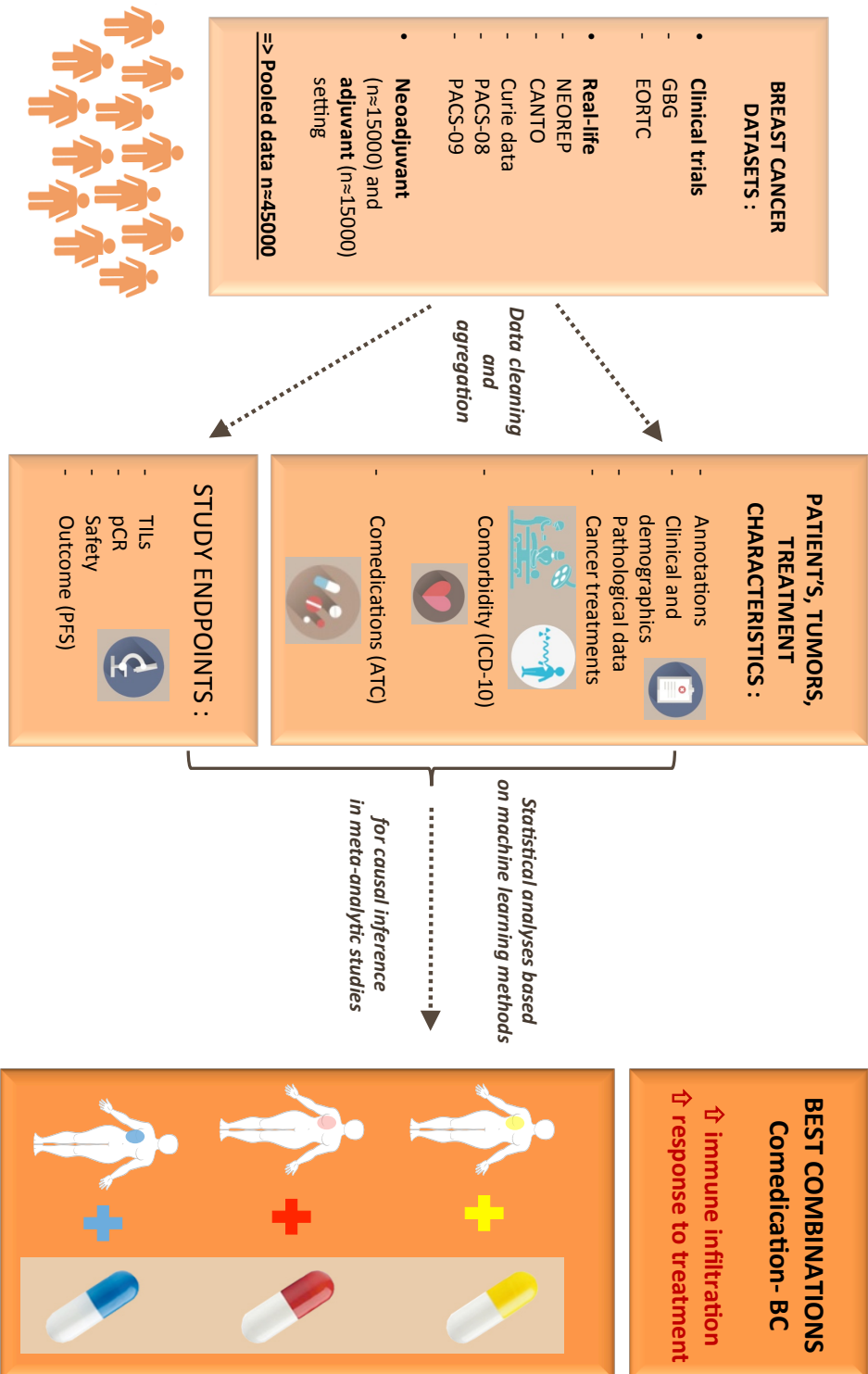


Figure 7 : Outline of the COMBIMMUNO research program (COMMedications and comorBIdities in breast cancer: Deciphering interactions between IMMUNe infiltration, response to treatment and prOgnosis (COMBIMMUNO)).

In the same vein, we scaled this project to the national level using data from the French national reimbursement system. We first conducted a feasibility study to investigate whether the reimbursement data for comedication faithfully reflect data retrieved in electronic health records (**Hamy AS., unpublished**). We analyzed a subset of 1101 Curie patients from the institutional database diagnosed for a primary invasive breast cancer without distant metastasis between 2009 and 2012. The SNDS data of these patients have been identified by matching the tracks of the acts directly sent by Curie hospital to the CNAM (Caisse Nationale d'Assurance Maladie) to the PMSI in the SNDS data. Overall, 2,938 drugs were reported in our study. In 2,304 cases, SNDS data matched with Curie records (78.4%) and in 634 cases, the drug was reported only in one out of the two sources (21.6%) (Figure 8).

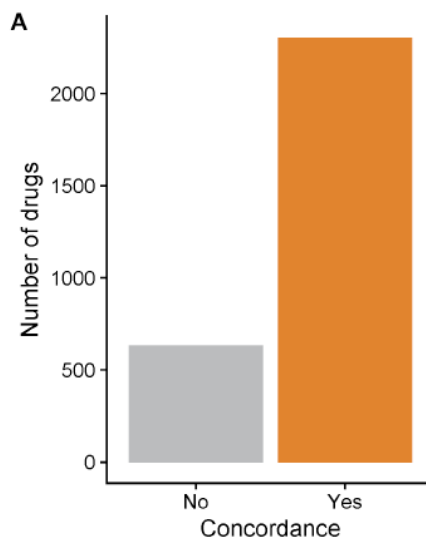


Figure 8: Concordance of comedications reported in Curie Data and in the SNDS data

The concordance between comedications reported in Curie and in SNDS data was class-dependent, with high concordance rates observed for lipid modifying agents, agents acting on the renin-angiotensin system, and thyroid therapy, whereas the concordance was poor for drugs for acid related disorders.

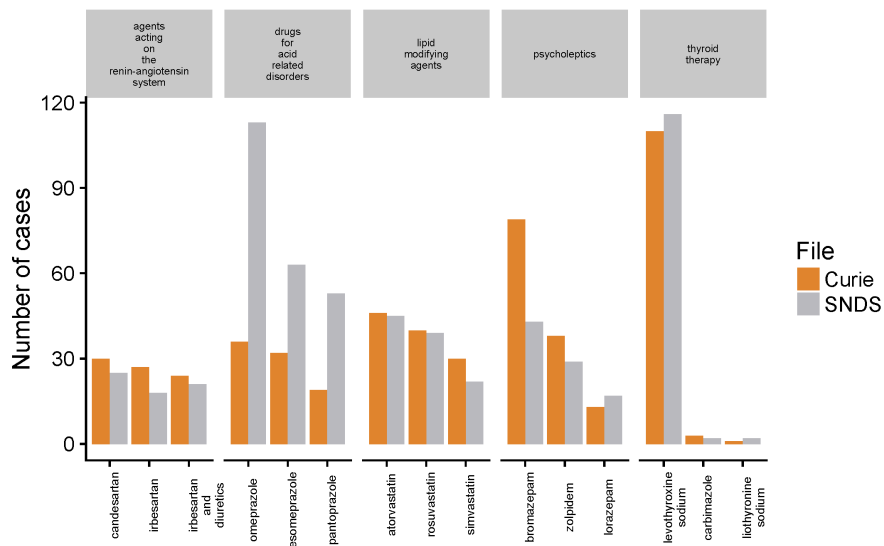


Figure 9: Concordance of comedications reported in Curie Data and in the SNDS data, according to the class of drugs.

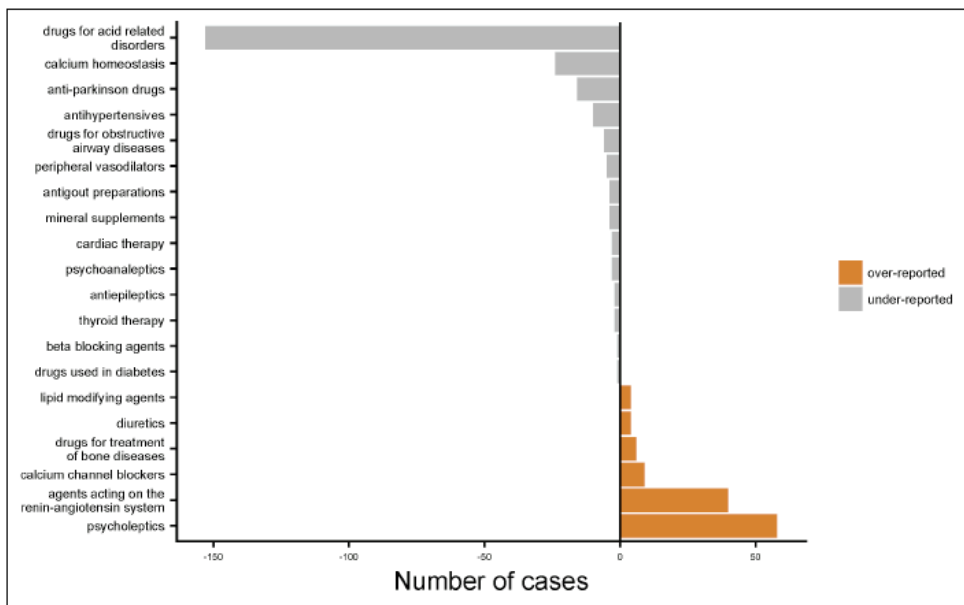


Figure 10: Repartition of drugs not reported in Curie electronic health records (EHR), but retrieved in the SNDS data (underreported), and drugs reported in Curie EHR, but not retrieved in the SNDS data, according to the class of drugs.

⇒ *These data suggest that concordance between EHR and SNDS data should be carefully evaluated within drug classes. In both cases (overreporting due to poor compliance or underreporting due to under-declaration), using SNDS data seems more relevant to consider than EHR data.*

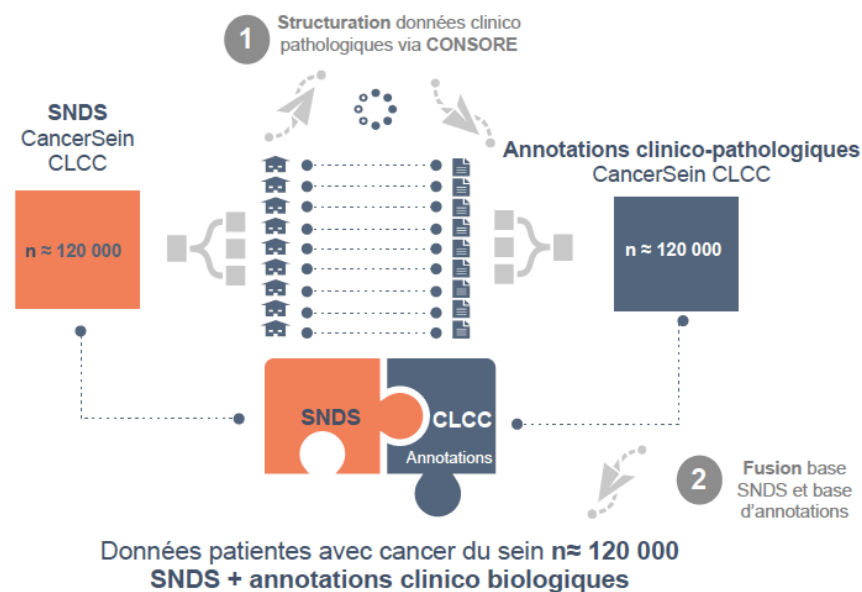
Based on these findings, we next designed the **COMBICANCER-SEIN** research project.

The objective of this project was to analyse the relationships between comorbidity, comediations, and oncologic outcome at the scale of the exhaustive French population from BC patients treated between 2007 and 2018. This project has been selected as a pilot project for the Health data Hub from the French government. This work will be organized according to 4 work packages :

WP #1. Creation of a fully annotated database of BC patients with clinical and pathological annotation (dataset SNDS-CLCC-SEIN, n≈120 000).

Patients will be identified from 9 cancer centers with a search engine (Consore). Clinical annotations (stage, BC subtype, pathological patterns, outcomes etc...) will be automatically structured from electronic health records (EHRs) and will be aggregated into a unique database. SNDS data will be retrieved and merged to the previous cohort (Set SNDS-CLCC-SEIN, n≈120 000)

WP1 : Création jeu données “Cancer du sein ”avec annotations clinico-pathologiques



WP #2. Analyses of **associations between comorbidities-comedications-response to treatment – prognostic** of BC in dataset SNDS-CLCC-SEIN.

WP #3: Evaluation of the concordance between clinical patterns between clinical annotations and SNDS data.

We will notably evaluate the *direct concordance* for the following patterns : age, dates and treatment regimen ; endocrine therapy / oral chemotherapy prescription and release; non anticancerous comedication ; type of surgery ; radiation therapy ; date of deaths.

For other items, we will *build algorithms* to test whether we estimate from SNDS data: local relapse, distant metastases, BC subtype, comorbidity. If this approach is successful, these items would further be used as proxy in further analyses in the SNDS.

WP #4. Analyses of **associations between comorbidities-comedications-response to treatment – prognostic of BCs in the complete dataset of breast cancer** from the SNDS ($n \approx 500\ 000$).

Perspectives derived from this program include the identification of comedications harming / improving BC prognosis. The associations identified might in turn be tested within drug repositioning trials, to evaluate if they might translate into clinical benefits for breast cancer patients.

Discussion and conclusion

In this thesis, we highlighted that the neoadjuvant model is a unique opportunity to explore resistance to treatment and represents an information mine to generate hypotheses to improve BC care. Performing basic analyses on 2 datasets of breast cancer patients treated by NAC, we successively demonstrated that :

- (i) The clinical significance of immune infiltration was modified by chemotherapy ;
- (ii) Comedication use modified response to treatment ;
- (iii) Post-NAC LVI was a major prognostic factor outperforming several classical clinico pathological variables;
- (iv) The reanalysis of clinical trials published as negative could unmask hidden clinically relevant effects;
- (v) Finally, by answering clinically or biologically-driven questions, we unmasked multiple hidden interactions : (a) Interaction between nodal axillar involvment – BMI- menopausal status in TNBC on DFS; (b) Interaction between LVI and BC subtype on DFS ; (c) Interaction between pre-NAC TILs and BC subtype on pCR; (d) Interaction between pre-NAC TILs and BC subtype on DFS ; (e) Interaction between post-NAC TILs and BC subtype on DFS ; (f) Interaction between post-NAC TILs and BC subtype on DFS ; (g) Interaction between post-NAC TILs and RCB on DFS ; (h) Interaction between post-NAC axillary involvment and BC subtype on DFS ; (i) Interaction between *PTGS2* expression and celecoxib use on pCR; (j) Interaction between both ER and *PTGS2* expression and celecoxib use on DFS, (k) Interaction between age and BC subtype on DFS ; (l) Interaction between comedications of class N and C and BC subtype on pCR etc....

⇒ *Alltogether, these findings suggest that digging deeply into breast cancer datasets of patients treated with NAC could enable to decipher subsets of patients deriving different benefits from different treatments, and we hypothesize that many associations remain to discover.*

In this aim, releasing data of BC patients treated by NAC as an open-access resource, *i.e.* an agile platform considered as a « Neoadjuvant hub », could enable agile research in the neoadjuvant field. To build such ambitious tool, efforts should be paid in:

1. **Improving data collection**: Increasing the *number of data sources* (connected devices, health insurance reimbursement data), the *quality of data* (collecting standardized patients reported outcomes, putting efforts on interoperability), and the *number of patients* would allow to generate large real life datasets. Creating an active community of BC patients treated with NAC represents a substantial meaningful challenge.
2. **Standardizing the translational framework of neoadjuvant treatment before and during treatment**: The standardization of *biobanking* (tumor and nodal resampling, peripheral blood), of *imaging evaluation* (PET-FDG, MRI), the automatization of *pathological slides scanning* would ensure comparability of endpoints, and enable central review.
3. **Enlarging research fields to find ways to optimize response to treatment**: while clinical trials mostly focus on the evaluation of new drugs, several other aspects remain to explore. They notably include dose and intensity modulation, number of cycles, sequence and order of treatment, schedules of administration and their links with circadian rhythm and clock genes. In addition, the modification of behaviours

(modulation of gut microbiota, physical activity, comedication use, impact of diet) could be investigated.

4. **Developing new statistical, analytic, and visualization methods** : As many modalities carry predictive or prognostic informations (Biological parameters, genomic and transcriptomic alterations, imaging data, pathological records, free full text of electronic health records etc...), all patterns can be integrated within this platform, and methods should be developed to explore and analyze massive data, to systematically detect interactions, and to visualize results.

Such an open-access platform would enable to quickly generate hypotheses, that could be validated in (or embedded within) clinical trials (Non pharmacological intervention trials, combination trials, early switch trials, de-escalation, second line trials).

References

1. Rouzier R, Perou CM, Symmans WF, Ibrahim N, Cristofanilli M, Anderson K, et al. Breast cancer molecular subtypes respond differently to preoperative chemotherapy. *Clin Cancer Res Off J Am Assoc Cancer Res.* 2005;11:5678–85.
2. Carey LA, Dees EC, Sawyer L, Gatti L, Moore DT, Collichio F, et al. The triple negative paradox: primary tumor chemosensitivity of breast cancer subtypes. *Clin Cancer Res Off J Am Assoc Cancer Res.* 2007;13:2329–34.
3. Kwan ML, Habel LA, Flick ED, Quesenberry CP, Caan B. Post-diagnosis statin use and breast cancer recurrence in a prospective cohort study of early stage breast cancer survivors. *Breast Cancer Res Treat.* 2008;109:573–9.
4. Goodwin PJ, Stambolic V, Lemieux J, Chen BE, Parulekar WR, Gelmon KA, et al. Evaluation of metformin in early breast cancer: a modification of the traditional paradigm for clinical testing of anti-cancer agents. *Breast Cancer Res Treat.* 2011;126:215–20.
5. AACR Cancer Prevention Summit. Shaping the Future of Cancer Prevention: A Roadmap for Integrative Cancer Science and Public Health [Internet]. [cited 2016 Dec 16]. Available from: <http://www.aacr.org:80/Research/Research/Pages/aacr-cancer-prevention-summit.aspx>
6. Zhao Y, Zhu S, Li X, Wang F, Hu F, Li D, et al. Association between NSAIDs use and breast cancer risk: a systematic review and meta-analysis. *Breast Cancer Res Treat.* 2009;117:141–50.
7. Ahern TP, Pedersen L, Tarp M, Cronin-Fenton DP, Garne JP, Silliman RA, et al. Statin prescriptions and breast cancer recurrence risk: a Danish nationwide prospective cohort study. *J Natl Cancer Inst.* 2011;103:1461–8.
8. Kwan ML, Habel LA, Slattery ML, Caan B. NSAIDs and breast cancer recurrence in a prospective cohort study. *Cancer Causes Control CCC.* 2007;18:613–20.
9. Powe DG, Voss MJ, Zänker KS, Habashy HO, Green AR, Ellis IO, et al. Beta-blocker drug therapy reduces secondary cancer formation in breast cancer and improves cancer specific survival. *Oncotarget.* 2010;1:628–38.
10. Barron TI, Connolly RM, Sharp L, Bennett K, Visvanathan K. Beta blockers and breast cancer mortality: a population- based study. *J Clin Oncol Off J Am Soc Clin Oncol.* 2011;29:2635–44.
11. Haukka J, Niskanen L, Auvinen A. Risk of Cause-Specific Death in Individuals with Cancer-Modifying Role Diabetes, Statins and Metformin. *Int J Cancer.* 2017;
12. van Leeuwen RWF, Brundel DHS, Neef C, van Gelder T, Mathijssen RHJ, Burger DM, et al. Prevalence of potential drug-drug interactions in cancer patients treated with oral anticancer drugs. *Br J Cancer.* 2013;108:1071–8.
13. Parsad S, Ratain MJ. Drug-Drug Interactions With Oral Antineoplastic Agents. *JAMA Oncol.* 2017;3:736–8.
14. Jiralerspong S, Palla SL, Giordano SH, Meric-Bernstam F, Liedtke C, Barnett CM, et al. Metformin and pathologic complete responses to neoadjuvant chemotherapy in diabetic patients with breast cancer. *J Clin Oncol Off J Am Soc Clin Oncol.* 2009;27:3297–302.
15. Pierga J-Y, Delaloge S, Espié M, Brain E, Sigal-Zafrani B, Mathieu M-C, et al. A multicenter randomized phase II study of sequential epirubicin/cyclophosphamide followed by docetaxel with or without celecoxib or trastuzumab according to HER2 status, as primary chemotherapy for localized invasive breast cancer patients. *Breast Cancer Res Treat.* 2010;122:429–37.
16. Giacchetti S, Hamy A-S, Delaloge S, Brain E, Berger F, Sigal-Zafrani B, et al. Long-term outcome of the REMAGUS 02 trial, a multicenter randomised phase II trial in locally advanced breast cancer patients treated with neoadjuvant chemotherapy with or without celecoxib or trastuzumab according to HER2 status. *Eur J Cancer Oxf Engl 1990.* 2017;75:323–32.

References of the papers published during PhD

Article n°1: Reyal F, **Hamy AS**, Piccart MJ. Neoadjuvant treatment: the future of patients with breast cancer. *ESMO Open*. 2018;3:e000371.

Article n°2: Brandao M, Reyal F, **Hamy A**, Piccart M. Neoadjuvant treatment for intermediate/high-risk HER2-positive and triple-negative breast cancers: no longer an “option” but an ethical obligation. *ESMO Open*. 2019;

Article n°3: Giacchetti S, **Hamy A-S**, Delalogue S, Brain E, Berger F, Sigal-Zafrani B, et al. Long-term outcome of the REMAGUS 02 trial, a multicenter randomised phase II trial in locally advanced breast cancer patients treated with neoadjuvant chemotherapy with or without celecoxib or trastuzumab according to HER2 status. *Eur J Cancer*. 2017;75:323–32.

Article n°4: **Hamy AS**, Bieche I, Lehmann-Che J, Scott V, Bertheau P, Guinebretière JM, et al. BIRC5 (survivin): a pejorative prognostic marker in stage II/III breast cancer with no response to neoadjuvant chemotherapy. *Breast Cancer Res Treat*. 2016;159:499–511.

Article n°5: Bonsang-Kitzis H, Chaltier L, Belin L, Savignoni A, Rouzier R, **Hamy AS**, Sablin M-P, et al. Beyond Axillary Lymph Node Metastasis, BMI and Menopausal Status Are Prognostic Determinants for Triple-Negative Breast Cancer Treated by Neoadjuvant Chemotherapy. *PloS One*. 2015;10:e0144359.

Article n°6: **Hamy-Petit A-S**, Belin L, Bonsang-Kitzis H, Paquet C, Pierga J-Y, Lerebours F, et al. Pathological complete response and prognosis after neoadjuvant chemotherapy for HER2-positive breast cancers before and after trastuzumab era: results from a real-life cohort. *Br J Cancer*. 2016;114:44–52.

Article n°7: Morel et al, in revision, Plos one

Article n°8: Labrosse J, Abdennebi I, Thibault L, Laas E, Merckelbagh H, Morel C, et al. Chemosensitivity, tumor infiltrating lymphocytes (TILs), and survival of postpartum PABC patients treated by neoadjuvant chemotherapy. *The Breast*. 2018;42:61–7.

Article n°9: **Hamy A-S**, Lam G-T, Laas E, Darrigues L, Balezeau T, Guerin J, et al. Lymphovascular invasion after neoadjuvant chemotherapy is strongly associated with poor prognosis in breast carcinoma. *Breast Cancer Res Treat*. 2018;

Article n°10: Sadacca B, **Hamy A-S**, Laurent C, Gestraud P, Bonsang-Kitzis H, Pinheiro A, et al. New insight for pharmacogenomics studies from the transcriptional analysis of two large-scale cancer cell line panels. *Sci Rep*. 2017;7:15126.

Article n°11: Bonsang-Kitzis H, Sadacca B, **Hamy-Petit AS**, Moarii M, Pinheiro A, Laurent C, et al. Biological network-driven gene selection identifies a stromal immune module as a key determinant of triple-negative breast carcinoma prognosis. *Oncoimmunology*. 2016;5:e1061176.

Article n°12: **Hamy A-S**, Bonsang-Kitzis H, Lae M, Moarii M, Sadacca B, Pinheiro A, et al. A Stromal Immune Module Correlated with the Response to Neoadjuvant Chemotherapy,

Prognosis and Lymphocyte Infiltration in HER2-Positive Breast Carcinoma Is Inversely Correlated with Hormonal Pathways. *PloS One*. 2016;11:e0167397.

Article n°13: Hamy A-S, Pierga J-Y, Sabaila A, Laas E, Bonsang-Kitzis H, Laurent C, et al. Stromal lymphocyte infiltration after neoadjuvant chemotherapy is associated with aggressive residual disease and lower disease-free survival in HER2-positive breast cancer. *Ann Oncol Off J Eur Soc Med Oncol*. 2017;28:2233–40.

Article n°14: Hamy A-S, H el ene Bonsang-Kitzis, Diane De Croze, Enora Laas, Lauren Darrigues, Lucian Topciu, Emmanuelle Menet, Anne Vincent-Salomon, Florence Lerebours, Jean-Yves Pierga, Etienne Brain, Jean-Guillaume Feron, Gabriel Benchimol, Giang-Thanh Lam, Marick La e, Fabien Reyald., Interaction between molecular subtypes, stromal immune infiltration before and after treatment in breast cancer patients treated with neoadjuvant chemotherapy. *CCR*, in press

Article n°15: Cremoux PD, Hamy A-S, Lehmann-Che J, Scott V, Sigal B, Mathieu M-C, et al. COX2/PTGS2 Expression Is Predictive of Response to Neoadjuvant Celecoxib in HER2-negative Breast Cancer Patients. *Anticancer Res*. 2018;38:1485–90.

Article n°16: Hamy A-S, Tury S, Wang X, Gao J, Pierga J-Y, Giacchetti S, et al. Celecoxib With Neoadjuvant Chemotherapy for Breast Cancer Might Worsen Outcomes Differentially by COX-2 Expression and ER Status: Exploratory Analysis of the REMAGUS02 Trial. *J Clin Oncol*. 2019;JCO.18.00636.

Article n°17: Anne-Sophie Hamy, Lisa Derosa, Constance Valdeli evre, Satoru Yonekura, Paule Opolon, Ma el Priour, Julien Guerin, Jean-Yves Pierga, Bernard Asselain, Diane de Croze, Alice Pinheiro, Marick Lae, Laure-Sophie Talagrand, Enora Laas, Lauren Darrigues, Elisabetta Marangoni, Elodie Montaudon, Guido Kroemer, Laurence Zitvogel, Fabien Reyald. Comedications influence immune infiltration and pathological response to neoadjuvant chemotherapy in breast cancer. Submitted to *Oncoimmunology*

Chapter 1 Neoadjuvant treatment



Neoadjuvant treatment: the future of patients with breast cancer

F Reyat,^{1,2} AS Hamy,² Martine J Piccart³

To cite: Reyat F, Hamy AS, Piccart MJ. Neoadjuvant treatment: the future of patients with breast cancer. *ESMO Open* 2018;3:e000371. doi:10.1136/esmoopen-2018-000371

Received 5 April 2018
Accepted 6 April 2018

In a recent paper published in the *British Medical Journal* by Vaidya and colleagues,¹ the authors call for reconsidering the routine use of neoadjuvant chemotherapy in patients with breast cancer. Their main arguments are (1) the increased risk of locoregional recurrence, (2) the imperfect correlation between the response to primary chemotherapy and overall survival, and (3) the complexity of surgery after neoadjuvant chemotherapy. We strongly disagree with this opinion paper.

First, great care should be applied in interpreting 15-year locoregional recurrence rates published in the recent Early Breast Cancer Trialists' Collaborative Group (EBCTCG) meta-analysis,² as considerable changes have occurred in the past 15 years in terms of diagnosis and staging (MRI, ultrasound, PET/CT), medical treatments (chemotherapy/targeted therapy/endocrine therapy), surgical techniques and radiation protocols. Similar hot debates on the increased risk of local relapse emerged in the transition period from radical Halsted mastectomy to breast conserving surgery plus radiation therapy, with the latter no longer being controversial as a standard of care.

Second, in response to the inconsistent relationship between treatment response and survival, the authors advocate to limit the use of neoadjuvant chemotherapy (NAC). Contrarily, we see this theme as a great opportunity to better understand breast cancer. Since National Surgical Adjuvant Breast and Bowel Project (NSABP)-18,³ a large body of literature has proved survival equivalence between chemotherapy administered in the neoadjuvant and in the adjuvant setting. Beyond the binary character of pathological complete response (pCR), a variety of post-NAC scores have been developed and validated, allowing to sharply refine individual prognosis. Residual cancer burden score by Symmans and colleagues⁴ proved to be a robust and reproducible tool to identify a group of patients with very poor prognosis.

To our knowledge, no such powerful prognostic marker has been validated so far in the adjuvant setting.

On the other hand, critical beneficial aspects of neoadjuvant treatment should be highlighted and can be grouped into three main axes: (1) patient benefits/care pathway, (2) access to innovation and (3) research and development.

Regarding patient benefits, the unequivocal increase in conservative surgery rates is associated with an improved quality of life and a reduction of the need for breast reconstruction. Primary chemotherapy also prevents from rushed oncogenetic screening and enables in case of BRCA1–2 mutation a much needed reflexion period to discuss surgical curative treatment options (total mastectomy/conservative surgery) and/or prophylactic procedures (contralateral breast/ovaries). Finally, beginning oncological treatment sequence with chemotherapy avoids delays in systemic treatment caused by surgical complications; the latter become more and more frequent as the complexity of surgical techniques increases (sentinel lymph node vs axillary dissection, lumpectomy vs oncoplasty, mastectomy vs mastectomy plus immediate breast reconstruction).

The second distinct feature of NAC is more rapid *access to innovation*. Many neoadjuvant clinical trials are currently opening due to the FDA-accelerated approval path for drugs achieving a higher rate of pathological response. Great progress has been made in identifying tumours unlikely to reach pCR, and patients can be offered 'early switch' trials. At NAC completion, patients with an excellent response can be enrolled into de-escalation trials, whereas patients with high tumour burden can be included in 'salvage therapy' trials testing new drugs. The residual tumour burden can be submitted to next-generation sequencing in order to identify actionable mutations or may be used to generate patient-derived xenograft.

¹Residual Tumor and Response to Treatment Laboratory, RT2Lab, PSL Research University, INSERM, U932 Immunity and Cancer, Institut Curie, Paris, France

²Breast and Gynecologic Cancer and Reconstructive Surgery Team, Institut Curie, Paris, France

³Institut Jules Bordet, Université Libre de Bruxelles, Bruxelles, Belgium

Correspondence to

Professor Martine J Piccart, Institut Jules Bordet, Université Libre de Bruxelles, Bruxelles, Belgium; martine.piccart@bordet.be



Third, in terms of *research and development*, neoadjuvant therapy is a strategic opportunity. It gives access successively to intrinsic baseline tumour characteristics, in vivo analysis of the sensitivity to treatment and to final postoperative evaluation of the residual tumour, making it the optimal framework for translational research. It enables serial tumour and blood biobanking, as well as iterative imaging procedures to lead comprehensive research programmes aimed at understanding tumour dynamics and resistance to treatments. In addition, the neoadjuvant setting allows the testing of new hypotheses and the identification of new predictive biomarkers. Let us just mention a few illustrative examples: the superiority of weekly paclitaxel over a three weekly administration,⁵ of aromatase inhibitors over tamoxifen^{6,7} and of sequential anthracycline–taxane over anthracycline alone⁸ has first been shown in neoadjuvant trials with subsequent confirmation in large adjuvant studies. The same is true for the dynamic biomarker Ki67, the drop of which after 2 weeks of endocrine therapy predicts endocrine sensitivity.⁹ Finally, neoadjuvant treatment makes it possible to investigate the role various factors play in modulating the response to treatment such as the microbiota, patient comorbidities and comedications, or other extrinsic factors. Decades of adjuvant clinical trials with needs of high number of patients to observe few ‘events’, long follow-up times to obtain mature survival data and huge costs have led to the conclusion that this model is no longer sustainable for drug development. In contrast, the neoadjuvant modal represents a more flexible setting, with shorter treatment durations, hundreds instead of thousands of patients who enrol and reduced costs. In the era of personalised oncology, adaptive trial designs such as those promoted by the I-SPY two group are remarkable templates for efficient and cost-effective drug development strategies.¹⁰

In conclusion, NAC is a not-to-be-missed opportunity for patients, physicians and researchers, and should in fact be the preferred approach for the majority of patients bearing aggressive forms of the disease (namely luminal B, triple negative and *HER2*-positive subtypes).

Contributors All authors contributed equally.

Funding The authors have not declared a specific grant for this research from any funding agency in the public, commercial or not-for-profit sectors.

Competing interests None declared.

Provenance and peer review Not commissioned; internally peer reviewed.

Open Access This is an Open Access article distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

© European Society for Medical Oncology (unless otherwise stated in the text of the article) 2018. All rights reserved. No commercial use is permitted unless otherwise expressly granted.

REFERENCES

- Vaidya JS, Massarut S, Vaidya HJ, *et al*. Rethinking neoadjuvant chemotherapy for breast cancer. *BMJ* 2018;360:j5913.
- Early Breast Cancer Trialists' Collaborative Group (EBCTCG). Long-term outcomes for neoadjuvant versus adjuvant chemotherapy in early breast cancer: meta-analysis of individual patient data from ten randomised trials. *Lancet Oncol* 2018;19:27–39.
- Fisher B, Bryant J, Wolmark N, *et al*. Effect of preoperative chemotherapy on the outcome of women with operable breast cancer. *J Clin Oncol* 1998;16:2672–85.
- Symmans WF, Peintinger F, Hatzis C, *et al*. Measurement of residual breast cancer burden to predict survival after neoadjuvant chemotherapy. *J Clin Oncol* 2007;25:4414–22.
- Green MC, Buzdar AU, Smith T, *et al*. Weekly paclitaxel improves pathologic complete remission in operable breast cancer when compared with paclitaxel once every 3 weeks. *J Clin Oncol* 2005;23:5983–92.
- Eiermann W, Paepke S, Appfelstaedt J, *et al*. Preoperative treatment of postmenopausal breast cancer patients with letrozole: a randomized double-blind multicenter study. *Ann Oncol* 2001;12:1527–32.
- Dowsett M, Ebbs SR, Dixon JM, *et al*. Biomarker changes during neoadjuvant anastrozole, tamoxifen, or the combination: influence of hormonal status and HER-2 in breast cancer—a study from the IMPACT trialists. *J Clin Oncol* 2005;23:2477–92.
- Smith IC, Heys SD, Hutcheon AW, *et al*. Neoadjuvant chemotherapy in breast cancer: significantly enhanced response with docetaxel. *J Clin Oncol* 2002;20:1456–66.
- Dowsett M, Smith IE, Ebbs SR, *et al*. Prognostic value of Ki67 expression after short-term presurgical endocrine therapy for primary breast cancer. *J Natl Cancer Inst* 2007;99:167–70.
- Bartsch R, de Azambuja E. I-SPY 2: optimising cancer drug development in the 21st century. *ESMO Open* 2016;1:e000113.



Neoadjuvant treatment for intermediate/high-risk HER2-positive and triple-negative breast cancers: no longer an 'option' but an ethical obligation

Mariana Brandão,¹ Fabien Reyat,^{2,3} Anne-Sophie Hamy,³ Martine Piccart-Gebhart¹

To cite: Brandão M, Reyat F, Hamy A-S, *et al.* Neoadjuvant treatment for intermediate/high-risk HER2-positive and triple-negative breast cancers: no longer an 'option' but an ethical obligation. *ESMO Open* 2019;4:e000515. doi:10.1136/esmoopen-2019-000515

Received 14 March 2019
Accepted 17 March 2019

© Author (s) (or their employer(s)) 2019. Re-use permitted under CC BY-NC. No commercial re-use. Published by BMJ on behalf of the European Society for Medical Oncology.

¹Université Libre de Bruxelles, Institut Jules Bordet, Bruxelles, Belgium

²Residual Tumor & Response to Treatment Laboratory, PSL Research University, Paris, France

³Breast and Gynecologic Cancer and Reconstructive Surgery Team, Institut Curie, Paris, France

Correspondence to
Professor Martine Piccart-Gebhart; martine.piccart@bordet.be

Last year, we published an editorial in this journal, advocating the use of neoadjuvant treatment (NAT) in patients with breast cancer, especially for those bearing aggressive tumours (luminal B, triple-negative and HER2-positive subtypes).¹ With the recent publication of important practice-changing data, we argue now that the use of NAT is the only ethical strategy for around one-third of women with early breast cancer.

The first reason for using NAT is that it allows surgical de-escalation, as it increases the rates of breast-conserving surgery.² It may also avoid a full axillary dissection in selected patients who 'convert' from cN1 to a negative sentinel lymph-node biopsy.³ Another very important reason is that it identifies patients at a higher risk of relapse, for whom additional 'salvage' options are now available. Two large meta-analyses have demonstrated that patients who do not achieve a pathological complete response (pCR) after NAT have worse long-term survival, especially in triple-negative breast cancer (TNBC) and HER2-positive disease.^{4,5} Yet, it has recently been shown that their outcome may be improved by escalating post-NAT.

The CREATE-X trial, conducted in Asia, included both patients with oestrogen receptor (ER)-positive/HER2-negative disease and TNBC, who were randomised to receive standard postsurgical treatment either with or without capecitabine.⁶ Among patients with TNBC, capecitabine significantly improved 5-year disease-free survival: it was 69.8% in the capecitabine group versus 56.1% in the control group (HR 0.58; 95% CI 0.39 to 0.87); it also improved overall survival (HR 0.52; 95% CI 0.30 to 0.90). In patients with ER-positive/HER2-negative disease, the HR for disease free-survival was more modest: 0.81 (95% CI 0.55 to 1.17). Despite concerns

on the extrapolation of CREATE-X results to non-Asian patients, international guidelines adopted adjuvant capecitabine as a possible treatment for patients with TNBC and invasive residual disease after NAT.^{7,8}

More recently, the KATHERINE trial randomised 1486 patients with residual invasive HER2-positive disease following NAT to adjuvant T-DM1 or trastuzumab for 14 cycles.⁹ Results were impressive: the 3-year invasive disease-free survival rate was 88.3% in the T-DM1 group versus 77.0% in the trastuzumab group (HR 0.50; 95% CI 0.39 to 0.64), making it clear that these patients with suboptimal responses to standard chemotherapy and anti-HER2 monoclonal antibodies (trastuzumab ± pertuzumab) should receive adjuvant T-DM1 instead of continuing trastuzumab. Nonetheless, there is space for further improvement in the ER-negative/HER2-positive subgroup, as 3-year invasive disease-free survival rate was 82.1% with T-DM1. Overall survival data are still immature.

Of note, there are several ongoing phase III trials testing the postneoadjuvant use of other drugs in patients with residual disease after NAT, like the PENELOPE-B trial in ER-positive/HER2-negative patients (standard endocrine therapy with/without 1 year of palbociclib; ClinicalTrials.gov identifier: NCT01864746) or the SWOG S1418/NRG BR006 trial in TNBC (1 year of pembrolizumab or placebo; NCT02954874).

Considering the above results—and particularly those of the very robust international KATHERINE trial—we advocate that clinicians must use tumour's response to NAT as a way to tailor adjuvant treatment of patients with intermediate to high-risk HER2-positive disease or TNBC, instead of blindly prescribing chemotherapy and/or targeted agents after surgery.



NAT becomes the ‘standard of care’ for these women and not only an ‘option’ to discuss for the purpose of increasing the probability of less aggressive surgery, as it has an impact on disease-free survival and, possibly, on overall survival as well. A number of remaining questions will need to be addressed, like which adjuvant anti-HER2 therapy to prescribe to patients who achieve pCR after neoadjuvant chemotherapy with trastuzumab and pertuzumab and whether or not biomarkers evaluated after one or two courses of NAT might reliably identify patients who will not reach a pCR and who could benefit from an earlier introduction of a ‘salvage’ treatment.

The NAT strategy could also become a standard of care for high-risk luminal B disease in the near future, if it is demonstrated that those patients who do not achieve a pCR after NAT may benefit from the addition of targeted therapy to endocrine treatment. Beyond pCR ‘yes or no’, other prognostic markers can be used to identify high-risk patients, like the residual cancer burden¹⁰ or the PEPI score.¹¹ More recently, prognostic markers like tumour-infiltrating lymphocytes in the residual tumour¹² or the persistence of circulating tumour DNA (ctDNA)¹³ have also been explored. These markers may also be important for patients who achieve pCR, as we know that a part of these patients still relapse afterwards and we should find ways of identifying them.

Even though the use of NAT helps tailoring adjuvant therapy, in patients who do not achieve pCR (who are still the majority), the duration of neoadjuvant plus adjuvant treatment can be very long—for example, up to 18 months in HER2-positive disease. Thus, an earlier identification of patients who are benefiting or not from NAT is necessary in order to (de)escalate therapy accordingly. One possibility is the use of imaging during the course of NAT, like MRI^{14,15} and/or ¹⁸F-FDG PET/CT,^{16,17} which have shown to be associated with achievement of pCR. Other possibilities are measuring the drop of Ki67 after 2–4 weeks of treatment^{18–21} or assessing the fall in ctDNA levels during NAT.^{22,23} Today, however, there is no proven benefit of changing the type of regimen used throughout NAT according to these markers, but there are ongoing trials testing this hypothesis (ie, ALTERNATE [NCT01953588] and ADAPT HR+/HER2- [NCT01779206]).

It should also be realised that the use of NAT demands a highly organised team of pathologists, radiologists, surgeons, medical oncologists, radiation oncologists and other professionals specialised in breast cancer care. As already recommended by the European Society for Medical Oncology,²⁴ we are strong believers that the model of ‘breast cancer units’ should now be fully implemented in Europe and abroad, as failing to do so might compromise patients’ survival.²⁵ A courageous way of accelerating its dissemination would be to restrict breast cancer treatment reimbursement to hospitals which have an accredited breast cancer unit.

In conclusion, we claim that patients with intermediate to high-risk TNBC or HER2-positive disease ($\geq T2$ and/or lymph-node positive tumours) must receive NAT, as

this strategy not only increases the chance of less aggressive surgery, but identifies patients who will benefit from ‘salvage’ adjuvant therapy with an impact on long-term outcomes.

Funding The authors have not declared a specific grant for this research from any funding agency in the public, commercial or not-for-profit sectors.

Competing interests MB: Travel expenses: Roche-Genentech. Research grants to the institute: AstraZeneca, Lilly, MSD, Novartis, Pfizer, Roche-Genentech, Synthron, Radius, Servier. FR: Consultant (honoraria): Agendia. Research grant: Roche-Genentech. MJP: Board member of Radius. Consultant (honoraria): AstraZeneca, Lilly, MSD, Novartis, Odonate, Pfizer, Roche-Genentech, Camel-IDS, Crescendo Biologics, Periphagen, Huya, Debiopharm, PharmaMar, G1 Therapeutics, Menarini, Seattle Genetics, Immunomedics, Oncolytics. Research grants to the institute: AstraZeneca, Lilly, MSD, Novartis, Pfizer, Roche-Genentech, Synthron, Radius, Servier.

Patient consent for publication Not required.

Provenance and peer review Not commissioned; internally peer reviewed.

Open access This is an open access article distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited, any changes made are indicated, and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>.

REFERENCES

- Reyal F, Hamy AS, Piccart MJ. Neoadjuvant treatment: the future of patients with breast cancer. *ESMO Open* 2018;3:e000371.
- Asselain B, Barlow W, Bartlett J, *et al*. Long-term outcomes for neoadjuvant versus adjuvant chemotherapy in early breast cancer: meta-analysis of individual patient data from ten randomised trials. *Lancet Oncol* 2018;19:27–39.
- Simons JM, van Nijnatten TJA, van der Pol CC, *et al*. Diagnostic accuracy of different surgical procedures for axillary staging after neoadjuvant systemic therapy in node-positive breast cancer: a systematic review and meta-analysis. *Ann Surg* 2019;269:432–42.
- Cortazar P, Zhang L, Untch M, *et al*. Pathological complete response and long-term clinical benefit in breast cancer: the CTNeoBC pooled analysis. *The Lancet* 2014;384:164–72.
- Spring LM, Fell G, Arfe A, *et al*. Abstract GS2-03: pathological complete response after neoadjuvant chemotherapy and impact on breast cancer recurrence and mortality, stratified by breast cancer subtypes and adjuvant chemotherapy usage: individual patient-level meta-analyses of over 27,000 patients. *Cancer Res* 2019;79.
- Masuda N, Lee S-J, Ohtani S, *et al*. Adjuvant capecitabine for breast cancer after preoperative chemotherapy. *N Engl J Med* 2017;376:2147–59.
- Denduluri N, Chavez-MacGregor M, Telli ML, *et al*. Selection of optimal adjuvant chemotherapy and targeted therapy for early breast cancer: ASCO clinical practice guideline focused update. *J Clin Oncol* 2018;36:2433–43.
- Gradishar WJ, Anderson BO, Abraham J. NCCN clinical practice guidelines in oncology. *Breast Cancer* 2019;215.
- von Minckwitz G, Huang C-S, Mano MS, *et al*. Trastuzumab emtansine for residual invasive HER2-positive breast cancer. *N Engl J Med* 2019;380:617–28.
- Symmans WF, Peintinger F, Hatzis C, *et al*. Measurement of residual breast cancer burden to predict survival after neoadjuvant chemotherapy. *J Clin Oncol* 2007;25:4414–22.
- Ellis MJ, Tao Y, Luo J, *et al*. Outcome prediction for estrogen receptor-positive breast cancer based on postneoadjuvant endocrine therapy tumor characteristics. *J Natl Cancer Inst* 2008;100:1380–8.
- Loi S, Drubay D, Adams S, *et al*. Tumor-infiltrating lymphocytes and prognosis: a pooled individual patient analysis of early-stage triple-negative breast cancers. *J Clin Oncol* 2019;37:559–69.
- Garcia-Murillas I, Schiavon G, Weigelt B, *et al*. Mutation tracking in circulating tumor DNA predicts relapse in early breast cancer. *Sci Transl Med* 2015;7.
- Taourel P, Pages E, Millet I, *et al*. Magnetic resonance imaging in breast cancer management in the context of neo-adjuvant chemotherapy. *Crit Rev Oncol Hematol* 2018;132:51–65.
- Gu Y-L, Pan S-M, Ren J, *et al*. Role of magnetic resonance imaging in detection of pathologic complete remission in breast cancer



- patients treated with neoadjuvant chemotherapy: a meta-analysis. *Clin Breast Cancer* 2017;17:245–55.
16. Gebhart G, Gámez C, Holmes E, *et al.* 18F-FDG PET/CT for early prediction of response to neoadjuvant lapatinib, trastuzumab, and their combination in HER2-positive breast cancer: results from Neo-ALTO. *J Nucl Med* 2013;54:1862–8.
 17. Ha S, Park S, Bang J-I, *et al.* Metabolic Radiomics for pretreatment 18F-FDG PET/CT to characterize locally advanced breast cancer: histopathologic characteristics, response to neoadjuvant chemotherapy, and prognosis. *Sci Rep* 2017;7.
 18. Ma CX, Gao F, Luo J, *et al.* NeoPalAna: neoadjuvant Palbociclib, a cyclin-dependent kinase 4/6 inhibitor, and anastrozole for clinical stage 2 or 3 estrogen receptor-positive breast cancer. *Clin Cancer Res* 2017;23:4055–65.
 19. Guarneri V, Dieci MV, Bisagni G, *et al.* De-escalated therapy for HR+/HER2+ breast cancer patients with Ki67 response after 2 weeks letrozole: results of the PerELISA neoadjuvant study. *Ann Oncol* 2019. doi:10.1093/annonc/mdz055. [Epub ahead of print: 18 Feb 2019].
 20. Nitz UA, Gluz O, Christgen M, *et al.* De-escalation strategies in HER2-positive early breast cancer (EBC): final analysis of the WSG-ADAPT HER2+/HR– phase II trial: efficacy, safety, and predictive markers for 12 weeks of neoadjuvant dual blockade with trastuzumab and pertuzumab ± weekly paclitaxel. *Ann Oncol* 2017;28:2768–72.
 21. Harbeck N, Gluz O, Christgen M, *et al.* De-Escalation Strategies in Human Epidermal Growth Factor Receptor 2 (HER2)-Positive Early Breast Cancer (BC): Final Analysis of the West German Study Group Adjuvant Dynamic Marker-Adjusted Personalized Therapy Trial Optimizing Risk Assessment and Therapy Response Prediction in Early BC HER2- and Hormone Receptor-Positive Phase II Randomized Trial—Efficacy, Safety, and Predictive Markers for 12 Weeks of Neoadjuvant Trastuzumab Emtansine With or Without Endocrine Therapy (ET) Versus Trastuzumab Plus ET. *J Clin Oncol* 2017;35:3046–54.
 22. Kim J-Y, Park D, Son D-S, *et al.* Circulating tumor DNA shows variable clonal response of breast cancer during neoadjuvant chemotherapy. *Oncotarget* 2017;8:86423–34.
 23. Riva F, Bidard F-C, Houy A, *et al.* Patient-specific circulating tumor DNA detection during neoadjuvant chemotherapy in triple-negative breast cancer. *Clin Chem* 2017;63:691–9.
 24. Senkus E, Kyriakides S, Ohno S, *et al.* Primary breast cancer: ESMO clinical practice guidelines for diagnosis, treatment and follow-up. *Ann Oncol* 2015;26(suppl 5):v8–30.
 25. Kesson EM, Allardice GM, George WD, *et al.* Effects of multidisciplinary team working on breast cancer survival: retrospective, comparative, interventional cohort study of 13 722 women. *BMJ* 2012;344:e2718.



ELSEVIER

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.ejcancer.com



1
2
3
4
5
6
7
8
9
10
11
12

13
14
15
16
17
18
19
20
21
22
23
24
25

Highlights

Q6

- Celecoxib, an anti Cox2, does not improve pathological complete response (pCR) nor outcome in HER2 negative breast cancer patients receiving sequential neoadjuvant chemotherapy.
- Long term outcome of luminal B breast cancer can be worse than triple negative breast cancer while HER2 positive breast cancer patients have a better prognosis than other subtypes with the addition of trastuzumab in neoadjuvant or adjuvant setting.

UNCORRECTED PROOF

<http://dx.doi.org/10.1016/j.ejca.2017.01.008>

0959-8049/© 2017 Elsevier Ltd. All rights reserved.

Please cite this article in press as: Giacchetti S, et al., Long-term outcome of the REMAGUS 02 trial, a multicenter randomised phase II trial in locally advanced breast cancer patients treated with neoadjuvant chemotherapy with or without celecoxib or trastuzumab according to HER2 status, European Journal of Cancer (2017), <http://dx.doi.org/10.1016/j.ejca.2017.01.008>

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.ejcancer.com

Clinical Trial

Long-term outcome of the REMAGUS 02 trial, a multicenter randomised phase II trial in locally advanced breast cancer patients treated with neoadjuvant chemotherapy with or without celecoxib or trastuzumab according to HER2 status

Sylvie Giacchetti^{a,*}, Anne-Sophie Hamy^b, Suzette Delalogue^c, Etienne Brain^d, Frédérique Berger^e, Brigitte Sigal-Zafrani^f, Marie-Christine Mathieu^g, Philippe Bertheau^h, Jean Marc Guinebretière^f, Mahasti Saghatchian^c, Florence Lerebours^d, chafouny mazouniⁱ, Olivier Tembo^k, Marc Espié^a, Fabien Reyal^{b,j}, Michel Marty^{a,k}, Bernard Asselain^l, Jean-Yves Pierga^{d,m}

^a AP-HP, Hôpital Saint-Louis, Breast Disease Unit, University Paris Diderot, 75475 Paris, France

^b Institut Curie, PSL Research University, Translational Research Department, INSERM, U932 Immunity and Cancer, Residual Tumor & Response to Treatment Laboratory (RT2Lab), Paris, France

^c Medical Oncology Department, Gustave Roussy, Cancer Center Villejuif, France

^d Medical Oncology Department, Institut Curie, Saint Cloud, Paris, France

^e Biostatistics Department, Institut Curie, Paris, France

^f Tumor Biology Department, Institut Curie, Saint Cloud, Paris, France

^g Pathology Department, Gustave Roussy, Cancer Center Villejuif, France

^h AP-HP, Hôpital Saint-Louis, Pathology Department, University Paris Diderot, Paris, France

ⁱ Department of Surgery, Gustave Roussy, Cancer Center Villejuif, France

^j Department of Surgery, Institut Curie, Paris, France

^k Center for Therapeutic Innovations in Oncology and Haematology (CITOH), APHP, Hôpital Saint-Louis, Paris, France

^l UMR 8081 'IR4M', Université Paris-Sud, 91400 Orsay, France

^m Université Paris Descartes, Sorbonne Paris Cite, Paris, France

Received 22 December 2016; accepted 11 January 2017

Available online ■ ■ ■

* Corresponding author.

E-mail address: sylvie.giacchetti@aphp.fr (S. Giacchetti).

<http://dx.doi.org/10.1016/j.ejca.2017.01.008>

0959-8049/© 2017 Elsevier Ltd. All rights reserved.

Please cite this article in press as: Giacchetti S, et al., Long-term outcome of the REMAGUS 02 trial, a multicenter randomised phase II trial in locally advanced breast cancer patients treated with neoadjuvant chemotherapy with or without celecoxib or trastuzumab according to HER2 status, European Journal of Cancer (2017), <http://dx.doi.org/10.1016/j.ejca.2017.01.008>

KEYWORDS

Neoadjuvant chemotherapy;
 Celecoxib;
 Trastuzumab;
 Breast cancer;
 Long-term outcome

Abstract Background: The REMAGUS-02 multicenter randomised phase II trial showed that the addition to neoadjuvant chemotherapy (NAC) of trastuzumab in patients with localised HER2-positive breast cancer (BC) increased the pathological complete response (pCR) rate and that the addition of celecoxib in HER2-negative cases did not increase the pCR rate. We report here the long-term follow-up results for disease-free survival (DFS) and overall survival (OS).

Patients and methods: From 2004 to 2007, 340 stage II–III BC patients were randomly assigned to receive neoadjuvant EC-T (four cycles of epirubicin–cyclophosphamide followed by four cycles of docetaxel) +/- celecoxib in HER2-negative cases (n = 220) and ± trastuzumab in HER2-positive cases (n = 120). From September 2005, all patients with HER2-positive BC received adjuvant T (n = 106).

Results: Median follow-up was nearly 8 years (94.4 months, 20–127 m). In the HER2-negative subgroup, addition of celecoxib was not associated with a DFS benefit. Favourable factors were smaller tumour size, expression of progesterone receptor status (PgR) and pCR. In the HER2-positive population, neoadjuvant trastuzumab was not associated with a DFS benefit. Axillary pCR was the only prognostic factor associated with DFS in this group [HR = 0.44, 95% CI = 0.2–0.97], p = 0.035]. To note, DFS and OS were significantly higher in the HER2-positive than in HER2-negative BC patients (HR = 0.58 [0.36–0.92], p = 0.021).

Conclusion: Celecoxib combined with NAC provided neither pCR nor survival benefit in patients with HER2-negative BC. Absence of PgR is a major prognostic factor. Neoadjuvant trastuzumab increased pCR rates without translation into a DFS or OS benefit compared with adjuvant trastuzumab only. Axillary pCR could be a more relevant surrogate of survival than in the breast in HER2-positive population. A retrospective comparison shows that patients with HER2-positive tumours have a better outcome than HER2-negative BC patients showing the impact of trastuzumab on the natural history of BC.

© 2017 Elsevier Ltd. All rights reserved.

Key message: Celecoxib, an anti-Cox2, neither improves pCR nor the outcome in HER2-negative BC patients receiving sequential NAC. The long-term outcome of luminal B BC can be worse than triple-negative BC, whereas HER2-positive BC patients have a better prognostic than other subtypes with the addition of trastuzumab in neoadjuvant or adjuvant setting.

Q1

1. Introduction

Neoadjuvant chemotherapy (NAC) was initially developed for non-resectable breast cancers, but is now widely used in localised breast cancer not eligible for breast-conserving surgery [1]. However, randomised trials and meta-analyses have not shown any improvement in disease-free survival (DFS) or overall survival (OS) compared with adjuvant approaches [1–4]. Despite the lack of demonstrated survival gain with the neoadjuvant strategy, several advantages are recognised. Tumour regression induced by NAC allows breast conservation in a proportion of patients with initially large tumours non-accessible to immediate conservative surgery. In patients with possible breast conservation from the beginning, preoperative treatment may also result in a better cosmetic outcome [5]. In particular, neoadjuvant therapy offers the advantage of rapidly

assessing the response to both standard treatment and novel therapies and a research platform to evaluate the predictive factors of response to treatment [6,7]. Such frontline treatment defined pathological complete response (pCR, i.e. absence of infiltrating tumour in breast and lymph nodes) as the key end-point in predicting the long-term survival especially in patients with oestrogen receptor (ER)-negative and triple-negative (TN) tumours [7–9].

We previously reported the pCR results of this multicenter randomised phase II study aiming to determine the impact of adding celecoxib or trastuzumab to NAC in stage II–III breast cancers [10]. We showed that celecoxib did not improve the pCR rates in HER2-negative population, whereas trastuzumab added to NAC was associated with an increased pCR rate in patients with HER2-positive tumours [10]. Here, we report the long-term outcome of the patients treated in

1 this study and evaluate the prognostic factors associated with DFS and OS.

2. Patients and methods

2 From May 2004 to October 2007, 340 patients were randomly assigned to receive NAC ± celecoxib in HER2-negative patients (Stratum A, n = 220), +/- trastuzumab in HER2-positive patients (Stratum B, n = 120) [10] (Fig. 1).

2.1. Patients selection

3 Main eligibility criteria were female patients over the age of 18 and under the age of 65 with histologically proven non-metastatic invasive breast carcinoma (Stage II and III), not amenable to breast-conserving surgery (diameter > 3 cm, central) or with risk factors making NAC the preferred treatment (i.e. N2–N3, rapid growth rate). Inflammatory breast cancers and clinical T4 stage were allowed. HER2 status was centrally reviewed for all patients and HER2 positivity confirmed by FISH in all cases.

2.2. Treatment

4 All patients were to receive epirubicin (75 mg/m²)–cyclophosphamide (750 mg/m²) intravenously every 3 weeks for four cycles followed by docetaxel (100 mg/m²)

5 every 3 weeks for four cycles. During neoadjuvant docetaxel sequence, HER2-negative tumour patients (stratum A) were randomised between without (arm1) or with (arm 2) celecoxib 400 mg twice daily orally. In HER2-positive tumour, patients were randomised between without (arm 3) or with (arm 4) trastuzumab every 3 weeks. Surgery was performed 21–45 d after cycle eight, based on initial and post-chemotherapy assessment. Surgery was followed by local and regional radiotherapies when indicated. The administration of adjuvant trastuzumab for a total of 18 3-weekly i.v. infusions was amended for HER2-positive cancer patients from September 2005, when adjuvant trastuzumab was available in France. Trastuzumab was thus started after surgery for patients randomised to control and pursued for patients randomised to neoadjuvant arm. Patients with hormone receptor-positive tumours received adjuvant tamoxifen or aromatase inhibitors according to their menopausal status. Adjuvant chemotherapy according to centres' preferences in patients with residual axillary nodal involvement (pN+) could be delivered based on 5-fluorouracil and vinorelbine combination (four cycles), concomitantly or not with radiotherapy.

2.3. Objectives

6 The primary end-point of the study was pCR rates. pCR was defined as absence of residual invasive cancer cells

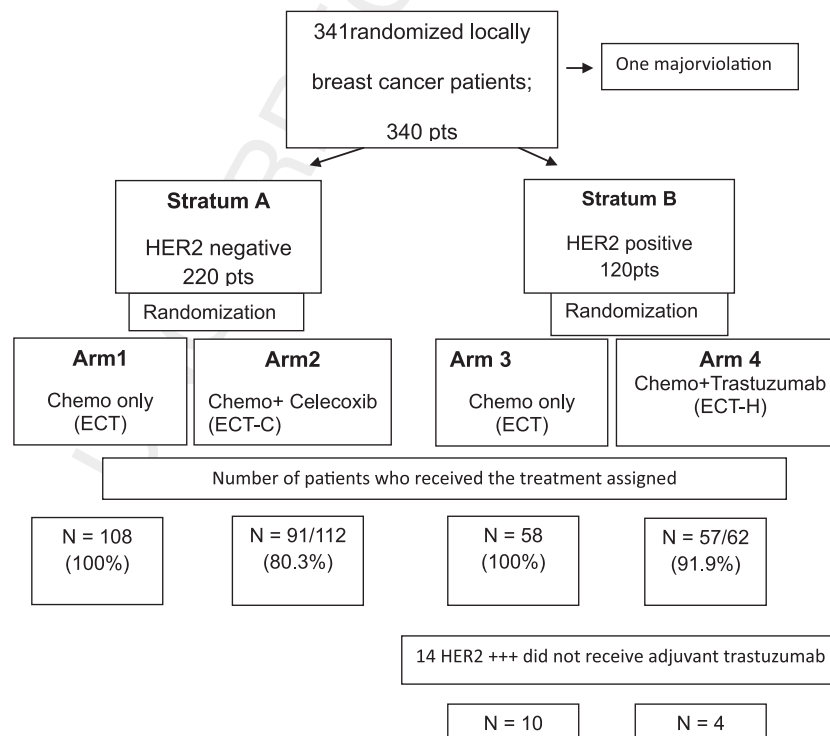


Fig. 1. Consort diagram.

1 in the breast and axillary lymph nodes (grade 1 and 2 of
2 Chevallier’s classification) [11]. Both DFS and OS were
3 predefined secondary objectives. DFS was defined as the
4 time from surgery to death, loco-regional or distant
5 recurrence, or contralateral cancer, whichever occurred
6 first. OS was defined as the time from surgery to death.
7 Patients for whom none of these events were recorded
8 were censored at the date of their last known contact.
9 Results are described in the whole population, and in
10 each stratum.
11

12
13 **2.4. Statistical analysis**

14
15 The study population was described in terms of fre-
16 quencies for qualitative variables or medians and asso-
17 ciated ranges for quantitative variables. The cutoff date
18 for the analysis was May 1st 2015. Hazard ratios (HRs)
19 and their associated 95% confidence intervals (CIs) were
20 calculated with the Cox proportional hazard model. Age
21 class, menopausal status, clinical tumour size, clinical
22 nodal status, histological type, SBR Grade, lympho-
23 vascular invasion, ER, PgR, HER2 status, p53 and
24 randomised treatment were included in the univariate
25 analysis. Variables with a *p*-value for the likelihood ratio
26 test lower than 0.15 in univariate analysis were included
27 in the multivariate model. Backward selection was used
28 to establish the final multivariate model. The signifi-
29 cance threshold was 5%. Survival probabilities were
30 estimated by the Kaplan–Meier method, and survival
31 curves were compared with log-rank tests. Analyses
32 were performed with R software, version 3.1.2 (R
33 Development Core Team, 2011).
34
35
36

37
38 **3. Results**

39
40 Among 340 patients included in the study, 220 had
41 HER2-negative tumours (stratum A) and 120 had
42 HER2-positive tumours (stratum B). All the patients
43 received the first four courses of epi-
44 rubicin–cyclophosphamide. In the stratum A, 21 pa-
45 tients who were assigned to celecoxib arm (arm 2) did
46 not receive it (19.6%). In the stratum B, five patients
47 who were assigned in the neoadjuvant trastuzumab arm
48 (arm 4) did not receive it (8%), whereas two patients in
49 the arm without trastuzumab (arm 3) received neo-
50 adjuvant trastuzumab. All patients but 14 received
51 adjuvant trastuzumab (Fig. 1), 10 in arm 3 (17.2%) and
52 four in arm 4 (6.5%), *p* = 0.07. A total of 14 patients
53 were not included in the DFS analysis [no surgery
54 *n* = 9; distant metastases during NAC (*n* = 4); protocol
55 violation (*n* = 1)].
56
57

58 Patient’s characteristics are described in Table 1.
59 Overall, the population had poor prognostic factors.
60 Near half of the patients had T3–T4 (47.4%) and grade
61 SBR 3 tumours (49.8%); 60% had lymph nodes involved
62 clinically at baseline (N1, N2 and N3). In the HER2-

negative population, almost 40% of the patients had TN
tumours. The median follow-up was 94.5 months [range:
19.9–126.9].

3.1. Whole population

A total of 111 patients experienced relapse and 74 patients died. The median DFS as and the median OS are not reached for the whole population. The 8-year DFS and OS were 67.9% [62.8–73.6] and 77.5% [72.7–82.6], respectively. After univariate analysis, ER expression was not (Fig. 2A) significantly associated with DFS, whereas clinical tumour size, clinical nodal status, histological type, lymphovascular invasion, progesterone receptor (PgR) (Fig. 2B), HER2 status (Fig. 2C) and pCR were prognostic for DFS. Tumour size (T2 versus T3 and T4; HR = 1.75 [1.15–2.66], *p* = 0.009); PgR status (positive versus negative; HR = 0.47 [0.3–0.72], *p* = 0.001), HER2 status (positive versus negative; HR = 0.58 [0.36–0.92], *p* = 0.021) and pCR (yes versus no; HR = 0.38 [0.17–0.84], *p* = 0.016) remained significantly associated with DFS after multivariate analysis (Table 2). All four factors were also associated with OS (clinical tumour size HR = 2.69 [1.61–4.49], *p* < 0.001, PgR, HR = 0.3 [0.17–0.53], *p* < 0.001, HER2; HR = 0.58 [0.34–0.99], *p* = 0.046, pCR; HR = 0.38 [0.15–0.94], *p* = 0.037).

3.2. Stratum A: HER2-negative tumours

Of 220 patients with HER2-negative tumours, 82 patients experienced recurrences and 53 patients died. Median DFS and OS were not reached. The 8-year DFS and OS are, respectively, 64.4% [57.7–71.7] and 76.5% [70.7–82.9]. No effect of the addition of celecoxib to NAC on DFS was observed, neither in intent to treat (ITT) (celecoxib versus no, HR = 1.23 [0.77–1.96], *p* = 0.38) nor in the per protocol analysis (HR = 1.27 [0.8–2.02], *p* = 0.32).

Due to the differences of the shapes of the Kaplan Meier curves for ER and PgR status, the heterogeneity of luminal breast cancer [12,13], we combined ER and PgR status into three classes (ER+/PgR+, ER+/PgR-, ER-/PgR-) (Fig. 3). Only two patients were ER-/PgR+ in the stratum A and were pooled with the ER+/PgR+ population into a PR+ group in the analysis. Overall, ER+/PR-status was associated with a worse long-term DFS when compared with ER+/PR+ (reference class, HR = 1); ER+/PR-: HR: 2.3 [1.28–4.12], *p* = 0.005, whereas TN status was not HR = 1.49 (95% CI (0.86–2.6), *p* = 0.16).

After univariate analysis, clinical tumour size, histological type, PgR and pCR were significantly associated with DFS and all factors, but histological type remained associated with DFS after multivariate analysis (Table 2). Histological type, ER status, tumour size, PgR status and pCR were significantly associated with

63
64
65
66
67
68
69
70
71
72
73
74
75
76
77
78
79
80
81
82
83
84
85
86
87
88
89
90
91
92
93
94
95
96
97
98
99
100
101
102
103
104
105
106
107
108
109
110
111
112
113
114
115
116
117
118
119
120
121
122
123
124

Table 1
Patients' characteristics.

		Stratum A: <i>HER2</i> negative		Stratum B: <i>HER2</i> positive		Total	p Value
		CT alone (arm 1)	CT + celecoxib (arm 2)	CT alone (arm 3)	CT + trastuzumab (arm 4)		
		n = 108 (%)	n = 112 (%)	n = 58 (%)	n = 62 (%)	340 (%)	
Age (years)	<40	23 (21.3)	25 (22.3)	13 (22.4)	15 (24.2)	76 (22.4)	0.8191
	40–49	42 (38.9)	49 (43.8)	25 (43.1)	20 (32.3)	136 (40.0)	
	≥50	43 (39.8)	38 (33.9)	20 (34.5)	27 (43.5)	128 (37.6)	
Menopausal status	No	69 (65.1)	76 (67.9)	41 (70.7)	40 (64.5)	226 (66.9)	0.8645
	Yes	37 (34.9)	36 (32.1)	17 (29.3)	22 (35.5)	112 (33.1)	
Clinical tumour size	T2	55 (50.9)	65 (58)	27 (46.6)	32 (51.6)	179 (52.6)	0.508
	T3 and T4	53 (49.1)	47 (42)	31 (53.4)	30 (48.4)	161 (47.4)	
Clinical nodal status	N0	42 (39.6)	45 (40.2)	18 (31.6)	25 (40.3)	130 (38.6)	0.699
	N1, N2, N3	64 (60.4)	67 (59.8)	39 (68.4)	37 (59.7)	207 (61.4)	
Histological type	Ductal	83 (76.9)	90 (80.4)	56 (96.6)	57 (91.9)	286 (84.1)	0.0059
	Lobular	19 (17.6)	13 (11.6)	1 (1.7)	2 (3.2)	35 (10.3)	
	Others	6 (5.6)	9 (8)	1 (1.7)	3 (4.8)	19 (5.6)	
SBR grade	I	13 (12.6)	7 (6.4)	0 (0)	0	20 (6.1)	0.0042
	II	41 (39.8)	56 (50.9)	22 (40.7)	25 (41.7)	144 (44%)	
	III	49 (47.6)	47 (42.7)	32 (59.3)	35 (58.3)	163 (49.8)	
LVI	No	90 (84.9)	93 (85.3)	41 (71.9)	43 (74.1)	267 (80.9)	0.0668
	Yes	16 (15.1)	16 (14.7)	16 (28.1)	15 (25.9)	63 (19.1)	
ER	Negative	35 (32.4)	45 (40.2)	23 (39.7)	30 (48.4)	133 (39.1)	0.2271
	Positive	73 (67.6)	67 (59.8)	35 (60.3)	32 (51.6)	207 (60.9)	
PR	Negative	55 (51.4)	64 (58.2)	37 (63.8)	41 (67.2)	197 (58.6)	0.1867
	Positive	52 (48.6)	46 (41.8)	21 (36.2)	20 (32.8)	139 (41.4)	
HR	Negative	34 (31.5)	44 (39.3)	21 (36.2)	28 (45.2)	127 (37.4)	0.3316
	Positive	74 (68.5)	68 (60.7)	37 (63.8)	34 (54.8)	213 (62.6)	
Triple negative	Yes	34 (31.5)	44 (39.3)				
	No	74 (68.5)	68 (60.7)				
HR (4 classes)	ER-/PR-	34 (31.8)	44 (40)	21 (36.2)	28 (45.9)	127 (37.8)	0.303
	ER+/PR-	21 (19.6)	20 (18.2)	16 (27.6)	13 (21.3)	70 (20.8)	
	ER-/PR+	1 (0.9)	1 (0.9)	2 (3.4)	2 (3.3)	6 (1.8)	
	ER+/PR+	51 (47.7)	45 (40.9)	19 (32.8)	18 (29.5)	133 (39.6)	
p53	WT	20 (54.1)	26 (63.4)	10 (47.6)	9 (39.1)	65 (53.3)	0.2815
	Mutated	17 (45.9)	15 (36.6)	11 (52.4)	14 (60.9)	57 (46.7)	
Per protocol neoadjuvant Treatment	No celecoxib	108 (100)	21 (18.8)				
	Celecoxib	0	91 (81.2)				
	No trastuzumab			56 (96.6)	5 (8.1)		
	Trastuzumab			2 (3.4)	57 (91.9)		
Surgery	No	4 (3.7)	4 (3.6)	1 (1.7)	0	9 (2.6)	0.4374
	Yes	104 (96.3)	108 (96.4)	57 (98.3)	62 (100)	331 (97.4)	
Adjuvant chemotherapy	No	77 (71.3)	82 (73.2)	46 (79.3)	49 (79)	254 (74.7)	0.5598
	Yes	31 (28.7)	30 (26.8)	12 (20.7)	13 (21)	86 (25.3)	
Adjuvant trastuzumab	No			10 (17.2)	4 (6.5)		0.0658
	Yes			48 (82.8)	58 (93.5)		
Hormonotherapy	No	28 (27.7)	38 (36.5)	26 (48.1)	32 (51.6)	124 (38.6)	0.0084
	Yes	73 (72.3)	66 (63.5)	28 (51.9)	30 (48.4)	197 (61.4)	
Radiotherapy	No	1 (1)	3 (2.9)	0	5 (8.1)	9 (2.8)	0.0276
	Yes	101 (99)	101 (97.1)	54 (100)	57 (91.9)	313 (97.2)	
pCR	No	93 (89.4)	94 (87)	46 (80.7)	46 (74.2)	279 (84.3)	0.0458
	Yes	11 (10.6)	14 (13)	11 (19.3)	16 (25.8)	52 (15.7)	
pCR (breast)	No	89 (85.6)	91 (85)	42 (73.7)	46 (74.2)	268 (81.2)	0.0917
	Yes	15 (14.4)	16 (15)	15 (26.3)	16 (25.8)	62 (18.8)	
pCR (axilla)	No	69 (67)	64 (60)	30 (52.6)	28 (45.2)	191 (58.2)	0.0361
	Yes	34 (33)	42 (39.6)	27 (47.4)	34 (54.8)	137 (41.8)	
Relapses		37	45	15	14	111	
Deaths		23	30	10	11	74	

*Missing data: menopausal status (n = 2), IMC (n = 1), nodal status (n = 3), grade (n = 13), LVI (n = 10), RP (n = 4), p53 (n = 218),
hormonotherapy (n = 19), radiotherapy (n = 18), pCR (n = 9).

Abbreviations: LVI, lymphovascular invasion; pCR, pathological complete response.

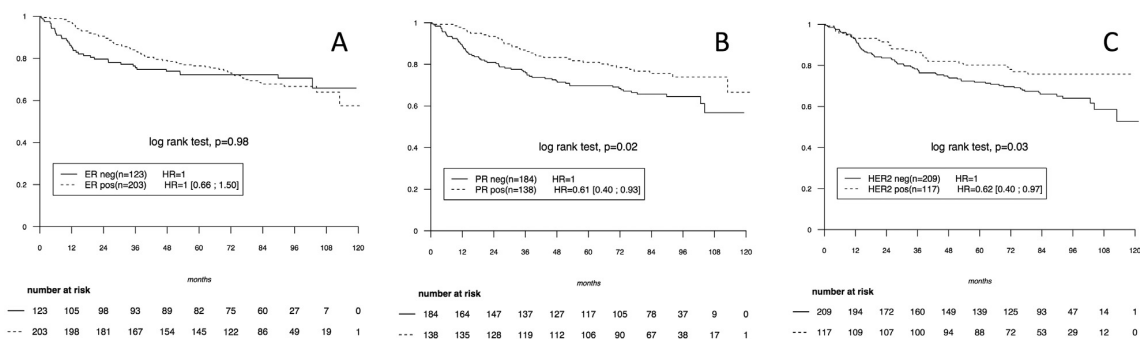


Fig. 2. DFS in the whole population. A: ER+ versus ER-, B: PgR+ versus PgR-, C: HER2-positive versus HER2 negative.

OS after univariate analysis. As for DFS, three of these factors remained significantly associated with OS after multivariate analysis: clinical tumour size (T3 and T4: HR = 2.64 [1.43–4.86] versus T2, p = 0.002); PgR positive (HR = 0.25 [0.12–0.49], p < 0.001) and pCR (HR = 0.23 [0.06–0.97], p = 0.046).

3.3. Stratum B: HER2-positive tumours

Of the 120 patients with HER-positive tumours, 29 patients experienced recurrences and 21 patients died. There was no association between neoadjuvant trastuzumab and DFS neither in intent to treat nor in the per

Table 2
Multivariate analysis for DFS in stratum A and B.

Variable	Stratum A (HER2 neg)			DFS			Stratum B (HER2+++)			DFS		
	Univariate analysis			Multivariate analysis			Univariate analysis			Multivariate analysis		
	HR	IC	p	HR	IC	p	HR	IC	p	HR	IC	p
Age	>40	1					1					
	40–49	0.81	[0.45–1.47]	0.78			1.4	[0.48–4.09]	0.583			
	≤50	0.91	[0.5–1.67]	0.78			1.71	[0.61–4.8]	0.583			
Menopausal status	No	1					1					
	Yes	1.07	[0.66–1.75]	0.787			1.42	[0.67–3.04]	0.36			
Clinical tumour size	T2	1		1	–	–	1					
	T3/T4	1.92	[1.21–3.07]	0.005	1.96	[1.2–3.19]	0.007	1.55	[0.72–3.3]	0.256		
Clinical nodal status	N0	1					1					
	N1/N2/N3	1.42	[0.86–2.34]	0.165			1.98	[0.8–4.9]	0.134			
Histological type	Ductal	1					1					
	Lobular	2	[1.14–3.52]	0.048								
	Others	1.25	[0.5–3.15]	0.048			1.3	[0.31–5.5]	0.718			
SBR Grade	I	1					1					
	II	1.79	[0.63–5.04]	0.41			1					
	III	2	[0.71–5.66]	0.41			1.99	[0.84–4.73]	0.113			
LVI	No	1					1					
	Yes	1.67	[0.93–3]	0.084			1.58	[0.72–3.46]	0.246			
ER	negative	1					1					
	positive	0.97	[0.59–1.58]	0.888			0.94	[0.44–1.99]	0.872			
PgR	negative	1		1	–	–	1					
	positive	0.56	[0.35–0.92]	0.019	0.41	[0.25–0.68]	0.001	0.65	[0.29–1.49]	0.307		
HR	negative	1					1					
	positive	0.91	[0.56–1.48]	0.698			0.8	[0.38–1.69]	0.557			
p53	WT	1					1					
	mutated	1.25	[0.57–2.77]	0.573			0.42	[0.1–1.78]	0.227			
pCR (breast and axilla)	No	1		1	–	–	1					
	Yes	0.28	[0.09–0.9]	0.023	0.24	[0.07–0.76]	0.016	0.53	[0.18–1.52]	0.229		
pCR (breast)	No	1					1					
	Yes	0.3	[0.11–0.81]	0.012			0.77	[0.31–1.9]	0.569			
pCR (axilla)	No	1					1			1		
	Yes	0.4	[0.22–0.72]	0.001			0.44	[0.2–0.97]	0.035	0.44	[0.2–0.97]	0.035

DFS as a function of HR status, 3 classes (HER2 negative population)

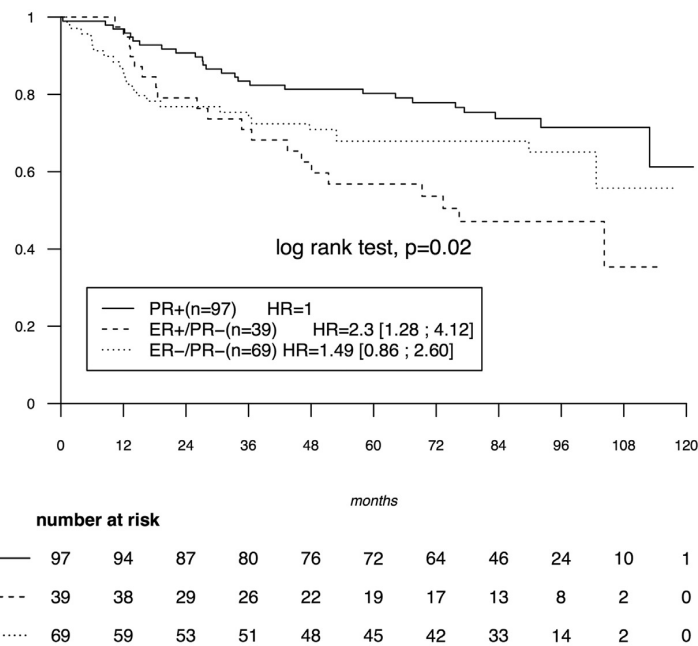


Fig. 3. DFS as a function of hormonal receptors status in patients with HER2-negative tumors.

protocol analysis in the *HER2*-positive stratum. Eight years DFS was 73.8% [62.9–86.8%] in the group randomised to receive NAC alone, versus 76.2% [65.9–88.1%] in the group randomised to receive NAC plus trastuzumab. After univariate analysis, none of the classical factors were significantly associated with DFS except axillary pCR: HR = 0.44 [0.2–0.97], $p = 0.035$. No factor was significantly associated with the OS after univariate analysis, although a trend to a better OS was observed when axillary pCR was reached [HR = 0.34 (0.11–1.07), $p = 0.053$].

4. Discussion

We report here the long-term outcome of patients with locally advanced breast cancer included in a randomised phase II trial studying the impact of adding NAC to celecoxib in *HER2*-negative population and trastuzumab in *HER2*-positive population. We showed that the addition of celecoxib was not associated with an increased DFS nor OS in *HER2*-negative patients irrespective of hormone receptors status. We previously published that the adjunction of celecoxib to NAC was not associated with higher pCR rates [10]. However, this study will provide the opportunity to study predictive genomic factors of celecoxib response as *PTGS2* overexpression.

In the *HER2*-positive population, neoadjuvant trastuzumab was associated with a higher pCR rate but this did not translate into a DFS or OS benefit. The REMAGUS 02 trial is the only neoadjuvant randomised trial including patients with *HER2*-positive tumors that compared neoadjuvant trastuzumab with no neoadjuvant trastuzumab in a population that received adjuvant trastuzumab in both arms. In the NOAH trial [14], patients received either trastuzumab given as neoadjuvant and adjuvant treatment or no trastuzumab at all. In other neoadjuvant trials as the GeparQuinto [15], NeoALTO [16] or Neosphere [17,18] trials, all control arms included neoadjuvant anti-*HER2*-targeted therapy. We acknowledge some weaknesses in our trial, possible lack of statistical power due to randomised phase II design, absence of adjuvant trastuzumab in a small subset of patients and a few discrepancies between treatment randomization and treatment allocation. However, these data arguing for no effect on DFS of an early introduction of trastuzumab in addition to NAC may ever remain the only trial to address such a question as neoadjuvant trastuzumab has become a standard of care in *HER2*-positive breast cancers.

In our study, pCR was not a surrogate of DFS and OS, regardless of the hormonal receptors in patients with *HER2*-positive tumors. pCR has been proposed as a surrogate end-point of long-term benefit such as event-free survival (EFS), DFS and OS. However, the association between pCR and long-term outcome is not

clearly demonstrated. The German Breast Group reported that in HER-positive patients, reaching pCR was associated with a DFS advantage in patients with ER negative, but not ER positive tumours [9]. Cortazar *et al.* on behalf of the FDA established an international working group (CTNeoBC) which aims to investigate the relation between pCR and long-term outcome via a pooled analysis of neoadjuvant trials [7]. They showed that pCR was positively associated with EFS and OS in all subgroups of breast cancers. However, at a trial level, the authors recorded little or no association between increases of pCR and the treatment's effect on EFS or OS. In a recent meta-analysis [19], only the NOAH trial has demonstrated that pCR achievement could be a surrogate end-point for EFS and OS [14]. The effect of pCR gain on survival in the NOAH trial could be seen only because the control group lacked an effective targeted therapy for *HER2*-positive breast cancer. In our trial, trastuzumab was given in both arms but earlier in the experimental group. The introduction of adjuvant trastuzumab could have compensated the use of neoadjuvant trastuzumab in terms of long-term DFS or OS. Our study, as well as other studies, emphasises that in future neoadjuvant studies in *HER2*-positive population, all patients will receive *HER2*-targeted therapy, and thus the gain of a new drug on pCR gain must be large enough to have an effect on EFS and survival [19].

The other point underlined in our series is the major role of post-NAC lymph nodes' involvement in patients with *HER2*-positive tumours. The analysis of the pathological response in the axilla is not or rarely studied independently of the breast, although we know that axilla involvement is a major predictor of DFS and/or OS in adjuvant. Patients with extensive nodal involvement after NAC have a very poor outcome [20–22]. The population included in our study has large locally advanced tumours with almost half of the population having T3/T4 tumours. The size of the tumours (T3 and T4 versus T2) is one of the major predictive factor in our series with a HR = 2.96, $p = 0.002$. Other authors have reported that the clinical size of the tumours is a major prognostic factor in breast cancers treated with NAC [23–26].

Another key observation from this study is the major impact of PgR receptors. Indeed, the long follow-up (nearly 8 years) allowed to report such mature data and to highlight the long-lasting risk of hormone-responsive breast cancers, as opposed to hormone receptor--negative BC. We already published that there is a non-constant effect in time for TN and non-TN breast cancers. Overall, TN status had the worst initial risk of recurrence that gradually decreased during the first 24 months of follow-up to disappear after 48 months of follow-up [27]. Here, ER+/PR-status was associated with a worse DFS when compared with ER+/PR+ (reference class, HR = 1); ER+/PR-: HR: 2.3 [1.28–4.12], $p = 0.005$, whereas TN status was not

HR = 1.49 (95% CI (0.86–2.6), $p = 0.16$). With a long follow-up, the prognosis of TNBC was not significantly different from ER+/PR-**positive** patients, whereas ER+/PR-**negative** BC patients had a nearly two-fold increased risk of relapse. These results are very important as chemotherapy is increasingly avoided for patients with ER+ tumours. At the last San Gallen conference, in patients with 'luminal B-like' (*HER2*-negative) tumours, the Panel was more closely divided, but only a minority would recommend chemotherapy for **most of the cases** [28]. In the **European Organisation for Research and Treatment of Cancer** recommendations [29], the luminal B *HER2*-negative cancers were considered as a population of the highest uncertainty regarding chemotherapy indications. Other authors explored the significance of PgR negativity [30–32]. Bae *et al.* [32] showed that patients with single HR+, negative *HER2* tumours were associated with poorer survival than ER + PR + tumours and had comparable poor survival than TN tumours. It has been recently shown that PgR is not merely an ER α -induced gene target, but is also an ER α -associated protein that modulates its behaviour [33]. PR functions as a molecular rheostat to control ER α chromatin binding and transcriptional activity. Our findings call for the necessity of PgR determination which has important implications for prognosis and therapeutic interventions, as considering chemotherapy in the Luminal B BC population.

In conclusion, the addition of celecoxib to NAC was not associated with an increase of DFS nor OS in patients with *HER2*-negative locally advanced tumours. Our study emphasised the role of progesterone receptors and their importance in distinguishing luminal A from luminal B breast cancers with worst prognostic.

Patients with *HER2*-positive tumours treated with trastuzumab had a statistically higher DFS and OS than patients with *HER2*-negative tumours, highlighting how targeted anti-*HER2* treatment has improved the initial poor prognostic of these cancers. This is the first study comparing neoadjuvant to adjuvant trastuzumab. Both DFS and OS were similar in the patients who received neoadjuvant versus adjuvant trastuzumab. It is not unlikely that adjuvant trastuzumab, compensate the lack of an early trastuzumab. Finally, in *HER2*-positive patients, only the pCR in the axilla was associated with a poor DFS. These patients with no pCR after NAC remain at high risk of relapse despite adjuvant trastuzumab treatment and should be considered for specific 'adjuvant-post neoadjuvant' studies.

Conflict of interest statement

jean Yves Pierga declared "Honoraria and research funding from Roche".the other authors declared no conflict of interest.

Funding

This work was funded by the Programme Hospitalier de Recherche Clinique: ISRCTN10059974, PHRC: AOM 02 11.

Sponsor

This work was sponsored by Roche Pharmaceutical, Pfizer Inc, France, and Sanofi Aventis.

Role of the funding sources

Academic fundings: Data manager and research technician salaries.

Industrial fundings: Provided drugs free of charge (Roche: trastuzumab, Pfizer: celecoxib, and Sanofi: taxotere).

Acknowledgement

This work has been presented as a poster at SABCS 2015 (Abstract number: P1-14-09).

References

- [1] Rastogi P, Anderson SJ, Bear HD, et al. Preoperative chemotherapy: updates of National Surgical Adjuvant Breast and Bowel Project Protocols B-18 and B-27. *J Clin Oncol* 2008;26:778–85.
- [2] Mauri D, Pavlidis N, Ioannidis JP. Neoadjuvant versus adjuvant systemic treatment in breast cancer: a meta-analysis. *J Natl Cancer Inst* 2005;97:188–94.
- [3] Mieog JS, van der Hage JA, van de Velde CJ. Neoadjuvant chemotherapy for operable breast cancer. *Br J Surg* 2007;94:1189–200.
- [4] Fisher CS, Ma CX, Gillanders WE, et al. Neoadjuvant chemotherapy is associated with improved survival compared with adjuvant chemotherapy in patients with triple-negative breast cancer only after complete pathologic response. *Ann Surg Oncol* 2012;19:253–8.
- [5] Ademuyiwa FO, Ellis MJ, Ma CX. Neoadjuvant therapy in operable breast cancer: application to triple negative breast cancer. *J Oncol* 2013;2013:219869.
- [6] Loibl S, Denkert C, von Minckwitz G. Neoadjuvant treatment of breast cancer—clinical and research perspective. *Breast* 2015;24(Suppl. 2):S73–7.
- [7] Cortazar P, Zhang L, Untch M, et al. Pathological complete response and long-term clinical benefit in breast cancer: the CTNeoBC pooled analysis. *Lancet* 2014;384:164–72.
- [8] Symmans WF, Peintinger F, Hatzis C, et al. Measurement of residual breast cancer burden to predict survival after neoadjuvant chemotherapy. *J Clin Oncol* 2007;25:4414–22.
- [9] von Minckwitz G, Untch M, Blohmer JU, et al. Definition and impact of pathologic complete response on prognosis after neoadjuvant chemotherapy in various intrinsic breast cancer subtypes. *J Clin Oncol* 2012;30:1796–804.
- [10] Pierga JY, Delaloge S, Espie M, et al. A multicenter randomized phase II study of sequential epirubicin/cyclophosphamide followed by docetaxel with or without celecoxib or trastuzumab according to HER2 status, as primary chemotherapy for localized invasive breast cancer patients. *Breast Cancer Res Treat* 2010;122:429–37.
- [11] Chevallier B, Roche H, Olivier JP, et al. Inflammatory breast cancer. Pilot study of intensive induction chemotherapy (FEC-HD) results in a high histologic response rate. *Am J Clin Oncol* 1993;16:223–8.
- [12] Skibinski A, Kuperwasser C. The origin of breast tumor heterogeneity. *Oncogene* 2015;34:5309–16.
- [13] Ignatiadis M, Sotiriou C. Luminal breast cancer: from biology to treatment. *Nat Rev Clin Oncol* 2013;10:494–506.
- [14] Gianni L, Eiermann W, Semiglazov V, et al. Neoadjuvant and adjuvant trastuzumab in patients with HER2-positive locally advanced breast cancer (NOAH): follow-up of a randomised controlled superiority trial with a parallel HER2-negative cohort. *Lancet Oncol* 2014;15:640–7.
- [15] Untch M, Loibl S, Bischoff J, et al. Lapatinib versus trastuzumab in combination with neoadjuvant anthracycline-taxane-based chemotherapy (GeparQuinto, GBG 44): a randomised phase 3 trial. *Lancet Oncol* 2012;13:135–44.
- [16] de Azambuja E, Holmes AP, Piccart-Gebhart M, et al. Lapatinib with trastuzumab for HER2-positive early breast cancer (Neo-ALTTO): survival outcomes of a randomised, open-label, multicentre, phase 3 trial and their association with pathological complete response. *Lancet Oncol* 2014;15:1137–46.
- [17] Gianni L, Pienkowski T, Im YH, et al. Efficacy and safety of neoadjuvant pertuzumab and trastuzumab in women with locally advanced, inflammatory, or early HER2-positive breast cancer (NeoSphere): a randomised multicentre, open-label, phase 2 trial. *Lancet Oncol* 2012;13:25–32.
- [18] Gianni L, Pienkowski T, Im YH, et al. 5-year analysis of neoadjuvant pertuzumab and trastuzumab in patients with locally advanced, inflammatory, or early-stage HER2-positive breast cancer (NeoSphere): a multicentre, open-label, phase 2 randomised trial. *Lancet Oncol* 2016;17:791–800.
- [19] Broglio KR, Quintana M, Foster M, et al. Association of pathologic complete response to neoadjuvant therapy in HER2-positive breast cancer with long-term outcomes: a meta-analysis. *JAMA Oncol* 2016;2:751–60.
- [20] Guarneri V, Broglio K, Kau SW, et al. Prognostic value of pathologic complete response after primary chemotherapy in relation to hormone receptor status and other factors. *J Clin Oncol* 2006;24:1037–44.
- [21] Pierga JY, Mouret E, Dieras V, et al. Prognostic value of persistent node involvement after neoadjuvant chemotherapy in patients with operable breast cancer. *Br J Cancer* 2000;83:1480–7.
- [22] Mamsang-Kitizis H, Chaltier L, Belin L, et al. Beyond axillary lymph node metastasis, BMI and menopausal status are prognostic determinants for triple-negative breast cancer treated by neoadjuvant chemotherapy. *PLoS One* 2015;10:e0144359.
- [23] Gonzalez-Angulo AM, McGuire SE, Buchholz TA, et al. Factors predictive of distant metastases in patients with breast cancer who have a pathologic complete response after neoadjuvant chemotherapy. *J Clin Oncol* 2005;23:7098–104.
- [24] Bodilsen A, Offersen BV, Christiansen P, Overgaard J. Pattern of relapse after breast conserving therapy, a study of 1519 early breast cancer patients treated in the Central Region of Denmark 2000–2009. *Acta Oncol* 2016;55:964–9.
- [25] Fei F, Messina C, Slaets L, et al. Tumour size is the only predictive factor of distant recurrence after pathological complete response to neoadjuvant chemotherapy in patients with large operable or locally advanced breast cancers: a sub-study of EORTC 10994/BIG 1-00 phase III trial. *Eur J Cancer* 2015;51:301–9.
- [26] Hamy-Petit AS, Belin L, Mamsang-Kitizis H, et al. Pathologic complete response and prognosis after neoadjuvant chemotherapy for HER2-positive breast cancers before and after trastuzumab era: results from a real-life cohort. *Br J Cancer* 2016;114:44–52.
- [27] Giacchetti S, Porcher R, Lehmann-Che J, et al. Long-term survival of advanced triple-negative breast cancers with a dose-intense cyclophosphamide/anthracycline neoadjuvant regimen. *Br J Cancer* 2014;110:1413–9.

- 1 [28] Coates AS, Winer EP, Goldhirsch A, et al. Tailoring therapies—improving the management of early breast cancer: St. Gallen international expert consensus on the primary therapy of early breast cancer 2015. *Ann Oncol* 2015;26:1533–46. 12
- 2 13
- 3 14
- 4 15
- 5 [29] Senkus E, Kyriakides S, Penault-Llorca F, et al. Primary breast cancer: ESMO clinical practice guidelines for diagnosis, treatment and follow-up. *Ann Oncol* 2013;24(Suppl. 6). vi7–23. 16
- 6 17
- 7 [30] Prat A, Cheang MC, Martin M, et al. Prognostic significance of progesterone receptor-positive tumor cells within immunohistochemically defined luminal a breast cancer. *J Clin Oncol* 2013; 31:203–9. 18
- 8 19
- 9 [31] Sun JY, Wu SG, Li FY, et al. Progesterone receptor loss identifies hormone receptor-positive and HER2-negative breast cancer subgroups at higher risk of relapse: a retrospective cohort study. *Onco Targets Ther* 2016;9:1707–13. 20
- 10 [32] Bae SY, Kim S, Lee JH, et al. Poor prognosis of single hormone receptor- positive breast cancer: similar outcome as triple-negative breast cancer. *BMC Cancer* 2015;15:138. 21
- 11 [33] Mohammed H, Russell IA, Stark R, et al. Progesterone receptor modulates ERalpha action in breast cancer. *Nature* 2015;523: 313–7. 22

UNCORRECTED PROOF

1 **Title**2 ***BIRC5* (survivin): a pejorative prognostic marker in stage II/III breast cancer with no**
3 **response to neoadjuvant chemotherapy**4 AS Hamy¹, I Bieche², J Lehmann-Che³, V Scott⁴, Ph Bertheau⁵, JM Guinebretière⁶, MC Matthieu⁷, B
5 Sigal-Zafrani⁸, O Tembo⁹, M Marty⁹, B Asselain¹, *F Spyrtatos², *P de Cremoux³

6

7 1 Department of Biostatistics, Institut Curie, Paris, France

8 2. Department of Genetics, Pharmacogenomics Unit, Institut Curie, Paris, France

9 3. APHP Molecular oncology Unit and Paris Diderot University, Hôpital Saint Louis, Paris, France

10 4. Biology Department, Institut Gustave Roussy, Villejuif, France.

11 5. APHP Pathology Department and Paris Diderot University, Hôpital Saint Louis, Paris, France

12 6. Pathology Department, Hôpital René-Huguenin, Institut Curie, Saint-Cloud, France.

13 7. Pathology Department, Institut Gustave Roussy, Villejuif, France.

14 8. Pathology Department, Institut Curie, Paris, France

15 9. Centre for Therapeutic Innovation, Saint-Louis Hospital, Paris, France

16

17 * Contributed equally to the study

18

19 Corresponding author: Dr P de Cremoux, Molecular Oncology unit, Hôpital Saint Louis, 1 avenue
20 Claude Vellefaux, 75010 Paris, France. patricia.de-cremoux@aphp.fr

21

22 **Key words:** breast cancer, neoadjuvant chemotherapy, predictive, *BIRC5*

23

24 **Abbreviations**

25 ER Estrogen receptor

26 HER2 Human epidermal growth factor receptor 2

27 DFS Disease-free survival

28 ER Estrogen receptor

29 NAC neoadjuvant chemotherapy

30 pCR pathological complete response

31 PgR Progesterone receptor

32

33

34 **Abstract**

35 Neoadjuvant systemic therapy (NAC) is currently used in the treatment of stage II/III breast
36 cancer. Pathological complete response as a surrogate endpoint for clinical outcomes is not
37 completely validated for all subgroups of breast cancers. Therefore, there is a need for reliable
38 predictive tests of the most effective treatment.

39 We used a combination of predictive clinical, pathological and gene-expression-based
40 markers of response to NAC in a prospective phase II multicentre randomized clinical trial in
41 breast cancer patients, with a long follow up (8 years). This study concerned the
42 subpopulation of 188 patients with similar levels of pathological response rates to sequential
43 epirubicin/cyclophosphamide and docetaxel to determine predictive marker of pCR and DFS.
44 We used a set of 45 genes selected from high throughput analysis and a standardized RT-
45 qPCR. We analyzed the predictive markers of pathological complete response (pCR) and DFS
46 in the overall population and DFS the subpopulation of 159 patients with no pCR.

47 In the overall population, combining both clinical and genomic variables, large tumor size,
48 low TFF1 and MYBL2 overexpression were significantly associated with pCR. T4 Stage,
49 lymphovascular invasion, negative PR status, histological type and high values of CCNB1
50 were associated with DFS. In the no pCR population, only lymphovascular invasion and high
51 values of BIRC5 were associated with DFS.

52

53 We confirm the importance of ER-related and proliferation genes in the prediction of pCR in
54 NAC-treated breast cancer patients. Furthermore, we identified BIRC5 (survivin) as a main
55 pejorative prognostic factor in patients with breast cancers with no pCR. These results also
56 open perspective for predictive markers of new targeted therapies.

57 **Introduction**

58 Breast cancer is a heterogeneous disease with regard to clinicopathological features,
59 biological behavior, molecular profiles, responses to treatment, and prognosis [1].

60 Neoadjuvant chemotherapy (NAC) is currently used in patients with early-stage and advanced
61 disease [2,3,4]. Its clinical benefits are: a) higher rates of breast-conserving surgery, b) similar
62 prognoses for breast cancer patients receiving a neoadjuvant and for those receiving an

63 adjuvant therapy regimen, c) a body of evidence showing that the achievement of a
64 pathological complete response (pCR) after neoadjuvant chemotherapy is associated with a
65 good prognosis in specific subgroups (triple-negative, HER2-positive) [5-6]. However, a
66 pCR is observed in only 10% to 25% of patients depending of the phenotype of the tumor,
67 and inversely, around 75 to 90% of patients do not respond. Consequently, there is a clinical
68 need for reliable predictive tests of the most effective treatment. Although some clinico-
69 pathological characteristics (e.g. estrogen receptor-negative status, high histological grades
70 and high proliferative status) and the simplified immuno-histochemical classification
71 according to intrinsic molecular subgroups [7-10], have already been demonstrated to be
72 associated with the sensitivity to chemotherapy, more precise classifications are required,
73 because intrinsic tumor sensitivity, for the same stage of disease and the same drug, may
74 differ according to tumor phenotype.

75 The pre-therapeutic breast cancer core biopsies provide naive tumor tissue and a basis for the
76 analysis of predictive biologic factors to identify patients either that will benefit from
77 chemotherapy but also to identify predictive marker of resistance to a chemotherapy regimen.

78 The present study aimed to identify predictive clinical, pathological and biological markers of
79 response to neoadjuvant chemotherapy and DFS in the context of a prospective randomized
80 clinical trial, the REMAGUS-02 trial (RO2), with a long follow up. The RO2 trial was a
81 phase II multicentre randomized trial designed to assess antitumor efficacy of sequential
82 epirubicin/cyclophosphamide followed by docetaxel in stage II and III operable breast cancer
83 patients +/- celecoxib for HER2- patients and +/- trastuzumab for HER2+ [11 This study
84 concerned the subpopulation of 188 patients with similar levels of pathological response rates
85 to NAC, (without neoadjuvant trastuzumab). We previously defined the optimal pre-analytical
86 conditions of samples treatment, quality criteria for RNA integrity assessment, calibration and
87 standardization of RT-qPCR to be able to analyze the multicentre samples with high accuracy

88 (de Cremoux, 2011). Two others publications (Spyratos et al, 2012; Valet et al, 2013)
89 concerned transcriptome analysis, ie high throughput data of unselected genes, analyzed on
90 the same samples. In the current study, we used a set of 45 genes selected from our previous
91 works and literature data, and a standardized RT-qPCR approach for the analysis of the
92 mRNA expression of genes to determine predictive markers of pCR. We also analyzed the
93 predictive markers of DFS in the overall population and in the sub population of 159 patients
94 with no pCR.

95

96 **Patients and Methods**

97 Patients

98 The phase II randomized REMAGUS-02 (R02) trial included 340 patients from 4 different
99 institutions, as previously described [11]. [The period of inclusion: was from May 2004 to](#)
100 [October 2007](#)). All patients received neoadjuvant chemotherapy for operable and locally
101 advanced breast cancer. After four cycles of epirubicin (75mg/m²)–cyclophosphamide
102 (750mg/m²), patients were randomized to four cycles of docetaxel (100mg/m²) with or
103 without celecoxib (400mg twice daily orally) for patients with HER2-negative tumors (arms
104 A and B, respectively), and docetaxel with or without trastuzumab (8mg/kg then 6mg/kg
105 every 3 weeks) for patients with HER2-positive tumors (arms C and D, respectively).
106 Adjuvant trastuzumab for a total of 18 three-weekly infusions was given for HER2-positive
107 cancer patients, starting either before surgery combined with docetaxel (arm C), or only after
108 surgery (arm D).

109 All patients were informed and prospectively gave their signed consent to participate in the
110 trial and ancillary studies, (ISRCTN 10059974, French ethics committee Paris-Bicêtre, n°03-
111 55).

112 The primary objective was pathological complete response (pCR), evaluated according to
113 Chevallier's criteria [12]. Secondary objectives were to define genomic profiles of success or
114 failure for response to chemotherapy (pCR / no pCR) and of survival for each type of
115 treatment.

116 Because of the known major impact of neoadjuvant trastuzumab on pCR rates in HER2-
117 positive tumors, we chose to study in the current work, the subpopulation of 188 patients with
118 adequate quality control criteria [13,14] and with similar levels of pathological response rates
119 [11]: arms A and B (both HER2-) and arm D (HER2+ without trastuzumab in the
120 neoadjuvant setting), with 11%, 16%, and 17% pCR respectively. We did not take into
121 account patients of group C ((HER2+ with trastuzumab in the neoadjuvant setting), that had
122 28% pCR.

123

124 **Tissue samples**

125 Frozen biopsy samples were processed under RNase-free conditions, as previously described
126 in detail [13,14]. Briefly, tumor cellularity was evaluated on frozen sections of biopsies
127 dedicated to RNA extraction by breast pathologists. Only biopsies with more than 30%
128 invasive epithelial tumor cells were analyzed. Total RNA was extracted from biopsies using a
129 TRIzol method followed by RNA clean-up using the NucleoSpin RNA II kit (Macherey
130 Nagel, Hoerdt, France). Evaluation of RNA integrity was performed on the Agilent
131 Bioanalyzer 2100 microfluidics-based platform using the RNA 6000 Nano Lab Chips kit
132 (Agilent, Santa Clara, CA, USA). Only samples with a RIN (RNA Integrity Number) greater
133 than 6 were analyzed. One μg total RNA was kept for RT-qPCR. All samples were tested for
134 albumin DNA contaminants using an intronic albumin gene design in qPCR. No amplification
135 of albumin DNA was observed in our series of samples ($\text{Ct} > 35$). Total RNA of human breast

136 cancer cell lines T47D and MDA-MB 231 were used to calibrate reverse transcription and
137 standardize real-time PCR.

138 **Real-time RT-qPCR analysis**

139 The list of selected genes belong to the main well-known pathways in breast cancer ie
140 estrogen receptor, growth factors/HER2 and proliferation pathways [15,16]. We also included
141 the classical genes considered in luminal (equivalent to ER pathway) and basal classification
142 [1]. We also included proteases genes while it is known that RNA expression of proteases is
143 less powerful than protein proteases expression to predict outcome [17,18]. Topoisomerase II,
144 MAPTau were added as target of chemotherapy (anthracyclines and taxanes [19,20]
145 PTGS2/COX2 was included because this may be a target for the celecoxib arm of the HER2
146 negative arm of the R02 trial [21]. ~~CD24 and CD 44 were included as markers of stem cells
147 and CD68 for macrophages.~~ Je propose de supprimer cette phrase car il faudrait des ref pour
148 tous les gènes non cités avant. On peut considérer qu'ils sont inclus dans la phrase suivante.
149 The Remagus 02 working group collegially discussed the definite choice of the limited list of
150 genes during the design of the trial and the ancillaries' studies.

151 First-strand cDNA synthesis was performed with 1µg total RNA using Superscript II Reverse
152 Transcriptase (Invitrogen Corporation) in a final volume of 20 µL, as previously described [6-
153 8]. Quantitative PCR analysis was performed on 6.25 ng cDNA in duplicate. A 5 µL diluted
154 sample of cDNA (6.25ng) was added to 10 µL of the PCR mix. The thermal cycling
155 conditions comprised an initial denaturation step at 95°C for 10 min, 45 cycles at 95°C for 15
156 sec, and annealing temperature, either 60°C or 65°C depending on the target, for 1 min.

157 All PCR reactions were performed using the ABI Prism 7900 Sequence Detection System
158 (Applied Biosystems Inc., Foster City, USA). The PCR Core reagent kit was used for
159 systems with Taqman probes (Eurogentec, Liège, Belgium), and the Power SYBR Green PCR
160 master Mix (Applied Biosystems Inc.) was used for systems without Taqman probe. Primers
161 and fluorescent probes were designed from published sequences using Primer express

162 software (Applied Biosystems Inc.). BLASTN searches against dbEST and nr (the
163 nonredundant set of the GenBank sequence database) were performed to confirm the total
164 gene specificity of the chosen nucleotide sequences and the absence of DNA polymorphisms.
165 Target sequences were 60-120 long. Forty-five cancer-related target genes (Table 1.
166 supplementary data) involved in the main signaling pathways associated with in breast cancer
167 development were studied (nucleotide and probe sequences available on request). *RPLPO*,
168 TATA Box binding protein (*TBP*), transferrin receptor (*TFR*), beta-actin, beta-glucuronidase
169 (*GUS*), and *GAPDH* were used as endogenous reference genes. Transferrin receptor-TFRC-5'
170 (Hs00951086_m1), *GAPDH*-5' (Hs99999905_m1) and *GUSB*-3' (Hs99999908_m1) were
171 obtained as Assays-on-Demand from Applied Biosystems. Human breast cancer cell lines
172 T47D and MDA-MB 231 cDNA were used to generate 8 points standard curves for each
173 gene. Target quantities were normalized to each of the reference genes and to the median of
174 the 6 reference genes and calibrated using the second point of each standard curve. Final
175 results were expressed as N-fold differences in target gene expression relative to the reference
176 genes and the calibrator and are expressed as:

$$177 E_{\text{target}}^{(Ct_{\text{calibrator}} - Ct_{\text{sample}})} / E_{\text{reference gene}}^{(Ct_{\text{calibrator}} - Ct_{\text{sample}})},$$

178 where E is the efficiency of PCR measured using the slope of the calibration curve, and Ct is
179 the cycle threshold.

180 No reverse-Transcription Controls (NTC) were included in each batch of samples. Only cases
181 with exploitable data obtained for the 6 reference genes and the 45 target genes were
182 submitted to further statistical analysis, i.e.

183 Samples needed to have linear amplification of the house keeping genes from 1/40 to 1/200
184 dilutions. NTC had to be greater than 40 in all cases. High RT-qPCR efficiencies (>90%) had
185 to be found for each gene transcript.

186 **Statistical analysis**

187 Clinico- pathological data (age, menopausal status, clinical tumor size and clinical nodal
188 status, histological type, grade, presence of lymphovascular invasion, Estrogen receptor status

189 (ER), Progesterone receptor status (PR), HER2 status were analyzed by classes as presented
190 in the different Tables.

191 Genes were first grouped into biological pathways categories (Estrogen Receptors genes
192 group, proliferation genes group, growth factor receptors genes group, basal genes group,
193 protease genes group, other genes). As no consensual threshold with RT-qPCR, analyses, and
194 [to ensure the robustness of the results](#), we chose to split the gene expression according to the
195 median (low versus high) or to the tertiles (low, intermediate, high). [The choice of analyzing
196 genes according to the tertiles or the median was based on the biological knowledge of the
197 gene function. For the genes analyzed by tertiles, we chose to group the two consecutive
198 tertiles with odds ratios for pCR of similar magnitude and we analyzed them versus the third
199 one.](#) If the three relative risks showed a continuous increase or decrease, suggesting a linear
200 association, we chose to analyze the genes within this family cutting them at the median
201 value.

202 To analyze the association between clinical, pathological, genomic variables and pCR, we
203 performed a univariate analysis using the chi-square test and a univariate logistic regression
204 model to estimate odds Ratios (OR) and their 95% confidence intervals.

205 DFS was defined as the time from surgery to death, loco-regional recurrence, or distant
206 recurrence, whichever occurred first. Patients for whom none of these events were recorded
207 were censored at the date of their last known contact. Survival probabilities were estimated by
208 the Kaplan–Meier method, and survival curves were compared with log-rank tests. Hazard
209 ratios and their associated 95% confidence intervals were calculated with the Cox
210 proportional hazard model.

211 For the multivariate analyses of gene expression, given the strong correlations [of most of the
212 genes](#) within a biological family ([data not shown](#)), we [chose to retain](#) retained in each family
213 the gene significantly associated to pCR or DFS with the lowest p -value. When no gene was

214 correlated to pCR or DFS with a *p*-value for the likelihood ratio test higher than 0.1, this
215 family was not retained. Variables with a *p*-value for the likelihood ratio test ≤ 0.10 in
216 univariate analysis were included in the multivariate model. Forward selection was used to
217 establish the final multivariate model.

218 To analyze the association between clinical, pathological and gene expression parameters and
219 pCR and DFS, we first analyzed separately clinico-pathological and genomic data, which
220 resulted in two different multivariate models (one “clinical model” and one “genomic”
221 model), finally, a mixed model was implemented after pooling clinico-pathological covariates
222 and genomic variables selected after the first two steps of the statistical analysis (final model).
223 Clinico-pathological variables were forced to be included in the final model irrespective of the
224 *p*-value for the association, and genomic variables were added to the model using a forward
225 selection procedure.

226 The cutoff date for the DFS analysis was May 1st 2015. The median follow-up was: 94.5
227 months, (range: 19.9-127 months).

228 Survival analyses were performed in the overall population and were also performed in the
229 “no pCR” population.

230 In this manuscript, we included the essential elements of "Reporting recommendations for
231 tumor marker prognostic studies (REMARK)" [22].

232

233 **Results**

234 **Patients' population**

235 Among the 188 patients from the arms A, B and D of Remagus 02 trial, 27 had complete
236 pathological response (14.4%) and 66 patients presented an event. Patients characteristics
237 were similar between this substudy and the whole population included in the REMAGUS02
238 trial regarding age, menopausal status, body mass index, clinical tumor size and nodal status,

239 ER, PR status. There was significantly less *HER2*-positive tumors (19.1% versus 54.6%
240 respectively, $p < 0.001$) in the studied subpopulation than in the remaining patients of the trial,
241 because patients from arm C who received neoadjuvant trastuzumab were excluded from the
242 current study.

243 For DFS analysis, 186 patients had available data. Among the 159 patients with no complete
244 pathological response and DFS data available, 56 patients presented an event. The
245 characteristics of the population according to the pathological complete response are
246 described in [Table 1](#). The occurrence of a pathological complete response was significantly
247 associated with a higher clinical tumor size, higher tumor grade and negativity of hormone
248 receptor status.

249

250 **Results of gene expression analyzed by RT-qPCR**

251 All RT-qPCR data were presented using the median of the housekeeping genes. Mean Ct
252 values were comprised between 20 and 27 for the majority of genes, and between 31 and 33
253 for *KRT 5*, *KRT17*, *PTGS2*, *TERT*, *PROM1* and *SERPINB5*. Forty-four of the 45 selected
254 genes were considered to be expressed in all samples; *GSTM1* was undetectable ($Ct > 35$) in
255 22% of samples. ([suppl table](#))

256

257 **Genes associated with the complete pathological response on the overall population**

258 The genomic variables in each pathway category were analyzed by univariate analysis.
259 Results are presented in supplemental Table 1. Only the most significant gene of each
260 subgroup (in bold) was selected for further multivariable analysis. High levels of proliferation
261 genes (*MKI67*, *AURKA*, *BIRC5*, *MYBL2*, *E2F1*, *TTK*) were strongly correlated with pCR. The
262 association between *MYBL2* expression and pCR reached the lowest p -value. In contrast, high
263 levels of genes of the oestrogen receptor pathway (*ESR1*, *PGR*, *SCUBE2*, *GATA3*, *FOXA1*,
264 *TTFI*), of *MAPT*, and *BCL2* were inversely correlated with pCR. *ERBB2* was not related to

265 pCR. High levels of the proteases *CTSL2* and *MMP9* were associated with pCR, as were high
266 levels of *PROM1*, *VEGF*, *HIF1 α* and *BRCA2*.

267

268 Table 2 represents the results of the multivariate analysis to predict pCR. In the first part of
269 the table, results present the model built with the clinical variables alone (clinical model,
270 Table 2a), then models were built with genomic variables alone (genomic model, Table 2b)
271 and the third part of the table shows the final model including both clinical and genomic
272 variables (Final model, Table 2c). Among clinical variables, high tumor size, low ER and PR
273 expression analyzed by IHC were associated with higher pCR. Among genomic variables,
274 higher pCR were observed in tumors with low *ESR1*-related genes represented by low *TFF1*
275 values and high proliferation- related genes represented by high *MYBL2* values and lower
276 *MAPT/tau* expression. Combining both clinical and genomic variables, only tumor size,
277 *TFF1* and *MYBL2* expression remains significantly associated with pCR.

278

279 **Genes predictive of the disease free survival in the overall population**

280 These results were obtained on 186 patients. After univariate analysis performed with clinical
281 variables, large tumor size, lobular histology, lymphovascular invasion and PR negativity,
282 were associated with a shorter DFS (Table 3). The pCR was not significantly predictive of
283 DFS in the overall population; however, pCR was predictive of longer DFS in the subgroup of
284 triple negative breast cancer patients (HR=0.28 [0.08-0.96]; p=0.03). After univariate analysis
285 with genomic variables, only high levels of proliferation's genes were correlated with a
286 shorter DFS (supplemental table 2). The two genes most significantly associated with DFS
287 were selected for further multivariable analysis (*BIRC5* and *CCNB1*).

288 Table 4a represents the results of the multivariate analysis of the clinical variables to predict
289 DFS. T3 and T4 stage, positive lymphovascular invasion, lobular histological subtype and

290 negative PR were associated with shorter DFS. Table 4b represents the results of the
291 multivariate analysis of genomic variables to predict DFS. Only high levels of *CCNBI* were
292 associated with a shorter DFS. In the final model including both clinical and genomic
293 variables (Table 4c) *CCNBI* remained associated with DFS in addition to stage of the disease,
294 lymphovascular invasion and histological type.

295 **Genes predictive of the disease free survival in patients with no pathological complete**
296 **response**

297 The same analyses were performed on the population of 159 patients with no pCR. After
298 univariate analysis of clinical variables, tumor size, histological subtype, lymphovascular
299 invasion and progesterone receptor status were associated with DFS (more T3, PR negative,
300 lobular or other subtypes with lymphovascular invasion had shorter DFS, Supplemental [Table](#)
301 [3](#)).

302 Supplemental [Table 4](#) represents the most significant genomic variables in each pathway
303 category selected by univariate analysis. We found that low levels of *ESR1* genes, high levels
304 of proliferation's genes mainly *BIRC5*, high level of the basal networks genes best
305 represented by *TRIM29* and low levels of protease gene *MMP11* and high levels of *TOP2A*
306 and *BRCA2* genes were correlated with shorter DFS.

307 Table 5a represents the results of the multivariate analysis of the clinical variables to predict
308 DFS. Patients with T stage 3 and 4, high tumor grade and lymphovascular invasion were
309 correlated with the shortest DFS. Table 5b represents the results of the multivariate analysis of
310 genomic variables to predict DFS. High levels of *BIRC5* and *TRIM29* predict shorter DFS in
311 the patients with no pCR. When clinicopathological variables were included in the
312 multivariate model using the same procedure (Table 5c), only lymphovascular invasion and
313 *BIRC5* levels remained significantly associated with DFS.

314

315 **Discussion**

316 This study was designed both to identify predictive clinical, pathological and biological
317 markers of response to chemotherapy and to identify predictive marker of DFS in the whole
318 population and, in subgroup of patients with no pCR, in the context of a prospective
319 randomized clinical trial [11]. We found that in neoadjuvant context, pCR and prognosis
320 prediction can be improved by adding a few genes selected after a biologically driven process,
321 to standard clinical and pathological variables. Indeed, few data have been reported to
322 determine prognostic biomarker in the context of no response to neoadjuvant chemotherapy
323 and particularly with such a long term follow up. [In addition, celecoxib treatment in the
324 HER-negative strate of the study, did not improve survival \(Giachetti et al, submitted\).](#)

325

326 Compared to a recent meta-analysis based on 11,695 subjects from 30 NAC eligible studies
327 from 2001 to 2005, our population is representative of a T2-T3 breast carcinoma particularly
328 in term of ER, PR, HER2 expression and pCR rate [23].

329 We used a set of genes selected from high throughput analysis that may have a biological role
330 in the response to treatment and genes involved in the major network activated in breast
331 carcinoma. They were then assembled from the literature [24,25]. We already published
332 qPCR quality control criteria for gene expression assessment [13,14].

333 In addition to the results of the whole population of patients with similar levels of
334 pathological response rate to NAC, we report results in the subpopulation of 159 patients with
335 no pCR. There is a great interest to identify clinical, pathological and biological factors
336 associated with a shorter DFS in this subgroup of patients, as it is known that patients with
337 pCR are very less likely to experience relapse than no pCR patients, notably in the TNBC and
338 the HER2-positive subgroup. Recently, a pooled analysis of 12 international neoadjuvant
339 clinical trials including 11.955 patients with breast carcinoma did not validate pCR as a
340 surrogate endpoint for improved EFS and DFS for all subtypes of breast carcinoma [2]. They

341 observed the strongest association between pCR and long-term outcome in patients with
342 aggressive breast cancer subtypes (triple negative; hormone-receptor-positive, high-grade, and
343 HER2-negative; and HER2-positive and hormone-receptor-negative).

344 In our study, we found an association between pCR and DFS only in the subgroup of triple
345 negative breast cancers patients. However, the discrepancy could be explained by the study
346 design (meta-analysis *versus* clinical trial), the small size of our series of patients and a longer
347 median follow up than in Cortazar's study [2] (near 8 years *versus* 5.40 years respectively).

348 As expected in this subpopulation of large breast carcinoma, we observed that tumor size and
349 hormone receptor status were the main determinants of the pathological response. We also
350 confirmed the major weight on pCR prediction, of a combined signature associating clinical
351 and genomic data, Trefoil factor 1 expression (*TFF1*, also known as pS2), from the ER
352 network, and *MYBL2*, from the proliferation network. In multivariate analysis, low level of
353 *TFF1*, and high level of *MYBL2* expression remained statistically significant in the prediction
354 of pCR. We previously demonstrated that probesets associated with *ESR1* provided one of
355 the best performances to predict pCR in a subgroup of patients' of the R02 trial, with
356 available material for transcriptome analysis [14,26]. Furthermore, the proliferation marker
357 network has been previously described as a predictive factor of response to neoadjuvant
358 chemotherapy either using transcriptomic analysis [27,28] and also genomic data from *in*
359 *silico* analysis [29,30].

360 In the subpopulation with no pCR, only two factors were predictive of a worse survival:
361 lymphovascular invasion and high level of *BIRC5*. Lymphovascular invasion is well known
362 factor of poor prognosis, ie breast cancer-specific survival and DFS, irrespective of the NAC
363 regimen [31]. To our knowledge, it is the first description of the prognostic role of *BIRC5*
364 expression after a long follow up in a large series of breast cancer patients that did not
365 respond to neoadjuvant chemotherapy and in addition, with a long follow up. *BIRC5* gene is

366 coding for survivin protein. Survivin is a member of the inhibitor of apoptosis (IAP) gene
367 family, which encodes negative regulatory proteins that prevent apoptotic cell death. It has a
368 dual role in regulation of cell division and apoptosis that are both involved in tumor
369 development [32]. It is not expressed in normal tissue and it is re-expressed in human cancer
370 cells at a frequency of 34-100% [33,34]. Prognostic and clinical significance of survivin in
371 breast cancers have been evaluated, mainly in the adjuvant setting and the results have been
372 published: overall, up- regulation of *BIRC5* is a frequent event in breast cancer, and its
373 expression in breast cancer tissue is significantly associated with a poor clinical outcome,
374 whether using immuno-histochemical analysis [35], quantitative analysis of the protein by
375 Elisa [36] or quantitative RT-PCR [37,38]. However, few studies did not find this prognostic
376 value. One retrospective study in 293 patients treated in an adjuvant setting, where only
377 nuclear staining of survivin was considered [39] and a small neoadjuvant study in 45 patients
378 treated by anthracyclins and taxane regimen, where the authors found a predictive value of
379 high levels of survivin for prediction of pCR, but no prognostic value [40]. However, in this
380 latter study, methodological concerns have been pointed out [41]. Finally, two recent meta
381 analyses [42,43] confirmed the prognostic role of survivin, revealing a high risk of recurrence
382 with higher survivin expressing tumors.

383 Furthermore, the impact of *BIRC5* (survivin) overexpression in the group of no pCR after
384 neoadjuvant chemotherapy not only is prognostic, but should also be theranostic. Recent
385 preclinical publications supported this hypothesis. Survivin overexpression may be
386 responsive to response to therapy, based on preclinical studies using organotypic human
387 breast tumors for doxorubicin treatment [44]. Preclinical *in vitro* study on TNBC cell line
388 (MDA-MB-231) demonstrated that repressing *BIRC5* expression by siRNA could
389 significantly inhibit the proliferation of TNBC cell lines [45]. Moreover, *in vitro* drug
390 sensitivity of breast cancer cell lines was restored by the decrease of survivin expression

391 induced by mir218 targeting [46]. Interestingly, a role for survivin in cancer cell protection
392 from DNA damage has been recently shown [47]. These interesting preclinical data, showing
393 the role for survivin in endogenous DNA damage repair by homologous recombination in
394 human breast cancer cell lines, were confirmed by the analysis of publically available
395 database from primary breast tumors. The authors showed a positive correlation between the
396 level of expression of *BIRC5* and other genes involved in HR pathways, namely *EME1*,
397 *RAD51* or *EXO1* mRNA, and that, high expression of these genes was independently
398 associated with pejorative prognostic. Finally, more recently, survivin has been discussed as a
399 potential target for cancer therapy with different promising therapeutic strategies, including a
400 phase II study in breast cancer using YM155, a small molecular suppressor of survivin
401 [48,49,50]. Interestingly, *BIRC5* gene is one of the common genes included in Oncotype
402 DX®, Endopredict® and PAM50, breast cancer signatures [51,52,53]. Our results reinforced
403 the need to identify biomarkers that could help to select patients who might benefit of specific
404 inhibitors of survivin.

405

406 In conclusion, we confirm the importance of ER-related and proliferation genes in the
407 prediction of pCR in breast cancer patients treated in neoadjuvant setting. We identified
408 survivin as a main pejorative prognostic factor in patients with breast cancers who did not
409 respond to neoadjuvant chemotherapy. As the survivin pathway seems potentially accessible
410 to drugs, these results open new thrilling perspective for not only prognostic, but also
411 predictive markers of new targeted therapies. If these results were confirmed, it would open a
412 way to modify the clinical course of breast cancer not responding to chemotherapy currently
413 associated with a poor prognosis. Following NAC, it is known that no pCR patients are at a
414 high risk of relapse. In the presence of poor prognostic factors, such as lymphovascular

415 invasion / high-grade tumors/ PR negativity, these patients could be enrolled in second line
416 clinical trials stratified on BIRC5 status. BIRC5-low patients would be allocated to
417 observation whereas BIRC5-high patients would be randomized to either observation or drugs
418 targeting BIRC5.

419

420

421 **Conflict of interest statement**

422 The authors declare that they have no competing interest.

423 **Acknowledgments**

424 We thank the members of the Remagus02 group (S Delalogue, M Espié, S Giacchetti, E Brain, JY Pierga) for
425 their contribution to this work. We thank K Tran-Perennou, C Barbaroux and S Vacher for their helpful technical
426 contribution. We thank Y de Rycke for his expert biostatistics contribution and helpful discussions. This work
427 was supported by Academic Grants (PHRC AOM/ 2002/02117) and Industrial Grants from Pfizer Inc., Roche,
428 Sanofi- Aventis ISRCTN100599. AS Hamy-Petit was supported by an ITMO-INSERM-AVIESAN cancer
429 translational research grant.

430

431 **References**

- 432 1. Sotiriou C, Wirapati P, Loi S, Harris A, Fox S et al (2006) Gene expression profiling in breast cancer:
433 272.
- 434 2. Fisher B, Bryant J, Wolmark N, Mamounas E, Brown A et al (1998) Effect of preoperative
435 chemotherapy on the outcome of women with operable breast cancer. *J Clin Oncol* 16:2672-2685.
- 436 3. Smith IC, Heys SD, Hutcheon AW, Miller ID, Payne S et al (2002) Neoadjuvant chemotherapy in
437 breast cancer: significantly enhanced response with docetaxel. *J Clin Oncol* 20:1456-1466.
- 438 4. Funt SA, Chapman PB (2016) The Role of Neoadjuvant Trials in Drug Development for Solid Tumors.
439 *Clin Cancer Res* 22:2323-2328.
- 440 5. Cortazar P, Zhang L, Untch M, Mehta K, Costantino JP et al (2014) Pathological complete response
441 and long-term clinical benefit in breast cancer: the CTNeoBC pooled analysis. *Lancet* 384:164-172.

- 442 6. Berruti A, Amoroso V, Gallo F, Bertaglia V, Simoncini E et al (2014) Pathologic complete response as
443 a potential surrogate for the clinical outcome in patients with breast cancer after neoadjuvant therapy: a
444 meta-regression of 29 randomized prospective studies. *J Clin Oncol* 32:3883-3891.
- 445 7. Darb-Esfahani S, Loibl S, Müller BM, Roller M, Denkert C et al (2009) Identification of biology-based
446 breast cancer types with distinct predictive and prognostic features: role of steroid hormone and HER2
447 receptor expression in patients treated with neoadjuvant anthracycline/taxane-based chemotherapy.
448 *Breast Cancer Res*. 11:R69.
- 449 8. Li XR, Liu M, Zhang YJ, Wang JD, Zheng YQ, et al (2011) CK5/6, EGFR, Ki-67, cyclin D1, and
450 nm23-H1 protein expressions as predictors of pathological complete response to neoadjuvant
451 chemotherapy in triple-negative breast cancer patients. *Med Oncol* 28 1:S129-134.
- 452 9. Lips EH, Mulder L, de Ronde JJ, Mandjes IA, Vincent A et al (2012) Neoadjuvant chemotherapy in
453 ER+ HER2- breast cancer: response prediction based on immunohistochemical and molecular
454 characteristics. *Breast Cancer Res Treat* 131:827-836.
- 455 10. von Minckwitz G, Fontanella C (2013) Selecting the neoadjuvant treatment by molecular subtype: how
456 to maximize the benefit? *Breast* 22 Suppl 2:S149-151.
- 457 11. Pierga JY, Delaloge S, Espié M, Brain E, Sigal-Zafrani B, et al (2010) A multicenter randomized phase
458 II study of sequential epirubicin/cyclophosphamide followed by docetaxel with or without celecoxib or
459 trastuzumab according to HER2 status, as primary chemotherapy for localized invasive breast cancer
460 patients. *Breast Cancer Res Treat* 122:429-437.
- 461 12. Chevallier B, Roche H, Olivier JP, Chollet P, Hurteloup P (1993) Inflammatory breast cancer Pilot
462 study of intensive induction chemotherapy (FEC-HD) results in a high histologic response rate. *Am J*
463 *Clin Oncol* 16:223-228.
- 464 13. de Cremoux P, Valet F, Gentien D, Lehmann-Che J, Scott V, et al (2011) Importance of pre-analytical
465 steps for transcriptome and RT-qPCR analyses in the context of the phase II randomised multicentre
466 trial REMAGUS02 of neoadjuvant chemotherapy in breast cancer patients. *BMC Cancer* 11:215.
- 467 14. Valet F, de Cremoux P, Spyrtos F, Servant N, Dujaric ME, et al (2013) Challenging single- and multi-
468 probesets gene expression signatures of pathological complete response to neoadjuvant chemotherapy
469 in breast cancer: experience of the REMAGUS 02 phase II trial. *Breast* 22:1052-1059.
- 470 15. Weigel MT, Dowsett M (2010) Current and emerging biomarkers in breast cancer: prognosis and
471 prediction. *Endocr Relat Cancer* 17:R245-262.
- 472 16. Toss A, Cristofanilli M (2015) Molecular characterization and targeted therapeutic approaches in breast
473 cancer. *Breast Cancer Res* 17:60.
- 474 17. Lankelma JM, Voorend DM, Barwari T, Koetsveld J, Van der Spek AH, et al (2010) Cathepsin L,
475 target in cancer treatment?. *Life Sci*. 86:225-233.
- 476 18. Ren F, Tang R, Zhang X, Madushi WM, Luo D, et al (2015) Overexpression of MMP Family Members
477 Functions as Prognostic Biomarker for Breast Cancer Patients: A Systematic Review and Meta-
478 Analysis. *PLoS One*.10:e0135544.
- 479 19. Smoter M, Bodnar L, Duchnowska R, Stec R, Grala B, et al (2011) The role of Tau protein in resistance
480 to paclitaxel. *Cancer Chemother Pharmacol* 68:553-557.
- 481 20. Brase JC, Schmidt M, Fischbach T, Sültmann H, Bojar H, et al (2010) ERBB2 and TOP2A in breast
482 cancer: a comprehensive analysis of gene amplification, RNA levels, and protein expression and their
483 influence on prognosis and prediction. *Clin Cancer Res*. 16:2391-2401.
- 484 21. Wang D, Dubois RN (2004) Cyclooxygenase-2: a potential target in breast cancer. *Semin Oncol* 31:64-
485 73.

- 486
487 22. McShane LM, Altman DG, Sauerbrei W, Taube SE, Gion M, et al (2006) REporting recommendations for tumor MARKer prognostic studies (REMARK). *Breast Cancer Res Treat.* 100:229-235.
- 488
489 23. Houssami N, Macaskill P, von Minckwitz G, Marinovich ML, Mamounas E (2012) Meta-analysis of
490 the association of breast cancer subtype and pathologic complete response to neoadjuvant
chemotherapy. *Eur J Cancer* 48:3342-3354.
- 491
492 24. Whitfield ML, George LK, Grant GD, Perou CM (2006) Common markers of proliferation. *Nat Rev
Cancer* 6:99-106.
- 493 25. Comprehensive molecular portraits of human breast tumours (2012) *Nature* 490:61-70.
- 494
495 26. Spyrtos F, Valet F, Bièche I, Scott V, Lehmann-Che J, et al (2012) Comments on the use of a single or
496 multiple probeset approach for microarray-based analyses of routine molecular markers in breast
cancer. *Breast Cancer Res Treat* 134:443-448.
- 497
498 27. Gianni L, Zambetti M, Clark K, Baker J, Cronin M, et al (2005) Gene expression profiles in paraffin-
499 embedded core biopsy tissue predict response to chemotherapy in women with locally advanced breast
cancer. *J Clin Oncol* 23:7265-7277.
- 500
501 28. Tordai A, Wang J, Andre F, Liedtke C, Yan K, et al (2008) Evaluation of biological pathways involved
in chemotherapy response in breast cancer. *Breast Cancer Res*10:R37.
- 502
503 29. Witkiewicz AK, Balaji U, Knudsen ES (2014) Systematically defining single-gene determinants of
response to neoadjuvant chemotherapy reveals specific biomarkers. *Clin Cancer Res* 20:4837-4848.
- 504
505 30. Liu R, Lv QL, Yu J, Hu L, Zhang LH, et al (2015) Correlating transcriptional networks with
506 pathological complete response following neoadjuvant chemotherapy for breast cancer. *Breast Cancer
Res Treat* 151:607-618.
- 507
508 31. Liu YL, Saraf A, Lee SM, Zhong X, Hibshoosh H, et al. Lymphovascular invasion is an independent
509 predictor of survival in breast cancer after neoadjuvant chemotherapy. *Breast Cancer Res Treat.* 2016
May 25.
- 510
511 32. Ambrosini G, Adida C, Altieri DC (1997) A novel anti-apoptosis gene, survivin, expressed in cancer
and lymphoma. *Nat Med* 3:917-921.
- 512 33. Altieri DC (2008) New wirings in the survivin networks. *Oncogene* 27:6276-6284.
- 513
514 34. Tanaka K, Iwamoto S, Gon G, Nohara T, Iwamoto M, et al (2000) Expression of survivin and its
relationship to loss of apoptosis in breast carcinomas. *Clin Cancer Res* 6:127-134.
- 515
516 35. Hinnis AR, Lockett JC, Walker RA (2007) Survivin is an independent predictor of short-term survival
in poor prognostic breast cancer patients. *Br J Cancer* 96:639-645.
- 517
518 36. Ryan BM, Konecny GE, Kahlert S, Wang HJ, Untch M, et al (2006) Survivin expression in breast
519 cancer predicts clinical outcome and is associated with HER2, VEGF, urokinase plasminogen activator
and PAI-1. *Ann Oncol* 17:597-604.
- 520
521 37. Yamashita S, Masuda Y, Kurizaki T, Haga Y, Murayama T, et al (2007) Survivin expression predicts
early recurrence in early-stage breast cancer. *Anticancer Res* 27:2803-2808.
- 522
523 38. Span PN, Sweep FC, Wiegerinck ET, Tjan-Heijnen VC, Manders P, et al (2004) Survivin is an
524 independent prognostic marker for risk stratification of breast cancer patients. *Clin Chem* 50:1986-
1993.
- 525
526 39. Kennedy SM, O'Driscoll L, Purcell R, Fitz-Simons N, McDermott EW, et al (2003) Prognostic
importance of survivin in breast cancer. *Br J Cancer* 88:1077-1083.

- 527
528
529 40. Petrarca CR, Brunetto AT, Duval V, Brondani A, Carvalho GP, et al (2011) Survivin as a predictive biomarker of complete pathologic response to neoadjuvant chemotherapy in patients with stage II and stage III breast cancer. *Clin Breast Cancer* 11:129-134.
- 530
531 41. Span PN, Tjan-Heijnen VC, Sweep FC (2007) Is survivin expression nevertheless related to disease outcome in breast cancer? *Breast Cancer Res Treat* 103:109.
- 532
533 42. Li Y, Ma X, Wu X, Liu X, Liu L (2014) Prognostic significance of survivin in breast cancer: meta-analysis. *Breast J* 20:514-524.
- 534
535 43. Song J, Su H, Zhou YY, Guo LL (2013) Prognostic value of survivin expression in breast cancer patients: a meta-analysis. *Tumour Biol* 34:2053-2062.
- 536
537
538 44. Favarsani A, Vaira V, Moro GP, Tosi D, Lopergolo A, et al (2014) Survivin family proteins as novel molecular determinants of doxorubicin resistance in organotypic human breast tumors. *Breast Cancer Res* 16:R55.
- 539
540
541 45. Wang S, Wang L, Chen M, Wang Y (2015) Gambogic acid sensitizes resistant breast cancer cells to doxorubicin through inhibiting P-glycoprotein and suppressing survivin expression. *Chem Biol Interact* 235:76-84.
- 542
543 46. Hu Y, Xu K, Yagüe E (2015) miR-218 targets survivin and regulates resistance to chemotherapeutics in breast cancer. *Breast Cancer Res Treat* 151:269-280.
- 544
545 47. Véquaud E, Desplanques G, Jézéquel P, Juin P, Barillé-Nion S (2016) Survivin contributes to DNA repair by homologous recombination in breast cancer cells. *Breast Cancer Res Treat* 155:53-63.
- 546
547 48. Chen X, Duan N, Zhang C, Zhang W (2016) Survivin and Tumorigenesis: Molecular Mechanisms and Therapeutic Strategies. *J Cancer* 7:314-323.
- 548
549
550 49. Clemens MR, Gladkov OA, Gartner E, Vladimirov V, Crown J, et al (2015) Phase II, multicenter, open-label, randomized study of YM155 plus docetaxel as first-line treatment in patients with HER2-negative metastatic breast cancer. *Breast Cancer Res Treat* 149:171-179.
- 551
552 50. Nestal de Moraes G, Delbue D, Silva KL, Robaina MC, Khongkow P, et al (2015) FOXM1 targets XIAP and Survivin to modulate breast cancer survival and chemoresistance. *Cell Signal* 27:2496-2505.
- 553
554 51. Paik S, Shak S, Tang G, Kim C, Baker J, et al (2004) A multigene assay to predict recurrence of tamoxifen-treated, node-negative breast cancer. *N Engl J Med*. 51:2817-2826.
- 555
556
557 52. Filipits M, Rudas M, Jakesz R, Dubsy P, Fitzal F, et al (2011) A new molecular predictor of distant recurrence in ER-positive, HER2-negative breast cancer adds independent information to conventional clinical risk factors. *Clin Cancer Res* 17:6012-6020.
- 558
559 53. Wallden B, Storhoff J, Nielsen T, Dowidar N, Schaper C, et al (2015) Development and verification of the PAM50-based Prosigna breast cancer gene signature assay. *BMC Med Genomics* 8:54.

560

561

562

563

564

565

RESEARCH ARTICLE

Beyond Axillary Lymph Node Metastasis, BMI and Menopausal Status Are Prognostic Determinants for Triple-Negative Breast Cancer Treated by Neoadjuvant Chemotherapy

Hélène Bonsang-Kitzis^{1,6,7}, Léonor Chaltier², Lisa Belin², Alexia Savignoni², Roman Rouzier¹, Marie-Paule Sablin³, Florence Lerebours³, François-Clément Bidard³, Paul Cottu³, Xavier Sastre-Garau⁴, Marick Laé⁴, Jean-Yves Pierga^{3,5}, Fabien Reyat^{1,6,7*}

1 Department of Surgery, Institut Curie, Paris, France, **2** Department of Biostatistic, Institut Curie, Paris, France, **3** Department of Medical Oncology, Institut Curie, Paris, France, **4** Department of Tumor Biology, Institut Curie, Paris, France, **5** Paris Descartes University, Paris, France, **6** Residual Tumor and Response to Treatment Lab, Translational Research Department, Institut Curie, Paris, France, **7** UMR932 Immunity and Cancer, INSERM, Paris, France

* fabien.reyat@curie.fr



CrossMark
click for updates

OPEN ACCESS

Citation: Bonsang-Kitzis H, Chaltier L, Belin L, Savignoni A, Rouzier R, Sablin M-P, et al. (2015) Beyond Axillary Lymph Node Metastasis, BMI and Menopausal Status Are Prognostic Determinants for Triple-Negative Breast Cancer Treated by Neoadjuvant Chemotherapy. PLoS ONE 10(12): e0144359. doi:10.1371/journal.pone.0144359

Editor: William B. Coleman, University of North Carolina School of Medicine, UNITED STATES

Received: July 9, 2015

Accepted: November 17, 2015

Published: December 18, 2015

Copyright: © 2015 Bonsang-Kitzis et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: The authors have no support or funding to report.

Competing Interests: The authors have declared that no competing interests exist.

Abstract

Background

Triple-negative breast cancers (TNBC) are a specific subtype of breast cancers with a particularly poor prognosis. However, it is a very heterogeneous subgroup in terms of clinical behavior and sensitivity to systemic treatments. Thus, the identification of risk factors specifically associated with those tumors still represents a major challenge. A therapeutic strategy increasingly used for TNBC patients is neoadjuvant chemotherapy (NAC). Only a subset of patients achieves a pathologic complete response (pCR) after NAC and have a better outcome than patients with residual disease.

Purpose

The aim of this study is to identify clinical factors associated with the metastatic-free survival in TNBC patients who received NAC.

Methods

We analyzed 326 cT1-3N1-3M0 patients with ductal infiltrating TNBC treated by NAC. The survival analysis was performed using a Cox proportional hazard model to determine clinical features associated with prognosis on the whole TNBC dataset. In addition, we built a recursive partitioning tree in order to identify additional clinical features associated with prognosis in specific subgroups of TNBC patients.

Results

We identified the lymph node involvement after NAC as the only clinical feature significantly associated with a poor prognosis using a Cox multivariate model (HR = 3.89 [2.42–6.25], $p < 0.0001$). Using our recursive partitioning tree, we were able to distinguish 5 subgroups of TNBC patients with different prognosis. For patients without lymph node involvement after NAC, obesity was significantly associated with a poor prognosis (HR = 2.64 [1.28–5.55]). As for patients with lymph node involvement after NAC, the pre-menopausal status in grade III tumors was associated with poor prognosis (HR = 9.68 [5.71–18.31]).

Conclusion

This study demonstrates that axillary lymph node status after NAC is the major prognostic factor for triple-negative breast cancers. Moreover, we identified body mass index and menopausal status as two other promising prognostic factors in this breast cancer subgroup. Using these clinical factors, we were able to classify TNBC patients in 5 subgroups, for which pre-menopausal patients with grade III tumors and lymph node involvement after NAC have the worse prognosis.

Introduction

Breast cancer is a heterogeneous disease with regard to clinicopathological features, biological behavior, molecular profiles, responses to treatment, and prognosis [1]. Triple-negative breast (TNBC), defined by negative estrogen/progesterone (ER/PR) receptor expression and lack of HER2 overexpression/amplification, corresponds to 15%–20% of breast cancers. TNBC differs from other subtypes in terms of axillary lymph node involvement, local and regional recurrence, time to metastasis delay (early distant metastatic events before 5 years from initial diagnosis), and patterns of distant recurrence (high rates of brain, lung, and distant nodal metastasis) [2].

TNBC represents an important clinical challenge as no major improvement in treatment has occurred recently in this subgroup apart from adjuvant chemotherapy, which has reduced mortality by approximately 30% [3]. This is well demonstrated with neoadjuvant therapy where those that achieve pCR have almost a 40% absolute difference in survival compared to those that do not. As a whole, patients with TNBC have the worst outcome among breast cancer subgroups [4]. Moreover, their survival differs from others subgroups: there is a sharp decrease in survival during the first 3–5 years after diagnosis but distant relapse after this is much less common [4].

Given their poor prognosis, their assumed relative chemosensitivity, and absence of any alternative specific systemic therapy, chemotherapy remains the mainstay of TNBC treatment. Neoadjuvant therapy is being increasingly used for TNBC and can be effectively used as a research tool to assess the efficacy of new drugs and/or new schedules with a validated surrogate endpoint [5]. It also represents a model to evaluate relationships between treatments and tumor biomarkers with the analysis of patient tissues possible both before and after therapy [6]. Patients who achieve a complete pathologic response (pCR) after neoadjuvant chemotherapy (NAC) have an improved prognosis compared to those with residual disease; pCR is a good surrogate marker of long-term survival and cure. Despite their relative chemosensitivity, 30%–40% of TNBC patients treated with routine NAC achieve pCR. However, patients with

residual disease (no pCR) following neoadjuvant chemotherapy have worse prognosis and overall survival [7][8].

The aim of our study was to identify patient subgroups with different prognostic outcome in a large population of TNBC patients treated by NAC at the Institut Curie (Paris, France) and to generate insight for the development of targeted therapies for the poor prognosis group.

Materials and Methods

Population

We analyzed a retrospective cohort of 326 cT1-3N1-3M0 patients with triple-negative infiltrating ductal breast carcinoma (NEOREP Cohort) treated at the Institut Curie between 2002 and 2012. Unilateral, non-recurrent, non-metastatic tumors were only included with the exclusion of T4. All patients received NAC followed by surgery with or without radiotherapy.

Tumor samples

The following histological features were retrieved: tumor type, clinical initial tumor size and nodal status, grade (Elston and Ellis), Estrogen Receptor (ER) and Progesterone Receptor (PR) status, HER2 status, number of metastatic and total sentinel and non-sentinel nodes. ER and PR status were determined as follows. After rehydration and antigenic retrieval in citrate buffer (10 mM, pH 6.1), the tissue sections were stained for ER (clone 6F11, Novocastra, Leica Biosystems, Newcastle, UK; 1/200) and PR (clone 1A6, Novocastra, 1/200). Revelation of staining was performed using the Vectastain Elite ABC peroxidase mouse IgG kit (Vector, Burlingame, CA, USA) and diaminobenzidine (Dako A/S, Glostrup, Denmark) as chromogen. Positive and negative controls were included in each slide run. Cases were considered positive for ER and PR according to the standardised guidelines using a cutoff of greater than or equal to 10% stained tumor nuclei. Hormone receptors (HR) positivity was defined by positivity of either ER or PR, and HR negativity was defined by the negativity of both ER and PR. The determination of HER2 over-expression status was determined according to the American Society of Clinical Oncology (ASCO) guidelines [9].

Triple-negative breast tumors were defined as infiltrating tumors with estrogen receptor < 10%, progesterone receptor < 10% and 0 or 1+ in IHC test or 2+ in IHC test with a situ hybridization test negative for HER2 status.

Pathologic complete response was defined as no invasive and non-invasive residuals in the breast and axillary nodes (ypT0 ypN0).

Treatments

Patients were treated according to national guidelines. Neoadjuvant chemotherapy regimens varied with time period (anthracyclines based regimen or sequential anthracyclines-taxanes regimen). Surgery was performed four to six weeks after the end of the chemotherapy. Patients received adjuvant radiotherapy according to national guidelines.

Statistical analysis

The study population was described in terms of frequencies for qualitative variables or medians and associated range for quantitative variables. The cutoff date for analysis was July 13, 2013. Metastasis-free interval was defined as the time from NAC until first occurrence of metastasis. Patients free of metastasis were censored at the date of their last known contact. Survival analyses were performed using the Kaplan-Meier estimate. Comparison between survival curves was performed using the log-rank test. Estimation of hazard ratios (HR) and their associated 95%

confidence interval was carried out using the Cox proportional hazard model. Variables with *P*-value of the score test inferior to 0.15 in univariate analysis were included in the multivariate model. Backward selection was used to establish the final multivariate model.

R-Part Software, a method of applying classification and regression trees, was used to identify the most significant variable that drive prognosis. A decision tree was established to identify homogeneous subgroups of patients and have a better clinical representation of the model. The rules of the construction of the decision tree are: the log-rank test *p*-value has to be significant and each subgroup defined by the discrimination has to include at least ten patients. Between several factors we choose the factor which minimizes the log-rank test *p*-value. Metastasis free survival rates of the subgroups identified by the decision tree were estimated by the Kaplan–Meier method, and were compared using the log-rank test. All these estimations were then plotted.

Significance level was 0.05. Analyses were performed using R software, 2.13.2 version (<http://cran.rproject.org>) using the following packages: glm, survival and rpart.

Results

Population characteristics

Patient demographics and baseline characteristics of the NEOREP Cohort ($N = 326$) are shown in [Table 1](#). Median age was 47 years with 38 patients (11%) aged less than 35 years old and 81 (25%) aged more than 55 years old. 197 patients (61%) had a body mass index (BMI) inferior or equal to 25 and 40 (12%) had a BMI superior to 30. Association between patient age and their BMI is significant (*p*-value = 0.054) ([S1 Table](#)) and BMI was more likely to be lower among pre-menopausal women (*p*-value = 0.012) ([S2 Table](#)). Median tumor size was 40 mm (0–140); 91% of patients were T2 or T3; 88% of patients were Elston–Ellis grade III and 56% of the population had a clinical lymph node involvement. A descriptive analysis of demographic and baseline clinical characteristics according to pre-NAC lymph node status is detailed on [S3 Table](#).

All patients received NAC, which was generally based on an anthracycline/taxane regimen (77%) or a solely anthracycline regimen (15%). Surgery and histological data are also detailed in [Table 1](#). Seventy-nine percent of patients had conservative surgery and 90% had axillary dissection.

Median residual tumor size was 10 mm (0–130) and 25% of patients had lymph node disease. There was no association between tumor size at diagnosis and lymph node status after NAC ([S3 Table](#)). Histological response was considered as complete (pCR) in 33% of patients. 36% of patients had no residual disease in the breast and 75% had no residual disease in axillary lymph nodes ([Table 2](#)). Seven percent of patients had residual *in-situ* disease.

Median follow-up duration was 52 (range, 8–125) months. At 36 months, metastasis free survival is 79% CI95% [74;84] and the overall survival is 85% CI95% [81;89] ([S1 Fig](#)). 69 patients experienced at least one metastases. 10 patients had more than 2 metastases. The most common site of metastasis was lung (23%) then central neurologic system (21%) and lymph node (20%) following by liver (18%) and bony metastases (16%). At 36 months, locoregional recurrence free survival is 93% CI95% [90; 96] ([S1 Fig](#)).

Lymph node disease after NAC is a prognostic factor for metastasis-free survival

Three factors were significantly associated ($p < 0.05$) with metastasis-free survival (MFS) on univariate analysis: post-NAC breast tumor disease (HR = 2.05, CI95% [1.17; 3.59]); post-NAC

Table 1. Patient demographics and baseline characteristics for NEOREP Cohort (N = 326).

Age, y, median (range)	47 (25–76)
Age, n (%)	
≤35 y	38 (11)
36–45 y	100 (31)
46–55 y	107 (33)
>55 y	81 (25)
Body weight, kg, median (range)	63.5 (43–120)
Height, cm, median (range)	164 (145–178)
BMI, n (%)*	
≤25 kg/m ²	197 (61)
26–30 kg/m ²	88 (27)
>30 kg/m ²	40 (12)
Family history of breast cancer, n (%)	
No	246 (76)
Yes	79 (24)
Missing	1
Pregnancy <1 y prior to diagnosis, n (%)	
No	315 (97)
Yes	11 (3)
Menopausal status, n (%)	
Premenopausal	199 (62)
Postmenopausal	123 (38)
Missing	4
Tumor size, mm, median (range)	40 (0–140)
T stage, n (%)	
T1	29 (9)
T2	212 (65)
T3	85 (26)
N stage, n (%)	
N0	142 (44)
N1	163 (50)
N2	17 (5)
N3	4 (1)
Elston-Ellis grade, n (%)*	
I	4 (1)
II	36 (11)
III	282 (88)
Missing	4
Mitotic index, n, median (range)	27 (1–138)
Mitotic index, n (%)*	
≤10	45 (15)
11–22	79 (26)
>22	180 (59)
Missing	22
Neoadjuvant chemotherapy type, n (%)	
Anthracycline	49 (15)
Anthracycline/taxane	252 (77)
Other	25 (8)

(Continued)

Table 1. (Continued)

Neoadjuvant chemotherapy cycles, n, median (range)	8 (1–16)
Mammary surgery, n (%)	
Partial mastectomy	259 (79)
Total mastectomy	67 (21)
Mammoplasty, n (%)	
No	265 (81)
Yes	61 (19)
Axillary surgery, n (%)	
Sentinel node biopsy	16 (5)
Axillary dissection	293 (90)
Sentinel node biopsy + axillary dissection	17 (5)
Histological tumor size, mm, median (range)	10 (0–130)
Lymph nodes removed, n, median (range)	12 (1–28)
Positive lymph nodes, n (%)	
0	245 (75)
1–3	49 (15)
3–9	26 (8)
>9	6 (2)

Note

* Data were missing or not evaluable for some patients and are not included in the denominator for percent calculations.

doi:10.1371/journal.pone.0144359.t001

lymph node disease (HR = 3.89, CI95% [2.42; 6.25]), and post-NAC residual disease (breast + lymph node) (HR = 2.63, CI95% [1.41; 4.91]) as expected. Clinical tumor size, EE grade and pre NAC lymph node status were associated in the univariate analysis but the only prognostic factor which persisted on multivariate analysis was post-NAC lymph node disease (HR = 3.89, CI95% [2.42; 6.25]; $P < 0.0001$) (Table 3). When we stratified lymph node involvement according to the pTNM classification (pN0, pN1, pN2, pN3), the results remained significantly associated with MFS after univariate and multivariate survival analyses. Additionally, the magnitude of the impact of lymph node involvement on MFS increased with increasing nodal status according to TNM (data not shown).

Body mass index, EE grade and menopausal status could have a role in MFS: a decision tree analysis

Five homogeneous prognostic subgroups were identified (Fig 1): patients without lymph node disease after NAC and not obese (pN-/BMI $\leq 30\text{kg/m}^2$) (which represents the reference group), obese patients without lymph node disease after NAC (pN-/BMI $> 30\text{kg/m}^2$), patients with lymph node disease after NAC and grade I-II (pN+/EEgrade I-II), patients with lymph node disease after NAC grade III tumor and postmenopausal status (pN+/EEgrade III/postM) and finally patients with lymph node disease after NAC grade III tumor and pre-menopausal status (pN+/EEgrade III/preM). The last group has the poorest metastatic prognosis (MFS at 36 months 31% CI95% [18; 54] for pN+/EEgrade III/preM patients).

Lymph node disease after NAC remain the first discriminant prognostic factor of MFS: it has the strongest prognostic impact.

For calculation of the metastasis hazard ratio (HR) associated with each branch of the tree, we used pN-/BMI $\leq 30\text{ kg/m}^2$ as a reference group. Metastatic risk of pN-/BMI $> 30\text{ kg/m}^2$

Table 2. Response to neoadjuvant chemotherapy in the NEOREP Cohort (N = 326).

Clinical response to treatment, n (%)	
Complete response	189 (58)
Partial response >50%	76 (23)
Partial response <50%	37 (11)
Stable disease	15 (5)
Progressive disease	9 (3)
Pathological response to treatment, n (%)	
Lymph node disease	
No	245 (75)
Yes	81 (25)
Tumor disease	
No	118 (36)
Yes	208 (64)
Histological response	
pCR	107 (33)
Residual disease	219 (67)
<i>In-situ</i> disease	
No	301 (93)
Yes	24 (7)

doi:10.1371/journal.pone.0144359.t002

patients is 2.64 times more than pN⁻/BMI < 30 kg/m². (CI95% [1.28; 5.55]). Hazard ratio of the pN⁺/EE grade I–II patients is 1.29 (CI95% [0.39; 4.26]), the pN⁺/EE grade III/post-menopausal status patients is 3.57 (CI95% [1.69; 7.77]), and the pN⁺/EE grade III/pre-menopausal status patients is 9.68 (CI95% [5.71; 18.31]).

Table 3. Metastasis-free survival analysis following neoadjuvant therapy in the NEOREP Cohort (N = 326).

		Univariate Cox Model		Multivariate Cox Model	
		HR (95% CI)	P-value	HR (95% CI)	P-value
Age	≤45 y	1			
	46–55 y	0.96 (0.56–1.68)	0.95		
	>55 y	1.07 (0.59–1.94)			
Body mass index	≤30 kg/m ²	1		1	
	>30 kg/m ²	1.52 (0.81–2.83)	0.19	1.71 (0.92–3.21)	0.09
Menopausal status	Premenopausal	1			
	Postmenopausal	0.89 (0.55–1.46)	0.65		
Clinical tumor size	≤30 mm	1		1	
	>30 mm	1.56 (0.85–2.85)	0.15	1.51 (0.82–2.78)	0.18
Pre-NAC lymph node status	N ⁻	1		1	
	N ⁺	1.52 (0.93–2.48)	0.09	1.28 (0.77–2.14)	0.34
Elston-Ellis grade	I/II	1			
	III	1.23 (0.59–2.57)	0.56		
Mitotic index	≤22	1			
	>22	1.15 (0.70–1.88)	0.58		
Post-NAC tumor	No	1		1	
	Yes	2.05 (1.17–3.59)	0.01	1.35 (0.74–2.44)	0.33

(Continued)

Table 3. (Continued)

		Univariate Cox Model		Multivariate Cox Model	
		HR (95% CI)	P-value	HR (95% CI)	P-value
disease					
Post-NAC	No	1		1	
lymph node	Yes	3.89 (2.42–6.25)	<10⁻⁴	3.48 (2.08–5.84)	2.25 x 10⁻⁶
disease					
Histological	pCR	1			
response	Residual disease	2.63 (1.41–4.91)	0.002		
		Univariate Cox Model		Multivariate Cox Model	
		HR (95% CI)	P-value	HR (95% CI)	P-value
	≤45 y	1			
Age	46–55 y	0.96 (0.56–1.68)	0.95		
	>55 y	1.07 (0.59–1.94)			
Body mass	≤30 kg/m ²	1		1	
index	>30 kg/m ²	1.52 (0.81–2.83)	0.19	1.71 (0.92–3.21)	0.09
Menopausal	Premenopausal	1			
status	Postmenopausal	0.89 (0.55–1.46)	0.65		
Clinical	≤30 mm	1		1	
tumor size	>30 mm	1.56 (0.85–2.85)	0.15	1.51 (0.82–2.78)	0.18
Pre-NAC	N ⁻	1		1	
lymph node	N ⁺	1.52 (0.93–2.48)	0.09	1.28 (0.77–2.14)	0.34
status					
Elston-Ellis	I/II	1			
grade	III	1.23 (0.59–2.57)	0.56		
Mitotic index	≤22	1			
	>22	1.15 (0.70–1.88)	0.58		
Post-NAC	No	1		1	
tumor	Yes	2.05 (1.17–3.59)	0.01	1.35 (0.74–2.44)	0.33
disease					
Post-NAC	No	1		1	
lymph node	Yes	3.89 (2.42–6.25)	<10⁻⁴	3.48 (2.08–5.84)	2.25 x 10⁻⁶
disease					
Histological	pCR	1			
response	Residual disease	2.63 (1.41–4.91)	0.002		

CI, confidence interval; NAC, neoadjuvant chemotherapy; pCR, complete pathological response

doi:10.1371/journal.pone.0144359.t003

The Kaplan-Meier plot of metastasis-free survival for each subgroup is presented in [Fig 2](#). Description of the clinical and pathological characteristics of the five subgroups defined in the decision tree is presented in [S4 Table](#).

Discussion

Triple-negative breast cancer patients are more likely to achieve a pathologic complete response after neoadjuvant chemotherapy compared to others breast cancer subtypes but those who do not still have poor prognosis [7][8][10]. The aim of this study was to identify prognostic factors in triple-negative breast cancer patients receiving neoadjuvant chemotherapy.

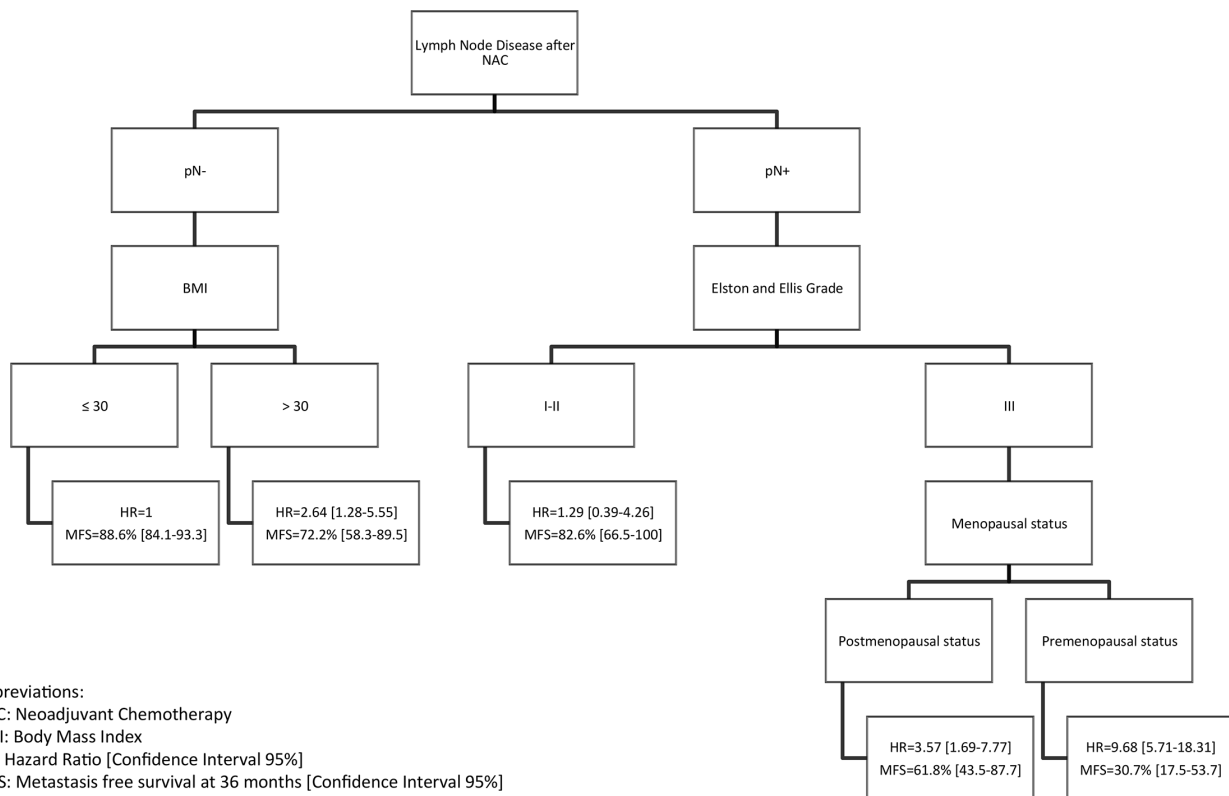


Fig 1. Decision tree algorithm. Abbreviations: NAC, neoadjuvant chemotherapy; BMI, body mass index; RR, relative risk [confidence interval 95%]; MFS, metastasis free survival at 36 months [confidence interval 95%].

doi:10.1371/journal.pone.0144359.g001

We found that axillary lymph node metastasis following NAC is the most important prognostic factor in women with TNBC. Lymph node response to NAC appears as the central determinant of the prognosis (metastasis-free survival) with a poorer prognosis when lymph nodes were involved (HR = 3.89, CI95% [2.42; 6.25]; $P < 0.0001$) while persistence of tumor disease in breast after NAC is not significant (HR = 1.35; CI95% [0.74; 2.44]; $P = 0.33$) in multivariate analysis.

Evidence from various studies has revealed that pCR after NAC is the most important prognostic factor for long-term outcome in TNBC [11]. However, there is no general agreement on the actual definition of pCR. Indeed, in both clinical trials and daily practice, different definitions of pCR are used, including absence of invasive cancer in the breast only or in both the breast and axillary lymph nodes, and absence of invasive and in-situ cancer in the breast only or in both the breast and axillary lymph nodes. The prognostic impact of pCR after NAC was only found to be true when pCR was defined as no residual disease present in both the breast and axilla (ypT0, ypN0 excluding ductal carcinoma in situ). In contrast patients with extensive nodal involvement after NAC have a very poor outcome [12]. Our study corroborates these results and highlights that eradication of lymph node disease is probably the major prognostic factor for pathological response in patients with node involvement. Lymph node disease after NAC should therefore be interpreted as a 'distant metastatic marker'.

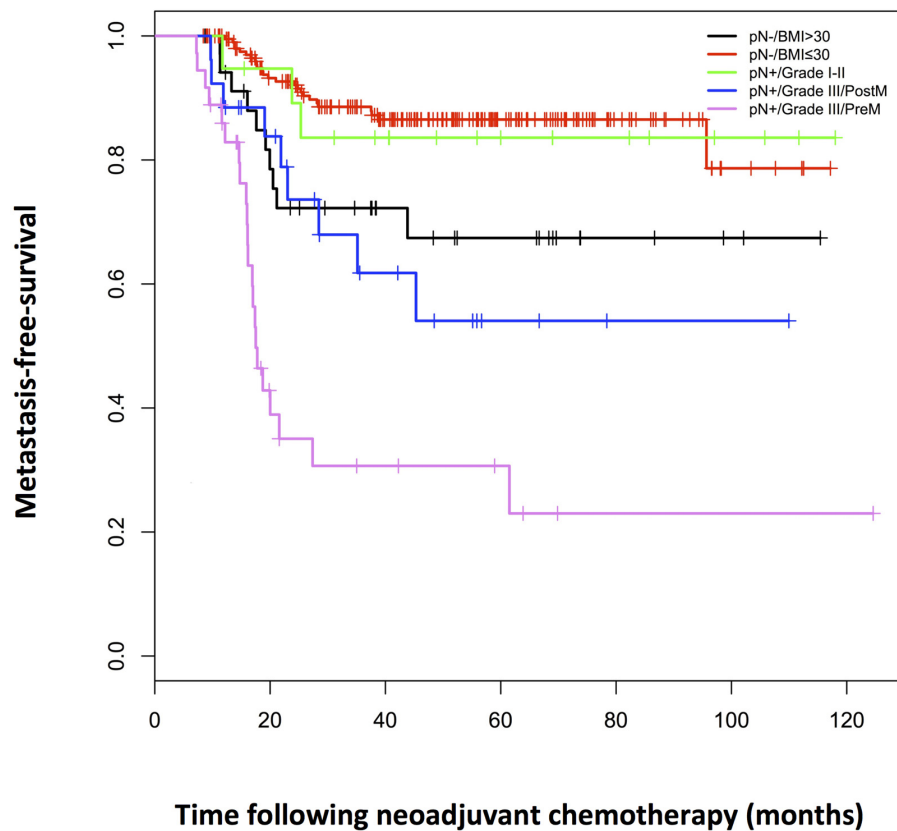


Fig 2. Kaplan-Meier plots of metastasis-free survival for each subgroup of the decision tree in the NEOREP Cohort. Abbreviations: BMI, body mass index; PostM, postmenopausal; PreM, premenopausal.

doi:10.1371/journal.pone.0144359.g002

There are conflicting results on the prevalence of lymph node metastasis at the time of diagnosis in TNBC patients [13]. Some studies described a higher prevalence of lymph node metastasis in TNBC [14] while others have found no statistical differences [15] or even an inverse association between TNBC and lymph node metastasis [16]. The main theory is that TNBC seems to disseminate to axillary nodes and bones less frequently than non-TNBC, presenting a preferential hematogenous dissemination [17][18] with a proclivity to develop metastatic deposition in the brain and lungs [13]. These different routes of metastatic spread may explain differences in recurrence and patient mortality rates [19].

Moreover, some authors have argued that basal-like tumors do not seem to obey the 'size-node' rule. For ER-negative, HER2-negative breast cancer, nodal status was almost independent of tumor size with a relatively constant trend for axillary metastases at ~20%. Conversely, for ER-positive or HER2-positive breast cancer, there was a strong, almost linear, correlation between tumor size and development of axillary metastasis [14][20]. Despite being in a neoadjuvant setting, our results appear to agree with this, as there was no association between tumor size and post-NAC lymph node disease for TNBC.

As previously demonstrated, we also showed that Elston and Ellis grade is a significant predictor of metastasis free survival in TNBC with poorer prognosis for grade III patients, even if TNBC are more likely to be high grade tumors [21][22][14] [23].

Markers of the hormonal environment such as BMI and menopausal status also seemed to have an important prognostic value in TNBC. For patients with no post-NAC lymph node disease, prognosis was poorer when BMI was $> 30 \text{ kg/m}^2$ (HR = 2.64, CI95% [1.28; 5.55]; $P = 0.0067$). Epidemiological investigations evaluating the relationship between TNBC and obesity have reported conflicting results leaving an open question in the understanding of the etiology of this aggressive tumor subtype. Some have reported an overall increase in the risk of TNBC in women with higher BMI [24][25] while others could not confirm this association [26][27]. There are several hypotheses on the mechanisms that link obesity to breast cancer. First, increased estrogen production availability by adipocytes (due to enhanced aromatase activity) may induce and stimulate the growth of abnormal ER-positive mammary cells [28]. Second, obesity, especially when associated with metabolic syndrome, presents increased levels of insulin and insulin-like growth factor, hormones with potent mitogenic activity toward epithelial cells [29]. Finally, paracrine secretion of interleukin-6 and tumor necrosis factor-alpha and the establishment of a pro-inflammatory micro-environment can induce the development of malignant phenotypes that are independent of hormonal secretion [30]. Since TNBC lacks expression of hormone receptors, distinct molecular mechanisms must link obesity to this subtype of breast cancer, for example insulin resistance, secretion of pro-angiogenic adipokines such as leptin, and chronic inflammation [31].

Alternatively, the detrimental effect of obesity on TNBC prognosis might be linked to sub-therapeutic treatment. Drug dosing has traditionally been based on a patient's estimated body surface area (BSA) in adults [32]. There is compelling evidence that reductions from standard dose and dose intensity may compromise disease-free and overall survival in the curative setting [33][34][35]. Despite studies confirming the safety and importance of full weight-based cytotoxic (intravenous and oral) chemotherapy dosing, many (up to 40%) overweight and obese patients continue to receive limited chemotherapy doses that are not based on actual body weight [36] [37][35]. Many oncologists continue to use either ideal body weight or adjusted ideal body weight, or to cap BSA at, for example, 2 m^2 rather than use actual body weight to calculate BSA.

Although regarded as an endocrine-insensitive disease, several hormonal alterations throughout a woman's life have been associated an increased risk of developing TNBC. For example, parity and young age at first full-term pregnancy increase the risk of developing TNBC in some studies, while a longer duration of breastfeeding and an increasing number of children breastfed reduce the risk of developing TNBC [24]. There is no strongly demonstrated association between menopausal status and TNBC prognosis in the literature. However, our study suggests that a premenopausal status is associated with a poorer prognosis for patients with post-NAC lymph node involvement (HR = 9.68, CI95% [5.71; 18.31]; $P = 5.22 \times 10^{-15}$), as though a hormonal pathway may be involved in such tumors in some way.

For example, aromatase receptors (ARs) are expressed in a subset of TNBC patients. The overall frequency of AR expression varies considerably among the studies from 0% to 53% of TNBC patients [38][39][25]. AR is a member of the family of steroid nuclear receptors, which also includes ER and PG receptors. Further studies demonstrated a correlation between AR and ER/PR pathways [40] and the potential role for AR in patient prognosis with TNBC [41] [39][25]. The prognostic value of these 'hormonal environmental' markers in TNBC may also be related in part to tumor heterogeneity, reinforcing the hypothesis that variation in ER, PR, and HER2 status between primary breast cancer and metastases [42] may actually reflect clonal genome evolution. Tumor heterogeneity may be attributable to tumor biological drift, selective

pressure of therapy leading to clonal selection with the development of a novel tumor cell clone, or the presence of small subclones routinely undetected within the primary tumor.

Conclusion

The identification of risk factors specifically associated with TNBC still represents a major challenge for the development of targeted and more efficient curative programs. This study highlights the strong association between the lymph node involvement after NAC and the worse prognostic outcome of patients with TNBC. This study confirms the association between the Elston and Ellis grade and the worse prognostic outcome of those patients. Moreover, it revealed two intrinsic factors of the « hormonal environment » of patients (BMI and menopausal status), which also play an important role in the prognosis of such tumors. This work needs to be validated, and will help in identifying individuals who are at a higher risk of developing an aggressive form of TNBC. Integration of monitoring of these factors now into NAC studies can help refine our understanding of high-risk TNBC patients (pN⁺/EE grade III/premenopausal) and generate ideas for new therapeutic solutions.

Ethical approval

All experiments were performed retrospectively and in accordance with the French Bioethics Law 2004–800, the French National Institute of Cancer (INCa) Ethics Charter and after approval by the Institut Curie review board and ethics committee (Comit de Pilotage of the Groupe Sein). In the French legal context, our institutional review board waived the need for written informed consent from the participants. Moreover, women were informed of the research use of their tissues and did not declare any opposition for such researches. Data were analyzed anonymously.

Supporting Information

S1 Fig. Histograms of time between diagnosis and start of neoadjuvant chemotherapy, and overall, metastasis-free, and relapse-free survival curves in the NEOREP Cohort.
(PDF)

S1 Table. Correlation between patient age and body mass index (BMI) in the NEOREP Cohort (N = 326).
(XLSX)

S2 Table. Correlation between patient menopausal status and body mass index (BMI) in the NEOREP Cohort (N = 326).
(XLSX)

S3 Table. Descriptive analysis according to post-NAC lymph node status in the NEOREP Cohort (N = 326).
(XLSX)

S4 Table. Descriptive analysis according to each subgroup of the decision tree in the NEOREP Cohort (N = 326).
(XLSX)

Acknowledgments

The authors acknowledge the support of Dr Nora Ady-Vago (Roche France) and Aline Gobillion (Department of Biostatistic, Institut Curie).

Author Contributions

Conceived and designed the experiments: RR JYP FR. Performed the experiments: HBK RR MPS FL FCB PC XSG ML JYP FR. Analyzed the data: HBK LC LB AS. Contributed reagents/materials/analysis tools: RR MPS FL FCB PC XSG ML JYP FR. Wrote the paper: HBK LB AS FR.

References

1. Sotiriou C, Wirapati P, Loi S, Harris A, Fox S, Smeds J, et al. Gene expression profiling in breast cancer: understanding the molecular basis of histologic grade to improve prognosis. *J Natl Cancer Inst.* 2006; 98: 262–272. doi: [10.1093/jnci/djj052](https://doi.org/10.1093/jnci/djj052) PMID: [16478745](https://pubmed.ncbi.nlm.nih.gov/16478745/)
2. Sorlie T. Molecular classification of breast tumors: toward improved diagnostics and treatments. *Meth-ods Mol Biol Clifton NJ.* United States; 2007; 360: 91–114. doi: [10.1385/1-59745-165-7:91](https://doi.org/10.1385/1-59745-165-7:91)
3. Carey LA. Directed therapy of subtypes of triple-negative breast cancer. *The oncologist.* United States; 2011; 16 Suppl 1: 71–78. doi: [10.1634/theoncologist.2011-S1-71](https://doi.org/10.1634/theoncologist.2011-S1-71) PMID: [21278443](https://pubmed.ncbi.nlm.nih.gov/21278443/)
4. Foulkes WD, Smith IE, Reis-Filho JS. Triple-negative breast cancer. *N Engl J Med.* 2010; 363: 1938–1948. doi: [10.1056/NEJMra1001389](https://doi.org/10.1056/NEJMra1001389) PMID: [21067385](https://pubmed.ncbi.nlm.nih.gov/21067385/)
5. Buchholz TA, Lehman CD, Harris JR, Pockaj BA, Khouri N, Hylton NF, et al. Statement of the Science Concerning Locoregional Treatments After Preoperative Chemotherapy for Breast Cancer: A National Cancer Institute Conference. 2008; 26: 791–797. doi: [10.1200/JCO.2007.15.0326](https://doi.org/10.1200/JCO.2007.15.0326) PMID: [18258988](https://pubmed.ncbi.nlm.nih.gov/18258988/)
6. Fisher CS, Ma CX, Gillanders WE, Aft RL, Eberlein TJ, Gao F, et al. Neoadjuvant chemotherapy is associated with improved survival compared with adjuvant chemotherapy in patients with triple-negative breast cancer only after complete pathologic response. *Ann Surg Oncol.* United States; 2012; 19: 253–258. doi: [10.1245/s10434-011-1877-y](https://doi.org/10.1245/s10434-011-1877-y) PMID: [21725686](https://pubmed.ncbi.nlm.nih.gov/21725686/)
7. Symmans WF, Peintinger F, Hatzis C, Rajan R, Kuerer H, Valero V, et al. Measurement of residual breast cancer burden to predict survival after neoadjuvant chemotherapy. *J Clin Oncol Off J Am Soc Clin Oncol.* 2007; 25: 4414–22. doi: [10.1200/JCO.2007.10.6823](https://doi.org/10.1200/JCO.2007.10.6823)
8. Liedtke C, Mazouni C, Hess KR, André F, Tordai A, Mejia J, et al. Response to neoadjuvant therapy and long-term survival in patients with triple-negative breast cancer. *J Clin Oncol Off J Am Soc Clin Oncol.* 2008; 26: 1275–81. doi: [10.1200/JCO.2007.14.4147](https://doi.org/10.1200/JCO.2007.14.4147)
9. Wolff AC, Hammond MEH, Schwartz JN, Hagerty KL, Allred DC, Cote RJ, et al. American Society of Clinical Oncology/College of American Pathologists guideline recommendations for human epidermal growth factor receptor 2 testing in breast cancer. *J Clin Oncol.* 2007; 25: 118–145. doi: [10.1200/JCO.2006.09.2775](https://doi.org/10.1200/JCO.2006.09.2775) PMID: [17159189](https://pubmed.ncbi.nlm.nih.gov/17159189/)
10. Rouzier R, Perou CM, Symmans WF, Ibrahim N, Cristofanilli M, Anderson K, et al. Breast cancer molecular subtypes respond differently to preoperative chemotherapy. *Clin Cancer Res.* 2005; 11: 5678–5685. doi: [10.1158/1078-0432.CCR-04-2421](https://doi.org/10.1158/1078-0432.CCR-04-2421) PMID: [16115903](https://pubmed.ncbi.nlm.nih.gov/16115903/)
11. Rastogi P, Anderson SJ, Bear HD, Geyer CE, Kahlenberg MS, Robidoux A, et al. Preoperative chemotherapy: updates of National Surgical Adjuvant Breast and Bowel Project Protocols B-18 and B-27. *J Clin Oncol Off J Am Soc Clin Oncol.* 2008; 26: 778–85. doi: [10.1200/JCO.2007.15.0235](https://doi.org/10.1200/JCO.2007.15.0235)
12. Pierga JY, Mouret E, Diéras V, Laurence V, Beuzeboc P, Dorval T, et al. Prognostic value of persistent node involvement after neoadjuvant chemotherapy in patients with operable breast cancer. *Br J Cancer.* 2000; 83: 1480–1487. doi: [10.1054/bjoc.2000.1461](https://doi.org/10.1054/bjoc.2000.1461) PMID: [11076657](https://pubmed.ncbi.nlm.nih.gov/11076657/)
13. Tutt ANJ. Triple negative tumours: a critical review. 2008; 108–118. doi: [10.1111/j.1365-2559.2007.02889.x](https://doi.org/10.1111/j.1365-2559.2007.02889.x)
14. Dent R, Trudeau M, Pritchard KI, Hanna WM, Kahn HK, Sawka CA, et al. Triple-negative breast cancer: clinical features and patterns of recurrence. *Clin Cancer Res Off J Am Assoc Cancer Res.* United States; 2007; 13: 4429–4434. doi: [10.1158/1078-0432.CCR-06-3045](https://doi.org/10.1158/1078-0432.CCR-06-3045)
15. Rakha E, El-Sayed ME, Green AR, Lee AHS, Robertson JF, Ellis IO. Prognostic markers in triple-negative breast cancer. *Cancer.* 2007; 109: 25–32. doi: [10.1002/ncr.22381](https://doi.org/10.1002/ncr.22381) PMID: [17146782](https://pubmed.ncbi.nlm.nih.gov/17146782/)
16. Tischkowitz M, Brunet J-S, Begin LR, Huntsman DG, Cheang MCU, Akslen LA, et al. Use of immunohistochemical markers can refine prognosis in triple negative breast cancer. *BMC Cancer.* England; 2007; 7: 134. doi: [10.1186/1471-2407-7-134](https://doi.org/10.1186/1471-2407-7-134) PMID: [17650314](https://pubmed.ncbi.nlm.nih.gov/17650314/)
17. Rodríguez-Pinilla SM, Sarrío D, Honrado E, Hardisson D, Calero F, Benitez J, et al. Prognostic significance of basal-like phenotype and fascin expression in node-negative invasive breast carcinomas. *Clin Cancer Res Off J Am Assoc Cancer Res.* 2006; 12: 1533–9. doi: [10.1158/1078-0432.CCR-05-2281](https://doi.org/10.1158/1078-0432.CCR-05-2281)

18. Fulford LG, Reis-filho JS, Ryder K, Jones C, Gillett CE, Hanby A, et al. Research article Basal-like grade III invasive ductal carcinoma of the breast : patterns of metastasis and long-term survival. 2007; 9: 1–11. doi: [10.1186/bcr1636](https://doi.org/10.1186/bcr1636)
19. Dent R, Hanna WM, Trudeau M, Rawlinson E, Sun P, Narod SA. Time to disease recurrence in basal-type breast cancers: effects of tumor size and lymph node status. *Cancer*. United States; 2009; 115: 4917–4923. doi: [10.1002/cncr.24573](https://doi.org/10.1002/cncr.24573) PMID: [19691094](https://pubmed.ncbi.nlm.nih.gov/19691094/)
20. Foulkes WD, Grainge MJ, Rakha EA, Green AR, Ellis IO. Tumor size is an unreliable predictor of prognosis in basal-like breast cancers and does not correlate closely with lymph node status. *Breast Cancer Res Treat*. Netherlands; 2009; 117: 199–204. doi: [10.1007/s10549-008-0102-6](https://doi.org/10.1007/s10549-008-0102-6) PMID: [18600446](https://pubmed.ncbi.nlm.nih.gov/18600446/)
21. Bauer KR, Brown M, Cress RD, Parise CA, Caggiano V. Descriptive analysis of estrogen receptor (ER)-negative, progesterone receptor (PR)-negative, and HER2-negative invasive breast cancer, the so-called triple-negative phenotype: a population-based study from the California cancer Registry. *Cancer*. United States; 2007; 109: 1721–1728. doi: [10.1002/cncr.22618](https://doi.org/10.1002/cncr.22618) PMID: [17387718](https://pubmed.ncbi.nlm.nih.gov/17387718/)
22. Blows FM, Driver KE, Schmidt MK, Broeks A, Van Leeuwen FE, Wesseling J, et al. Subtyping of Breast Cancer by Immunohistochemistry to Investigate a Relationship between Subtype and Short and Long Term Survival : A Collaborative Analysis of Data for 10, 159 Cases from 12 Studies. 2010;7. doi: [10.1371/journal.pmed.1000279](https://doi.org/10.1371/journal.pmed.1000279)
23. Yang XR, Sherman ME, Rimm DL, Lissowska J, Brinton LA, Peplonska B, et al. Differences in Risk Factors for Breast Cancer Molecular Subtypes in a Population-Based Study. 2007; 16: 439–444. doi: [10.1158/1055-9965.EPI-06-0806](https://doi.org/10.1158/1055-9965.EPI-06-0806) PMID: [17372238](https://pubmed.ncbi.nlm.nih.gov/17372238/)
24. Millikan RC, Newman B, Tse C, Moorman PG, Smith LV, Labbok MH, et al. Epidemiology of basal-like breast cancer. 2008; 109: 123–139. PMID: [17578664](https://pubmed.ncbi.nlm.nih.gov/17578664/)
25. Tang D, Xu S, Zhang Q, Zhao W. The expression and clinical significance of the androgen receptor and E-cadherin in triple-negative breast cancer. *Med Oncol Northwood Lond Engl*. 2012; 29: 526–33. doi: [10.1007/s12032-011-9948-2](https://doi.org/10.1007/s12032-011-9948-2)
26. Gaudet MM, Press MF, Haile RW, Lynch CF, Glaser SL, Schildkraut J, et al. Risk factors by molecular subtypes of breast cancer across a population-based study of women 56 years or younger. *Breast Cancer Res Treat*. Netherlands; 2011; 130: 587–597. doi: [10.1007/s10549-011-1616-x](https://doi.org/10.1007/s10549-011-1616-x) PMID: [21667121](https://pubmed.ncbi.nlm.nih.gov/21667121/)
27. Phipps AI, Chlebowski RT, Prentice R, McTiernan A, Stefanick ML, Wactawski-Wende J, et al. Body size, physical activity, and risk of triple-negative and estrogen receptor-positive breast cancer. *Cancer Epidemiol Biomark Prev Publ Am Assoc Cancer Res Cosponsored Am Soc Prev Oncol*. United States; 2011; 20: 454–463. doi: [10.1158/1055-9965.EPI-10-0974](https://doi.org/10.1158/1055-9965.EPI-10-0974)
28. Bulun SE, Chen D, Moy I, Brooks DC, Zhao H. Aromatase, breast cancer and obesity: a complex interaction. *Trends Endocrinol Metab TEM*. United States; 2012; 23: 83–89. doi: [10.1016/j.tem.2011.10.003](https://doi.org/10.1016/j.tem.2011.10.003) PMID: [22169755](https://pubmed.ncbi.nlm.nih.gov/22169755/)
29. Stephenson GD, Rose DP. Breast Cancer and Obesity: An Update. *Nutr Cancer*. 2003; 45: 1–16. doi: [10.1207/S15327914NC4501_1](https://doi.org/10.1207/S15327914NC4501_1) PMID: [12791499](https://pubmed.ncbi.nlm.nih.gov/12791499/)
30. Lorincz AM, Sukumar S. Molecular links between obesity and breast cancer. 2006; 279–292. doi: [10.1677/erc.1.00729](https://doi.org/10.1677/erc.1.00729) PMID: [16728564](https://pubmed.ncbi.nlm.nih.gov/16728564/)
31. Federico A, Morgillo F, Tuccillo C, Ciardiello F, Loguercio C. Chronic inflammation and oxidative stress in human carcinogenesis. 2007; 2386: 2381–2386. doi: [10.1002/jic.23192](https://doi.org/10.1002/jic.23192)
32. Freireich EJ, Gehan EA, Rall DP, Schmidt LH, Skipper HE. Quantitative comparison of toxicity of anti-cancer agents in mouse, rat, hamster, dog, monkey, and man. *Cancer Chemother Rep*. 1966; 50: 219–244. PMID: [4957125](https://pubmed.ncbi.nlm.nih.gov/4957125/)
33. Budman DR, Berry DA, Cirrincione CT, Henderson IC, Wood WC, Weiss RB, et al. Dose and Dose Intensity as Determinants of Outcome in the Adjuvant Treatment of Breast Cancer. 1998; 90: 1205–1211.
34. Lyman GH. Impact of chemotherapy dose intensity on cancer patient outcomes. *JNCCN J Natl Compr Cancer Netw*. 2009; 7: 99–108.
35. Lyman GH. Commentary: chemotherapy dosing in obese patients with cancer—the need for evidence-based clinical practice guidelines. *J Oncol Pract Am Soc Clin Oncol*. United States; 2011; 7: 17–18. doi: [10.1200/JOP.2010.000200](https://doi.org/10.1200/JOP.2010.000200)
36. Griggs JJ, Sorbero MES, Lyman GH. Undertreatment of obese women receiving breast cancer chemotherapy. *Arch Intern Med*. United States; 2005; 165: 1267–1273. doi: [10.1001/archinte.165.11.1267](https://doi.org/10.1001/archinte.165.11.1267) PMID: [15956006](https://pubmed.ncbi.nlm.nih.gov/15956006/)
37. Greenman CG, Jagielski CH, Griggs JJ. Breast cancer adjuvant chemotherapy dosing in obese patients: dissemination of information from clinical trials to clinical practice. *Cancer*. United States; 2008; 112: 2159–2165. doi: [10.1002/cncr.23416](https://doi.org/10.1002/cncr.23416) PMID: [18338813](https://pubmed.ncbi.nlm.nih.gov/18338813/)

38. Loibl S, Müller BM, von Minckwitz G, Schwabe M, Roller M, Darb-Esfahani S, et al. Androgen receptor expression in primary breast cancer and its predictive and prognostic value in patients treated with neoadjuvant chemotherapy. *Breast Cancer Res Treat.* 2011; 130: 477–87. doi: [10.1007/s10549-011-1715-8](https://doi.org/10.1007/s10549-011-1715-8) PMID: [21837479](https://pubmed.ncbi.nlm.nih.gov/21837479/)
39. He J, Peng R, Yuan Z, Wang S, Peng J, Lin G, et al. Prognostic value of androgen receptor expression in operable triple-negative breast cancer: a retrospective analysis based on a tissue microarray. *Med Oncol.* 2012; 29: 406–410. doi: [10.1007/s12032-011-9832-0](https://doi.org/10.1007/s12032-011-9832-0) PMID: [21264529](https://pubmed.ncbi.nlm.nih.gov/21264529/)
40. Brentani MM, Franco EL, Oshima CT, Pacheco MM. Androgen, estrogen, and progesterone receptor levels in malignant and benign breast tumors: a multivariate analysis approach. *Int J Cancer.* 1986; 38: 637–642. PMID: [3770993](https://pubmed.ncbi.nlm.nih.gov/3770993/)
41. Rakha EA, Green AR, Lee AHS, Robertson JF, Ellis IO. Prognostic Markers in Triple-Negative Breast Cancer. 2006; 2: 4–11. doi: [10.1002/cncr.22381](https://doi.org/10.1002/cncr.22381)
42. Aurilio G, Disalvatore D, Pruneri G, Bagnardi V, Viale G, Curigliano G, et al. A meta-analysis of oestrogen receptor, progesterone receptor and human epidermal growth factor receptor 2 discordance between primary breast cancer and metastases. *Eur J Cancer Oxf Engl* 1990. England; 2014; 50: 277–289. doi: [10.1016/j.ejca.2013.10.004](https://doi.org/10.1016/j.ejca.2013.10.004)

CORRECTION

Correction: Beyond Axillary Lymph Node Metastasis, BMI and Menopausal Status Are Prognostic Determinants for Triple-Negative Breast Cancer Treated by Neoadjuvant Chemotherapy

Hélène Bonsang-Kitzis, Léonor Chaltier, Lisa Belin, Alexia Savignoni, Roman Rouzier, Anne-Sophie Hamy, Marie-Paule Sablin, Florence Lerebours, François-Clément Bidard, Paul Cottu, Xavier Sastre-Garau, Marick Laé, Jean-Yves Pierga, Fabien Reyat

Dr. Anne-Sophie Hamy should be included in the byline as the sixth author. Her affiliations are 6: Residual Tumor and Response to Treatment Lab, Translational Research Department, Institut Curie, Paris, France, and 7: UMR932 Immunity and Cancer, INSERM, Paris, France. The contributions of this author are as follows: Wrote the manuscript.

The correct citation is: Bonsang-Kitzis H, Chaltier L, Belin L, Savignoni A, Rouzier R, Hamy AS, et al. (2015) Beyond Axillary Lymph Node Metastasis, BMI and Menopausal Status Are Prognostic Determinants for Triple-Negative Breast Cancer Treated by Neoadjuvant Chemotherapy. PLoS ONE 10(12): e0144359. doi:[10.1371/journal.pone.0144359](https://doi.org/10.1371/journal.pone.0144359)



Reference

1. Bonsang-Kitzis H, Chaltier L, Belin L, Savignoni A, Rouzier R, Sablin M-P, et al. (2015) Beyond Axillary Lymph Node Metastasis, BMI and Menopausal Status Are Prognostic Determinants for Triple-Negative Breast Cancer Treated by Neoadjuvant Chemotherapy. PLoS ONE 10(12): e0144359. doi:[10.1371/journal.pone.0144359](https://doi.org/10.1371/journal.pone.0144359) PMID: [26684197](https://pubmed.ncbi.nlm.nih.gov/26684197/)

OPEN ACCESS

Citation: Bonsang-Kitzis H, Chaltier L, Belin L, Savignoni A, Rouzier R, Hamy A-S, et al. (2016) Correction: Beyond Axillary Lymph Node Metastasis, BMI and Menopausal Status Are Prognostic Determinants for Triple-Negative Breast Cancer Treated by Neoadjuvant Chemotherapy. PLoS ONE 11(7): e0159123. doi:[10.1371/journal.pone.0159123](https://doi.org/10.1371/journal.pone.0159123)

Published: July 7, 2016

Copyright: © 2016 Bonsang-Kitzis et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

1 **Pathological complete response and prognosis after neoadjuvant**
2 **chemotherapy for *HER2*-positive breast cancers before and after trastuzumab**
3 **era: results from a real life cohort.**

4 Anne-Sophie Hamy-Petit^{1,2}, Lisa Belin³, H  l  ne Bonsang-Kitzis^{1,2,4}, Caroline Paquet⁵, Jean-
5 Yves Pierga⁵, Florence Lerebours⁵, Paul Cottu⁵, Roman Rouzier⁶, Alexia Savignoni³, Marick
6 Lae⁶, Fabien Reyal^{1,2,4}

7

8 **Running title:** Neoadjuvant chemotherapy in *HER2*+ breast cancer

9

10 **Affiliations:**

11 1. Residual Tumor & Response to Treatment Laboratory, RT2Lab, Translational Research
12 Department, Institut Curie, 26, rue d'Ulm, Paris, F-75248, France.

13 2. U932, Immunity and Cancer, INSERM, Institut Curie, Paris, F-75248, France.

14 3. Biostatistics Department, Institut Curie, France

15 4. Department of Surgery, Institut Curie, Paris, F-75248, France.

16 5. Department of Medical Oncology, Institut Curie, France

17 6. Department of Tumor Biology, Institut Curie, 92100 Saint Cloud

18

19 **Corresponding author:**

20 Dr Fabien REYAL, Institut Curie, Department of Surgery, 26 rue d'Ulm, 75005 Paris

21 00 33 615271980; fabien.reyal@curie.fr

22

1 **Abstract:**

2 **Introduction:** Trastuzumab was introduced a decade ago and has improved outcomes for
3 *HER2*-positive breast cancer. We investigated the factors predictive of pathological complete
4 response (pCR), prognostic factors for disease-free survival (DFS), and interactions between
5 pCR and DFS after neoadjuvant treatment.

6 **Material and Methods:** We identified 287 patients with primary *HER2*-positive breast
7 cancers given neoadjuvant chemotherapy (NAC) between 2002 and 2011. Univariate and
8 multivariate analyses of clinical and pathological factors associated with pCR and DFS were
9 performed.

10 **Results:** pCR rates differed between patients receiving neoadjuvant trastuzumab treatment or
11 not (47.7% versus 19.3%, $p < 0.0001$). DFS also differed significantly between patients
12 receiving adjuvant trastuzumab or not (hazard ratio=4.84, 95% CI [2.52; 9.31], $p < 0.001$). We
13 analyzed 199 patients given neoadjuvant and adjuvant trastuzumab. Multivariate analysis
14 identified older age and hormone receptor-negative tumors as independent predictors of pCR.
15 T stage (hazard ratio=2.55, 95% CI [1.01; 6.48], $p = 0.05$) and strict pCR (hazard ratio=9.15,
16 95% CI [1.22; 68.83], $p = 0.03$) were independent predictors of DFS. The latter association
17 was significant in the HR-negative subgroup ($p = 0.02$), but not in the HR-positive subgroup
18 ($p = 0.12$).

19 **Conclusion:** Major pCR and DFS gains in *HER2*-positive BC were observed since
20 “trastuzumab” era. Further improvements rely on the enrollment of accurately selected
21 patients into clinical trials.

22 **Keywords:** Breast cancer; *HER2*-positive; neoadjuvant chemotherapy; pathologic complete
23 response; prognostic factors; trastuzumab

24

1 Introduction

2 Breast cancer (BC) is the most frequently diagnosed cancer and the leading cause of cancer-
3 related death in women. *HER2*-positive breast carcinomas display amplification and
4 overexpression of the *HER2* tyrosine kinase receptor gene (17q12). This subgroup is defined
5 by aggressive pathological features and a high rate of early distant metastatic events.
6 Trastuzumab-based treatments have been used for the last decade and have improved
7 outcomes in patients with early or metastatic *HER2*-positive breast cancer.

8 Neoadjuvant treatment is currently being used in patients with early-stage and advanced
9 disease. Its clinical benefits are: a) higher rates of breast-conserving surgery, b) similar
10 prognoses for breast cancer patients receiving a neoadjuvant and for those receiving an
11 adjuvant therapy regimen, c) a body of evidence showing that the achievement of a
12 pathological complete response (pCR) after neoadjuvant chemotherapy is associated with a
13 good prognosis in specific subgroups (triple-negative, *HER2*-positive). Furthermore, it may
14 serve as a test of *in vivo* chemosensitivity, making it possible to evaluate the efficacy of
15 systemic therapy early and to discontinue ineffective treatment.

16 In parallel, interest has increased in the use of pCR as a surrogate marker for long-term
17 outcome, to accelerate the approval process for new drugs, since the publication by the FDA
18 of a set of guidelines entitled "Guidance for Industry. Pathologic Complete Response in
19 Neoadjuvant Treatment of High-Risk Early-Stage Breast Cancer: Use as an Endpoint to
20 Support Accelerated Approval".

21 In the last few years, the combination of trastuzumab with neoadjuvant chemotherapy (NAC)
22 has become standard, since two phase III trials comparing a regimen in which trastuzumab
23 was added to NAC and NAC alone reported higher pCR rates (MD Anderson Cancer center
24 trial: pCR rates: 26.3 vs. 65.2% with and without trastuzumab respectively (Buzdar *et al.*,
25 2007); NOAH trial: pCR rates: 19% versus 38% respectively) and longer DFS for the
26 combined treatment (NOAH trial: 3-year EFS, 71% vs. 56% with and without trastuzumab,
27 respectively (Gianni *et al.*, 2010)). In patients with *HER2*-positive breast tumors for whom

1 neoadjuvant treatment is indicated, trastuzumab is generally added to chemotherapy, and the
2 patient then receives one year of adjuvant trastuzumab treatment.

3 However, factors predictive of pCR and prognostic factors for survival have yet to be
4 identified, and there is still no robust demonstration of the correlation between pCR and
5 outcome in patients treated with optimal therapy. The aim of this study was to identify factors
6 predictive of pCR and prognostic factors in a large cohort of *HER2*-positive breast cancer
7 patients treated by neoadjuvant chemotherapy plus trastuzumab.

8 **Materials and methods**

9 *Patients*

10 We analyzed a cohort of 287 T1-3NxM0 patients with *HER2*-positive invasive breast
11 carcinoma (NEOREP Cohort, CNIL declaration number 1547270) treated at Institut Curie
12 between 2002 and 2012. We included only unilateral, non-recurrent, non-inflammatory, non-
13 metastatic tumors, excluding T4 and lobular tumors. All patients received NAC, followed by
14 surgery and radiotherapy. The study was approved by the Breast Cancer Study Group of
15 Institut Curie and was conducted according to institutional and ethical rules concerning
16 research on tissue specimens and patients. Informed consent from the patients was not
17 required.

18

19 *Tumor samples*

20 The following histological features were retrieved: tumor type, initial tumor size and nodal
21 status, grade (Elston and Ellis), estrogen receptor (ER) and progesterone receptor (PR) status,
22 *HER2* status, number of metastatic nodes and total sentinel and non-sentinel nodes. ER and
23 PR status were determined as follows. Tissue sections were rehydrated and antigen retrieval
24 was carried out in citrate buffer (10 mM, pH 6.1). The sections were then incubated with
25 antibodies against for ER (clone 6F11, Novocastra, Leica Biosystems, Newcastle, UK; 1/200)
26 and PR (clone 1A6, Novocastra, 1/200). The antibodies were then detected with the
27 Vectastain Elite ABC peroxidase-conjugated mouse IgG kit (Vector, Burlingame, CA, USA),
28 with diaminobenzidine (Dako A/S, Glostrup, Denmark) as the chromogen. Positive and

1 negative controls were included in each run. Cases were considered positive for ER and PR if
2 at least 10% of the tumor nuclei were stained, in accordance with standard guidelines used in
3 France (Harvey *et al*, 1999) (Recommandations pour la Pratique Clinique : Saint Paul de Vence
4 2007 « cancers du sein »). Tumors were considered to be hormone receptor (HR)-positive if
5 they were positive for either ER or PR, and HR-negative if they were negative for both ER
6 and PR. HER2 overexpression status was determined according to the American Society of
7 Clinical Oncology (ASCO) guidelines (Wolff *et al.*, 2007).

8

9 *Treatments*

10 Patients were treated according to national guidelines. Neoadjuvant chemotherapy regimens
11 changed over time (anthracycline-based regimen or sequential anthracycline-taxane regimen),
12 with trastuzumab used in an adjuvant and/or neoadjuvant setting since the middle of the last
13 decade. Endocrine therapy (tamoxifen, aromatase inhibitor, or GnRH agonists) was
14 prescribed when indicated. Surgery was performed four to six weeks after the end of the
15 chemotherapy. All patients received adjuvant radiotherapy. Trastuzumab treatments changed
16 over time and we splitted the whole cohort into 3 distinct groups, according to trastuzumab
17 use. Patients who did not receive any trastuzumab were indicated as cohort A ($n= 35$);
18 patients who received only adjuvant trastuzumab were indicated as cohort B ($n=53$); patients
19 who received both neoadjuvant and adjuvant trastuzumab were indicated as cohort C ($n=$
20 199).

21

22 *Pathology assessment at NAC completion*

23 A pathologic complete response (pCR) was defined as the absence of residual invasive cancer
24 cells in the breast and axillary lymph nodes (ypT0/is + / ypN0). Strict pCR (spCR) was
25 defined as an absence of invasive and non-invasive residuals in the breast, and invasive
26 disease in the axillary nodes (ypT0 ypN0).

27

28 *Disease free survival and overall survival*

1 Disease-free survival (DFS) was defined as the time from surgery to death, loco-regional
2 recurrence or distant recurrence, whichever occurred first, and overall survival (OS) was
3 defined as the time from surgery to death. Patients for whom none of these events were
4 recorded were censored at the date of their last known contact. Survival probabilities were
5 estimated by the Kaplan-Meier method, and survival curves were compared with log-rank
6 tests.

7

8 *Descriptive analysis of pCR and DFS rates according to the three cohorts*

9 For the pCR rates descriptive analysis, because of the known major impact on trastuzumab
10 use on pCR rates, we chose to pool cohort A and B (in both of which patients did not receive
11 neoadjuvant trastuzumab) and compared the resulting pooled cohort to the cohort C (in
12 which patients received neoadjuvant trastuzumab).

13 For the DFS and OS descriptive analysis, because of the known major impact on trastuzumab
14 on DFS rates, we chose to pool cohort B and C (in both of which patients received
15 trastuzumab) and compared the resulting pooled cohort to the cohort A (in which patients did
16 not receive any trastuzumab).

17

18 *Statistical analysis*

19 The study population was described in terms of frequencies for qualitative variables or
20 medians and associated ranges for quantitative variables. The cutoff date for the analysis was
21 March 13th 2013.

22 The statistical analyses of the factors predictive of pCR and prognostic for DFS were
23 performed in the cohort C only, as neoadjuvant trastuzumab in association to chemotherapy
24 followed by adjuvant trastuzumab represents the gold standard treatment in 2015.

25 Factors predictive of pCR were introduced into a univariate logistic regression model. A
26 multivariate logistic model was then implemented. The covariates selected for the
27 multivariate analysis were those with a likelihood ratio test p -value lower than 0.10 in
28 univariate analysis. A backward stepwise selection procedure was used.

1 Hazard ratios and their associated 95% confidence intervals were calculated with the Cox
2 proportional hazard model. Variables with a p -value for the likelihood ratio test lower than
3 0.10 in univariate analysis were included in the multivariate model. Backward selection was
4 used to establish the final multivariate model. The proportional hazards hypothesis was tested
5 for each factor, with Schoenfeld's residuals test and plotting. The significance threshold was
6 5%. Analyses were performed with R software, version 2.13.2 (R Development Core Team,
7 2011).

8

9

10 **Results**

11 Overall, 287 patients were identified in our database. The baseline characteristics of these
12 patients are summarized in Table 1. All 287 patients received neoadjuvant chemotherapy and
13 underwent surgery followed by radiotherapy. The median age of the patients was 48 years old
14 (27-79); 193 patients had T2 tumors (67.2%), and 169 had clinically involved nodes (58.9%).
15 In total, 129 patients had hormone receptor-negative breast cancer (44.9%). Trastuzumab
16 treatments changed over time and the characteristics of the patients are presented by treatment
17 (cohort A, $n= 35$, no trastuzumab at all; cohort B, $n=53$, adjuvant trastuzumab only; cohort C,
18 $n= 199$, both neoadjuvant and adjuvant trastuzumab) in Table 1. There were significant
19 differences between cohorts A, B, C for treatment period, number of nodes involved (patients
20 in cohort C were less likely to have nodes involved), and median follow-up. Strict pathologic
21 complete response (spCR) and pathologic complete response (pCR) rates differed
22 significantly between the three cohorts ($p<0.0001$). These rates were higher in patients given
23 neoadjuvant trastuzumab (cohort C, pCR rate: 47.7%) than in patients who did not receive
24 this treatment (cohort A and B, pCR rate: 19.3% $p<0.0001$), and in HR-negative subgroups
25 than in HR-positive subgroups (pCR rate: 48.8% versus 31.2%, $p=0.003$) (Table 2).
26 DFS (Figure 1A) also differed significantly between cohorts ($p< 0.001$). Patients treated
27 without trastuzumab (cohort A) had a higher risk of relapse (hazard ratio = 4.84 95% CI
28 [2.52; 9.31]) than patients receiving adjuvant trastuzumab with or without neoadjuvant
29 trastuzumab (cohort B and C pooled). Five years disease-free-survival rates were 48.6%

1 (95%CI [34.5-68.3], cohort A) versus 83.5% (95% CI [77.6-89.9], cohort B and C pooled),
2 and were not different between cohort B and C (cohort B: 80.0%, 95%CI [69.5-92.0] versus
3 cohort C 85.8%, 95%CI [79.0-93.3]).

4 Overall survival (Figure 1B) was also significantly lower in cohort A (hazard ratio=9.01, 95%
5 CI [2.95-27.52]) than in cohort B and C pooled ($p<0.001$; 5 years OS rates: 76.9%, 95% CI
6 [64.1-92.3]) versus 96.9; 95% CI [94.2-99.7] respectively).

7 Pathological complete response was predicted and prognostic analysis performed for cohort C
8 only (patients who received optimal neoadjuvant and adjuvant treatment, $n=199$). After
9 neoadjuvant treatment, 66 patients had no residual disease on the surgical specimen, and 29
10 patients had residual carcinoma *in situ* only (strict pCR rate: 33.2% (66/199); pCR:
11 47.7%(95/199)). The following results are given for strict pCR. Univariate logistic regression
12 analysis identified two factors correlated with spCR: age at diagnosis and hormone receptor
13 expression. Both factors remained significant in the multivariate logistic regression model
14 (Table 3). Strict pathologic complete response rates increased with age in both HR-positive
15 tumors (12.5, 18.6 and 28.6% for patients <45 years old, 45 to 55, and >55 years old
16 respectively), and in HR-negative ones (27.3, 36.0 and 50.0% respectively) (Figure 2).

17 After a median of 33 months of follow-up (range: 6-92), 18 patients experienced relapses (8
18 local, 3 regional, 7 distant). Two of these patients died. In univariate analysis, the factors
19 associated with DFS were age at diagnosis, spCR and pCR, menopausal status and initial
20 tumor stage. Tumor stage (T3: HR=2.55, 95%CI [1.01-6.48] versus T1-T2: HR=1, reference
21 class) and strict pathological complete response (No pCR: HR=9.15, 95%CI [1.22-68.83]
22 versus spCR (reference class), $p=0.03$) remained significantly associated with DFS in
23 multivariate analysis (Table 4), though the number of events was very low in patients whose
24 tumor achieved pCR after NAC. Five years DFS rates were 78% (95%CI [66.9-90.9]; No
25 pCR group) versus 95% (95%CI [89.4-100]; spCR group) respectively.

26 The persistence of *in situ* carcinoma after chemotherapy was not associated with shorter DFS
27 than the absence of any residual disease ($p=0.17$), or invasive disease only ($p=0.32$).

28 Pathologic complete response was positively associated with DFS in patients with HR-
29 negative tumors (Figure 3A), but not in those with HR-positive tumors (Figure 3B).

1

2 **Discussion**

3 Our retrospective longitudinal study highlights the major impact of the introduction of
4 trastuzumab on *HER2*-positive tumors, with a dramatic improvement in pCR (19.3% to
5 47.7%), DFS (5y DFS: 48.6% to 83.5%), and OS rates (5y OS: 76.9% to 96.9%) between
6 “pre-trastuzumab” and “trastuzumab” eras. In patients treated by neoadjuvant chemotherapy
7 plus trastuzumab, we identified age at diagnosis and hormone receptor status as predictive
8 factors for spCR, and pCR and tumor stage at diagnosis as prognostic factors for DFS.

9 Our study confirms that patients with pCR have excellent DFS and OS. Several studies
10 documented trastuzumab benefits in real-world practice in the adjuvant (Vici *et al.* , 2014)
11 (Matos *et al.* , 2014) (Inwald *et al.* , 2014) (Bonifazi *et al.* , 2014)(Seferina *et al.* , 2015)
12 (Jackisch *et al.* , 2014), and in the metastatic setting (Olson *et al.* , 2013) (Karam *et al.* , 2013)
13 (Park *et al.* , 2009)(Jackisch *et al.* , 2014). Most of these authors found that the magnitude of
14 trastuzumab benefits was equivalent to what was observed in clinical trials (improvement the
15 relative risk for DFS by approximately 50% and OS by 30%). Few-if any- observational
16 studies focused on the neoadjuvant setting. Our results suggest an even higher magnitude of
17 trastuzumab benefits in a population of *HER2*-positive breast tumors treated by NAC. Due to
18 the retrospective, non randomized design of the study, we can not conclude to the single role
19 of trastuzumab effect. Indeed, there were significant differences in the three cohorts in the
20 number of nodes involved as node negative patients represented 77.4% of the cohort C, versus
21 60% and 47.2% of the cohort A and B respectively. As it is known that the prognostic of
22 breast carcinoma following neoadjuvant chemotherapy is largely driven by nodal
23 status(Hennessy *et al.*, 2005), we can assume that the dramatic differences in DFS between the
24 3 cohorts are not solely explained by the trastuzumab treatment, but also by post NAC nodal
25 status.

26 As expected from previous studies of neoadjuvant treatment(Untch *et al.*, 2012)(Baselga *et*
27 *al.*, 2012; Gianni *et al.*, 2012), the absence of hormone receptor expression was an important
28 predictor of pCR. This relationship may be quantitative, as some authors have reported an
29 inverse correlation between the level of HR expression and pCR (Bhargava *et al.*, 2011).

1 Trastuzumab emtansine (T-DM1) is an antibody–drug conjugate composed of the cytotoxic
2 agent DM1 and trastuzumab, connected by a stable thioether linker. The ADAPT trial
3 (NCT01745965) is currently investigating if the concomitant adjunction of endocrine therapy
4 to T-DM1 neoadjuvant therapy would increase pCR rates in *HER2+*/HR+ operable breast
5 cancers.

6 In our cohort, older age was significantly associated with spCR. These finding are consistent
7 with those of a retrospective study of 229 *HER2*-positive tumors treated by NAC plus
8 trastuzumab, in which both being young and premenopausal status were significantly
9 associated with lower pCR rates (Kim *et al.*, 2013). By contrast, Huober *et al.* found no
10 difference in pCR rates between two age groups (<40 years versus ≥ 40 years) for 475 *HER2*-
11 positive tumors (Huober *et al.*, 2010). However, none of these patients were treated with
12 neoadjuvant trastuzumab. Similarly, the German Breast Group (GBG) and the AGO-B study
13 group published a meta-analysis focusing on the impact of age on neoadjuvant chemotherapy
14 outcomes. In 1820 patients with *HER2*-positive tumors, pCR rates did not differ significantly
15 with age, in either HR-positive or HR-negative tumors (Loibl *et al.*, 2015). Patients with
16 *HER2*-positive disease received anti-*HER2* treatment as part of the neoadjuvant treatment in 3
17 on 8 trials of this meta-analysis.

18 Initial T stage remained a significant prognostic factor. This finding is consistent with those
19 of several other studies, (Tanioka *et al.*, 2014; Kim *et al.*, 2013; Takada *et al.*, 2014), although
20 similar results were obtained only for the HR-negative subgroup in the study by Takada *et al.*

21 In a sub-study of EORTC 10994/BIG 1-00 phase III trial (Fei *et al.* , 2015) on 283 patients
22 with pCR achievement after NAC, only clinical tumor size independently predicted relapse.
23 Several hypotheses can be drawn to explain the independent impact of tumoral size. The first
24 one is that large tumors may be more likely to present intrinsic or acquired chemoresistance.
25 Causal factors may first include a variety of physical and mechanical effects (inefficient
26 distribution of the drug, central necrosis and hypoxia, anarchic neoangiogenesis etc...).
27 Second, the immune reaction appears to evolve with tumor progression, and it is known that
28 immune subpopulations densities change with increasing stage (Bindea *et al.* , 2013) (Fridman
29 *et al.* , 2012), potentially impairing the response to chemotherapy . Third, tumoral

1 heterogeneity increases with tumor size, leading to the potential emergence of drug-
2 multiresistant clones.

3 A second hypothesis considers the kinetics of the tumor growth. Mathematical modelings
4 (Hartung *et al.* , 2014) validate the link between primary tumor size and emission rate, ie,
5 metastatic spreading. In clinical practice, this relation between a large tumor size and the
6 presence of circulating tumor cells in peripheral blood has also been identified (Liao *et al.* ,
7 2014). Considering initial exponential growth phase of the Gompertz model (Benzekry *et al.* ,
8 2014) and the high proliferation rate of *HER2* positive breast cancers, it seems plausible that
9 these tumors may rapidly toggle from localized breast cancers to a micrometastatic disease.
10 The subsequent pivotal transition between micrometastases and macrometastases (namely the
11 metastatic colonization) is still poorly understood. It remains unknown if tumoral size may
12 impact this process. Both phenomena (chemoresistance and micrometastatic spreading) may
13 coexist and share pathways by complex homing interactions.

14 It remains a matter of debate whether pCR can be used as a surrogate for DFS in *HER2*-
15 positive breast carcinomas, particularly those that are HR-positive. In our cohort, residual
16 disease was associated with a hazard ratio for relapse of 9 relative to patients with spCR. This
17 effect was limited to HR-negative tumors. In a large meta-analysis of 6,377 patients with
18 primary breast cancer receiving neoadjuvant anthracycline-taxane-based chemotherapy in
19 seven randomized trials, Von Minckwitz (von Minckwitz *et al.*, 2012) identified pCR as a
20 surrogate marker for both DFS and OS in *HER2*-positive subgroups. In patients with *HER2*-
21 positive tumors treated with trastuzumab ($n=662$), pCR was associated with a hazard ratio of
22 2.85 ([1.69-4.83], $p<0.001$) for DFS, and of 14.11 ([1.93-103.03], $p<0.009$) for OS. However,
23 the prognostic impact of pCR was restricted to HR-negative tumors. It was not observed in
24 the luminal B/*HER2*-positive subgroup. In a recent pooled analysis of 12 international trials
25 and 11955 patients (CTNeoBC), Cortazar (Cortazar *et al.*, 2014) found a significant
26 association between pCR and event-free survival in both the HR-positive and HR-negative
27 subgroups, although the magnitude of this effect was greater in HR-negative tumors (HR-
28 positive, 0.58 [0.42-0.82]; HR-negative: 0.25 [0.18-0.34]). However, a subset of *HER2*-
29 positive breast cancer did not receive adjuvant trastuzumab. When the analysis was restricted

1 to patients who received trastuzumab, the association between pCR and overall survival was
2 not significant in HR-positive tumors (0.56 [0.23-1.37]). In addition, three multicenter
3 retrospective studies on *HER2*-positive breast tumors treated with NAC and trastuzumab
4 identified pCR as a surrogate marker for DFS in HR-negative disease (Tanioka *et al.*, 2014,
5 Takada *et al.*, 2014; Kim *et al.*, 2013), but the results for the HR-positive group were
6 discordant, with a positive association retrieved by some authors (Kim *et al.*, 2013), but not
7 others (Takada *et al.*, 2014; Tanioka *et al.*, 2014).

8 Our study adds weight to the findings of previous investigations, because it focuses on a
9 particular breast cancer subtype and reports results for a large population treated with
10 neoadjuvant chemotherapy and trastuzumab, the gold standard treatment in 2015. This study
11 provides a better representation of real-life experience than previous meta-analyses of clinical
12 trials, because, although meta-analysis provide an effective means of acquiring large amounts
13 of data, the patients included in clinical trials differ from the general population. Our data
14 confirm the association of pCR with DFS in *HER2*-positive HR-negative breast cancers and
15 provide new insight that could improve prognostic prediction. The absence of a significant
16 effect in the HR-positive subgroup might be due to biological differences though we can not
17 exclude a lack of statistical power.

18 The confirmation of a quantitative correlation between increments in pCR and gains in
19 survival in large datasets is of paramount importance for accelerated drug approval for the
20 neoadjuvant model. It is particularly important because the *HER2*-targeting drug pipeline
21 contains many candidates. The novel anti-*HER2* antibody pertuzumab has obtained
22 accelerated approval from the US Food and Drug Administration (FDA) (Prowell & Pazdur,
23 2012) for use in the neoadjuvant setting for *HER2*-positive breast cancer, based on the results
24 of the NEOSPHERE trial (Gianni *et al.*, 2012). Definitive approval for pertuzumab will
25 depend of the results of the APHINITY trial, evaluating the addition of pertuzumab to
26 adjuvant trastuzumab-based chemotherapy. Controversy concerning the legitimacy of pCR as
27 a surrogate reemerged with the results of the ALTTO trial in ASCO 2014 (Piccart-Gebhart *et*
28 *al.*, 2014). In this study, the addition of lapatinib to standard trastuzumab adjuvant therapy
29 was not found to improve survival in women with *HER2*-positive early breast cancer. This

1 result was unexpected, because the combination of lapatinib and trastuzumab was associated
2 with higher rates of pCR rates in the neoALTTO trial (Baselga *et al.*, 2012). Improving pCR
3 rates may theoretically: (i) increase conservative treatment probabilities; (ii) identify a
4 population at higher risk of relapse and thus help selecting patients likely to benefit from new
5 therapies. Accurate and sharp patients selection may avoid failure of all-comers trials as
6 ALTTO and MARIANNE (NCT01120184). The KATHERINE trial (NCT01772472) is
7 currently investigating TDM-1 as alternative adjuvant treatment to trastuzumab in *HER2*-
8 positive patients with residual disease following NAC. Our study supports this design for new
9 drug testing, bearing in mind that even in patients with residual disease, DFS rates were high
10 in our cohort.

11 **Conclusion**

12 Trastuzumab considerably modifies the prognosis of *HER2*-positive breast carcinomas. These
13 tumors have an excellent prognosis when pCR is achieved. However, it remains unclear
14 whether second-line *HER2*-targeted treatments with pertuzumab, lapatinib or TDM1
15 following neoadjuvant chemotherapy improve survival in selected patients. Our findings
16 suggest that patients with *HER2*-positive tumors of a large initial size, for which pCR is not
17 achieved at the end of neoadjuvant chemotherapy, remain at risk of relapse despite adjuvant
18 trastuzumab treatments. Such patients could be studied in second-line treatment trials.
19 However, there is a need to rethink future clinical trials designs, bearing in mind several
20 pitfalls: (i) sufficient recruitment of patients despite the scarcity of trastuzumab-resistant
21 patients; (ii) consider a different disease setting with possibly already micrometastatic
22 populations and thus consider new therapeutic targets to investigate (Mina & Sledge, 2011) ;
23 (iii). Finally, expected events may whatever be low, and only international collaborative
24 works will allow sufficient population size. The challenge still needs to be overcome.

25

26

27

28

29 **Acknowledgments**

1 We thank Roche* France for financial support for construction of the Institut Curie
2 neoadjuvant database (NEOREP).
3 AS Hamy-Petit was supported by an ITMO-INSERM-AVIESAN cancer translational
4 research grant.
5 Funding was also obtained from the Site de Recherche Intégrée en Cancérologie/Institut
6 National du Cancer (Grant No. INCa-DGOS-4654)
7

1 Figures captions:

2 **Figure 1:** Disease-free survival (Fig. 1A) and overall survival (Fig. 1B), by cohort.

3 **Figure 2:** pCR rates by age and hormone receptor status.

4 **Figure 3:** Association of pCR and DFS in patients with HR-negative tumors (Fig. 3A), and
5 patients with HR-positive tumors (Fig. 3B).

6

7 Table captions:

8 **Table 1:** Patients, tumors, and treatment characteristics, by cohort (cohort A, $n=35$, no
9 trastuzumab; cohort B, $n=53$, adjuvant trastuzumab only; cohort C, $n= 199$, both neoadjuvant
10 and adjuvant trastuzumab).

11 Abbreviations : tz*= trastuzumab.

12 **Table 2:** Pathological response rates, by definition, by cohort, and by hormone receptor
13 status.

14 **Table 3:** Odds ratios for predicting strict pCR (univariate and multivariate analyses)

15 **Table 4:** Hazard ratios for predicting DFS (univariate and multivariate analyses)

16

17

1 References:

- 2 Baselga J, Bradbury I, Eidtmann H, Di Cosimo S, de Azambuja E, Aura C, Gómez H, Dinh P,
3 Fauria K, Van Dooren V, Aktan G, Goldhirsch A, Chang T-W, Horváth Z, Coccia-Portugal
4 M, Domont J, Tseng L-M, Kunz G, Sohn JH, Semiglazov V, Lerzo G, Palacova M, Probachai
5 V, Puzstai L, Untch M, Gelber RD, Piccart-Gebhart M, NeoALTTTO Study Team (2012)
6 Lapatinib with trastuzumab for HER2-positive early breast cancer (NeoALTTTO): a
7 randomised, open-label, multicentre, phase 3 trial. *Lancet* **379**: 633–640,
8 doi:10.1016/S0140-6736(11)61847-3.
- 9 Benzekry S, Lamont C, Beheshti A, Tracz A, Ebos JML, Hlatky L, Hahnfeldt P (2014)
10 Classical mathematical models for description and prediction of experimental tumor
11 growth. *PLoS Comput Biol* **10**: e1003800, doi:10.1371/journal.pcbi.1003800.
- 12 Bhargava R, Dabbs DJ, Beriwal S, Yildiz IA, Badve P, Soran A, Johnson RR, Brufsky AM,
13 Lembersky BC, McGuire KP, Ahrendt GM (2011) Semiquantitative hormone receptor
14 level influences response to trastuzumab-containing neoadjuvant chemotherapy in
15 HER2-positive breast cancer. *Mod Pathol Off J U S Can Acad Pathol Inc* **24**: 367–374,
16 doi:10.1038/modpathol.2010.209.
- 17 Bindea G, Mlecnik B, Tosolini M, Kirilovsky A, Waldner M, Obenauf AC, Angell H,
18 Fredriksen T, Lafontaine L, Berger A, Bruneval P, Fridman WH, Becker C, Pagès F,
19 Speicher MR, Trajanoski Z, Galon J (2013) Spatiotemporal dynamics of intratumoral
20 immune cells reveal the immune landscape in human cancer. *Immunity* **39**: 782–795,
21 doi:10.1016/j.immuni.2013.10.003.
- 22 Bonifazi M, Franchi M, Rossi M, Zambelli A, Moja L, Zambon A, Corrao G, La Vecchia C,
23 Zocchetti C, Negri E (2014) Long term survival of HER2-positive early breast cancer
24 treated with trastuzumab-based adjuvant regimen: a large cohort study from clinical
25 practice. *Breast Edinb Scotl* **23**: 573–578, doi:10.1016/j.breast.2014.05.022.
- 26 Buzdar AU, Valero V, Ibrahim NK, Francis D, Broglio KR, Theriault RL, Puzstai L, Green
27 MC, Singletary SE, Hunt KK, Sahin AA, Esteva F, Symmans WF, Ewer MS, Buchholz TA,
28 Hortobagyi GN (2007) Neoadjuvant therapy with paclitaxel followed by 5-fluorouracil,
29 epirubicin, and cyclophosphamide chemotherapy and concurrent trastuzumab in human
30 epidermal growth factor receptor 2-positive operable breast cancer: an update of the
31 initial randomized study population and data of additional patients treated with the
32 same regimen. *Clin Cancer Res Off J Am Assoc Cancer Res* **13**: 228–233,
33 doi:10.1158/1078-0432.CCR-06-1345.
- 34 Cortazar P, Zhang L, Untch M, Mehta K, Costantino JP, Wolmark N, Bonnefoi H, Cameron
35 D, Gianni L, Valagussa P, Swain SM, Prowell T, Loibl S, Wickerham DL, Bogaerts J, Baselga
36 J, Perou C, Blumenthal G, Blohmer J, Mamounas EP, Bergh J, Semiglazov V, Justice R,
37 Eidtmann H, Paik S, Piccart M, Sridhara R, Fasching PA, Slaets L, Tang S, Gerber B, Geyer
38 CE, Pazdur R, Ditsch N, Rastogi P, Eiermann W, von Minckwitz G (2014) Pathological
39 complete response and long-term clinical benefit in breast cancer: the CTNeoBC pooled
40 analysis. *Lancet* **384**: 164–172, doi:10.1016/S0140-6736(13)62422-8.
- 41 Fei F, Messina C, Slaets L, Chakiba C, Cameron D, Bogaerts J, Bonnefoi H (2015) Tumour
42 size is the only predictive factor of distant recurrence after pathological complete
43 response to neoadjuvant chemotherapy in patients with large operable or locally
44 advanced breast cancers: a sub-study of EORTC 10994/BIG 1-00 phase III trial. *Eur J*
45 *Cancer Oxf Engl* **1990** **51**: 301–309, doi:10.1016/j.ejca.2014.11.023.
- 46 Fridman WH, Pagès F, Sautès-Fridman C, Galon J (2012) The immune contexture in
47 human tumours: impact on clinical outcome. *Nat Rev Cancer* **12**: 298–306,
48 doi:10.1038/nrc3245.
- 49 Gianni L, Eiermann W, Semiglazov V, Manikhas A, Lluch A, Tjulandin S, Zambetti M,

- 1 Vazquez F, Byakhov M, Lichinitser M, Climent MA, Ciruelos E, Ojeda B, Mansutti M,
2 Bozhok A, Baronio R, Feyereislova A, Barton C, Valagussa P, Baselga J (2010)
3 Neoadjuvant chemotherapy with trastuzumab followed by adjuvant trastuzumab versus
4 neoadjuvant chemotherapy alone, in patients with HER2-positive locally advanced
5 breast cancer (the NOAH trial): a randomised controlled superiority trial with a parallel
6 HER2-negative cohort. *Lancet* **375**: 377–384, doi:10.1016/S0140-6736(09)61964-4.
- 7 Gianni L, Pienkowski T, Im Y-H, Roman L, Tseng L-M, Liu M-C, Lluch A, Staroslawska E,
8 de la Haba-Rodriguez J, Im S-A, Pedrini JL, Poirier B, Morandi P, Semiglazov V,
9 Srimuninnimit V, Bianchi G, Szado T, Ratnayake J, Ross G, Valagussa P (2012) Efficacy
10 and safety of neoadjuvant pertuzumab and trastuzumab in women with locally
11 advanced, inflammatory, or early HER2-positive breast cancer (NeoSphere): a
12 randomised multicentre, open-label, phase 2 trial. *Lancet Oncol* **13**: 25–32,
13 doi:10.1016/S1470-2045(11)70336-9.
- 14 Hartung N, Mollard S, Barbolosi D, Benabdallah A, Chapuisat G, Henry G, Giacometti S,
15 Iliadis A, Ciccolini J, Faivre C, Hubert F (2014) Mathematical modeling of tumor growth
16 and metastatic spreading: validation in tumor-bearing mice. *Cancer Res* **74**: 6397–6407,
17 doi:10.1158/0008-5472.CAN-14-0721.
- 18 Harvey JM, Clark GM, Osborne CK, Allred DC (1999) Estrogen receptor status by
19 immunohistochemistry is superior to the ligand-binding assay for predicting response
20 to adjuvant endocrine therapy in breast cancer. *J Clin Oncol Off J Am Soc Clin Oncol* **17**:
21 1474–1481.
- 22 Hennessy BT, Hortobagyi GN, Rouzier R, Kuerer H, Sneige N, Buzdar AU, Kau SW,
23 Fornage B, Sahin A, Broglio K, Singletary SE, Valero V (2005) Outcome after pathologic
24 complete eradication of cytologically proven breast cancer axillary node metastases
25 following primary chemotherapy. *J Clin Oncol Off J Am Soc Clin Oncol* **23**: 9304–9311,
26 doi:10.1200/JCO.2005.02.5023.
- 27 Huober J, von Minckwitz G, Denkert C, Tesch H, Weiss E, Zahm DM, Belau A, Khandan F,
28 Hauschild M, Thomssen C, Högel B, Darb-Esfahani S, Mehta K, Loibl S (2010) Effect of
29 neoadjuvant anthracycline-taxane-based chemotherapy in different biological breast
30 cancer phenotypes: overall results from the GeparTrio study. *Breast Cancer Res Treat*
31 **124**: 133–140, doi:10.1007/s10549-010-1103-9.
- 32 Inwald EC, Ortmann O, Zeman F, Koller M, Hofstädter F, Gerstenhauer M, Klinkhammer-
33 Schalke M (2014) Guideline concordant therapy prolongs survival in HER2-positive
34 breast cancer patients: results from a large population-based cohort of a cancer registry.
35 *BioMed Res Int* **2014**: 137304, doi:10.1155/2014/137304.
- 36 Jackisch C, Schoenegg W, Reichert D, Welslau M, Selbach J, Harich H-D, Tesch H,
37 Wohlfarth T, Eustermann H, Hinke A (2014) Trastuzumab in advanced breast cancer--a
38 decade of experience in Germany. *BMC Cancer* **14**: 924, doi:10.1186/1471-2407-14-924.
- 39 Karam I, Hamilton S, Nichol A, Woods R, Speers C, Kennecke H, Tyldesley S (2013)
40 Population-based outcomes after brain radiotherapy in patients with brain metastases
41 from breast cancer in the Pre-Trastuzumab and Trastuzumab eras. *Radiat Oncol Lond*
42 *Engl* **8**: 12, doi:10.1186/1748-717X-8-12.
- 43 Kim MM, Allen P, Gonzalez-Angulo AM, Woodward WA, Meric-Bernstam F, Buzdar AU,
44 Hunt KK, Kuerer HM, Litton JK, Hortobagyi GN, Buchholz TA, Mittendorf EA (2013)
45 Pathologic complete response to neoadjuvant chemotherapy with trastuzumab predicts
46 for improved survival in women with HER2-overexpressing breast cancer. *Ann Oncol Off*
47 *J Eur Soc Med Oncol ESMO* **24**: 1999–2004, doi:10.1093/annonc/mdt131.
- 48 Liao Y, Wang S-Y, Meng X-Y, Yang J, Shi M-J, Liu H-L, Chen F-F, Xiong B (2014) Circulating
49 tumor cells in breast cancer and its association with tumor clinicopathological

- 1 characteristics: a meta-analysis. *Med Oncol Northwood Lond Engl* **31**: 343,
2 doi:10.1007/s12032-014-0343-7.
- 3 Loibl S, Jackisch C, Lederer B, Untch M, Paepke S, Kümmel S, Schneeweiss A, Huober J,
4 Hilfrich J, Hanusch C, Gerber B, Eidtmann H, Denkert C, Costa SD, Blohmer J-U,
5 Nekljudova V, Mehta K, von Minckwitz G (2015) Outcome after neoadjuvant
6 chemotherapy in young breast cancer patients: a pooled analysis of individual patient
7 data from eight prospectively randomized controlled trials. *Breast Cancer Res Treat*
8 doi:10.1007/s10549-015-3479-z.
- 9 Matos E, Zakotnik B, Kuhar CG (2014) Effectiveness of adjuvant trastuzumab in daily
10 clinical practice. *Radiol Oncol* **48**: 403–407, doi:10.2478/raon-2013-0081.
- 11 Mina LA, Sledge GW (2011) Rethinking the metastatic cascade as a therapeutic target.
12 *Nat Rev Clin Oncol* **8**: 325–332, doi:10.1038/nrclinonc.2011.59.
- 13 Von Minckwitz G, Untch M, Blohmer J-U, Costa SD, Eidtmann H, Fasching PA, Gerber B,
14 Eiermann W, Hilfrich J, Huober J, Jackisch C, Kaufmann M, Konecny GE, Denkert C,
15 Nekljudova V, Mehta K, Loibl S (2012) Definition and impact of pathologic complete
16 response on prognosis after neoadjuvant chemotherapy in various intrinsic breast
17 cancer subtypes. *J Clin Oncol Off J Am Soc Clin Oncol* **30**: 1796–1804,
18 doi:10.1200/JCO.2011.38.8595.
- 19 Olson EM, Najita JS, Sohl J, Arnaout A, Burstein HJ, Winer EP, Lin NU (2013) Clinical
20 outcomes and treatment practice patterns of patients with HER2-positive metastatic
21 breast cancer in the post-trastuzumab era. *Breast Edinb Scotl* **22**: 525–531,
22 doi:10.1016/j.breast.2012.12.006.
- 23 Park YH, Park MJ, Ji SH, Yi SY, Lim DH, Nam DH, Lee J-I, Park W, Choi DH, Huh SJ, Ahn JS,
24 Kang WK, Park K, Im Y-H (2009) Trastuzumab treatment improves brain metastasis
25 outcomes through control and durable prolongation of systemic extracranial disease in
26 HER2-overexpressing breast cancer patients. *Br J Cancer* **100**: 894–900,
27 doi:10.1038/sj.bjc.6604941.
- 28 Piccart-Gebhart MJ, Holmes AP, Baselga J, Azambuja ED, Dueck AC, Viale G, Zujewski JA,
29 Goldhirsch A, Santillana S, Pritchard KI, Wolff AC, Jackisch C, Lang I, Untch M, Smith IE,
30 Boyle F, Xu B, Gomez HL, Gelber RD, Perez EA (2014) First results from the phase III
31 ALTTO trial (BIG 2-06; NCCTG [Alliance] N063D) comparing one year of anti-HER2
32 therapy with lapatinib alone (L), trastuzumab alone (T), their sequence (T→L), or their
33 combination (T+L) in the adjuvant treatment of HER2-positive early breast cancer
34 (EBC). *J Clin Oncol* **32**:5s:
- 35 Prowell TM, Pazdur R (2012) Pathological complete response and accelerated drug
36 approval in early breast cancer. *N Engl J Med* **366**: 2438–2441,
37 doi:10.1056/NEJMp1205737.
- 38 R Development Core Team (2011) R: A Language and Environment for Statistical
39 Computing. R Foundation for Statistical Computing.
- 40 Recommandations pour la Pratique Clinique : Saint Paul de Vence 2007 « cancers du
41 sein ».
- 42 Seferina SC, Lobbezoo DJA, de Boer M, Dercksen MW, van den Berkmortel F, van
43 Kampen RJW, van de Wouw AJ, de Vries B, Joore MA, Peer PGM, Voogd AC, Tjan-Heijnen
44 VCG (2015) Real-Life Use and Effectiveness of Adjuvant Trastuzumab in Early Breast
45 Cancer Patients: A Study of the Southeast Netherlands Breast Cancer Consortium. *The*
46 *Oncologist* doi:10.1634/theoncologist.2015-0006.
- 47 Takada M, Ishiguro H, Nagai S, Ohtani S, Kawabata H, Yanagita Y, Hozumi Y, Shimizu C,
48 Takao S, Sato N, Kosaka Y, Sagara Y, Iwata H, Ohno S, Kuroi K, Masuda N, Yamashiro H,
49 Sugimoto M, Kondo M, Naito Y, Sasano H, Inamoto T, Morita S, Toi M (2014) Survival of

1 HER2-positive primary breast cancer patients treated by neoadjuvant chemotherapy
2 plus trastuzumab: a multicenter retrospective observational study (JBCRG-C03 study).
3 *Breast Cancer Res Treat* **145**: 143–153, doi:10.1007/s10549-014-2907-9.
4 Tanioka M, Sasaki M, Shimomura A, Fujishima M, Doi M, Matsuura K, Sakuma T,
5 Yoshimura K, Saeki T, Ohara M, Tsurutani J, Watatani M, Takano T, Kawabata H, Mukai H,
6 Naito Y, Hirokaga K, Takao S, Minami H (2014) Pathologic complete response after
7 neoadjuvant chemotherapy in HER2-overexpressing breast cancer according to
8 hormonal receptor status. *Breast Edinb Scotl* **23**: 466–472,
9 doi:10.1016/j.breast.2014.03.008.
10 Untch M, Loibl S, Bischoff J, Eidtmann H, Kaufmann M, Blohmer J-U, Hilfrich J, Strumberg
11 D, Fasching PA, Kreienberg R, Tesch H, Hanusch C, Gerber B, Rezai M, Jackisch C, Huober
12 J, Kühn T, Nekljudova V, von Minckwitz G, German Breast Group (GBG),
13 Arbeitsgemeinschaft Gynäkologische Onkologie-Breast (AGO-B) Study Group (2012)
14 Lapatinib versus trastuzumab in combination with neoadjuvant anthracycline-taxane-
15 based chemotherapy (GeparQuinto, GBG 44): a randomised phase 3 trial. *Lancet Oncol*
16 **13**: 135–144, doi:10.1016/S1470-2045(11)70397-7.
17 Vici P, Pizzuti L, Natoli C, Moscetti L, Mentuccia L, Vaccaro A, Sergi D, Di Lauro L, Trenta
18 P, Seminara P, Santini D, Iezzi L, Tinari N, Bertolini I, Sini V, Mottolese M, Giannarelli D,
19 Giotta F, Maugeri-Saccà M, Barba M, Marchetti P, Michelotti A, Sperduti I, Gamucci T
20 (2014) Outcomes of HER2-positive early breast cancer patients in the pre-trastuzumab
21 and trastuzumab eras: a real-world multicenter observational analysis. The RETROHER
22 study. *Breast Cancer Res Treat* **147**: 599–607, doi:10.1007/s10549-014-3133-1.
23 Wolff AC, Hammond MEH, Schwartz JN, Hagerty KL, Allred DC, Cote RJ, Dowsett M,
24 Fitzgibbons PL, Hanna WM, Langer A, McShane LM, Paik S, Pegram MD, Perez EA, Press
25 MF, Rhodes A, Sturgeon C, Taube SE, Tubbs R, Vance GH, van de Vijver M, Wheeler TM,
26 Hayes DF, American Society of Clinical Oncology, College of American Pathologists
27 (2007) American Society of Clinical Oncology/College of American Pathologists
28 guideline recommendations for human epidermal growth factor receptor 2 testing in
29 breast cancer. *J Clin Oncol Off J Am Soc Clin Oncol* **25**: 118–145,
30 doi:10.1200/JCO.2006.09.2775.
31
32

1

The presence of an *in situ* component on pre-treatment biopsy is not associated with response to neoadjuvant chemotherapy for breast cancer

Charlotte Morel¹¶, Than Lam²¶, Julie Labrosse¹, Enora Laas¹, Jean-Guillaume Feron¹, Florence Coussy³, Marick Lae⁴, Fabien Reyal^{1,5*}, Anne-Sophie Hamy^{3,5}

1 Department of Surgery, Institut Curie, Paris, France.

2 Service de Gynécologie, Hôpitaux universitaires de Genève, Switzerland.

3 Medical Oncology department, Centre René Huguenin, Saint Cloud, France.

4 Department of Tumor biology, Institut Curie, Paris, France.

5 Residual Tumor & Response to Treatment Laboratory, RT2Lab, PSL Research University, Translational Research Department, INSERM, U932 Immunity and Cancer, F-75248, Paris, France.

*** Corresponding author:**

E-mail: fabien.reyal@curie.fr (FR)

¶Charlotte Morel and Than Lam equally contributed to this work.

1 Abstract

2

3 **Background.** A ductal *in situ* (DCIS) component is often associated with invasive breast
4 carcinoma (BC). It is unknown whether this parameter affects response to treatment. In this
5 study, we assessed the predictive value of DCIS component on pathologic complete response
6 (pCR) after neoadjuvant chemotherapy (NAC).

7 **Method.** We analyzed a cohort of 1148 T1-3NxM0 breast cancer (BC) patients treated by NAC
8 at Institut Curie between 2002 and 2012. Presence of a DCIS component was retrospectively
9 retrieved both in pre-NAC biopsy pathological report and surgical specimen.

10 **Results.** 1148 BC patients treated with NAC who had available pre and post-NAC data on *in*
11 *situ* component were included. DCIS was present before NAC in 19.6% of the population.

12 Overall, 283 patients (19.4%) reached pCR after NAC. There were no significant association
13 between the presence of pre-NAC DCIS in biopsy and pCR. After multivariate analysis
14 including subtype, tumor size, grade, mitotic index, Ki67 index, only BC subtype
15 (luminal/TNBC/*HER2*-positive) and Ki67 were significantly associated with pCR.

16 **Conclusion.** Presence of a DCIS component on pre-NAC biopsy is not associated with pCR
17 and does not seem to be a critical factor to predict response to NAC.

Keywords: Breast cancer - Neoadjuvant chemotherapy (NAC)- Ductal carcinoma in situ

(DCIS) - Pathological complete response (pCR)

18 **Background**

19 Neoadjuvant or pre-operative chemotherapy (NAC) is administered to patients with
20 inflammatory or locally advanced breast cancer (BC). It is now used more widely, including in
21 early-stage breast cancers. On one hand, since it can reduce some tumors and make them
22 resectable [1-2], this strategy increases breast-conserving surgery rates; on the other hand, it
23 enables to study the effect of chemotherapy on the tumor itself by analyzing residual tumor
24 burden on surgical specimens. Furthermore, it was shown that patients who reached
25 pathological complete response (pCR) after neoadjuvant systemic treatment had a better long
26 term outcome [3-4]. Identifying markers for predicting response and resistance to NAC has
27 become an important research objective [5]. The major clinical and biological factors yet
28 identified to predict the chance of achieving pCR are age [6], body mass index (BMI) [7],
29 proliferation biomarker Ki-67 [8], estrogen receptor status [5], and more recently, tumor
30 infiltrating lymphocytes (TILs) [9].

31

32 Ductal carcinoma *in situ* (DCIS) is associated with invasive disease in more than half of
33 invasive BC cases [10]. It is defined as a neoplastic proliferation of epithelial cells confined to
34 the ductal-lobular system that can evolve to invasive breast cancer. Although it is generally
35 assumed that DCIS does not respond to NAC [11], the effect of chemotherapy on *in situ*
36 component has scarcely been studied. In this study, we assessed the predictive value of *in situ*
37 component on pre-NAC biopsy on pCR in a real-life cohort of patients treated by neoadjuvant
38 chemotherapy.

39

40 In this study, we assessed the predictive value of *in situ* component on pre-NAC biopsy on pCR
41 in a real-life cohort of patients treated by neoadjuvant chemotherapy.

42 **Methods**

43 **Patients**

44 We analyzed a cohort of 1148 T1-3NxM0 patients with invasive breast carcinoma (NEOREP
45 Cohort, CNIL declaration number 1547270) treated at Institut Curie, Paris, between 2002 and
46 2012. We included patients with only unilateral, non-recurrent, non-inflammatory, non-
47 metastatic tumors that had an indication of NAC and for whom pre and post NAC data on
48 DCIS component were available. Every patient received NAC, followed by surgery and
49 radiotherapy when indicated. The study was approved by the Breast Cancer Study Group of
50 Institut Curie and by the CNIL concerning data analysis for research purposes. It was
51 conducted according to institutional and ethical rules regarding research on tissue specimens
52 and patients. Written informed consent from the patients was not required by French
53 regulations. All data were anonymized prior to access and analysis.

54

55 **Treatments**

56 Patients were treated according to national guidelines. NAC regimens changed over time
57 (anthracycline-based regimen or sequential anthracycline-taxane regimen), with trastuzumab
58 used in an adjuvant and/or neoadjuvant setting since 2005 for *HER2*-positive breast cancer.
59 Surgery was performed four to six weeks after the end of chemotherapy. Most patients (98.2%,
60 n=1127) received adjuvant radiotherapy. Endocrine therapy (tamoxifen, aromatase inhibitor,
61 and/or GnRH agonists) was prescribed when indicated.

62

63 **Tumor samples**

64 Tumor samples were collected in routine care in the management of breast cancer at Institut
65 Curie. ER and PR status were determined as follows. Tissue sections were rehydrated and

66 antigen retrieval was carried out in citrate buffer (10 mM, pH 6.1). The sections were then
67 incubated with antibodies against ER (clone 6F11, Novocastra, Leica Biosystems, Newcastle,
68 UK; 1/200) and PR (clone 1A6, Novocastra, 1/200). Antibody binding was detected with
69 Vectastain Elite ABC peroxidase-conjugated mouse IgG kit (Vector, Burlingame, CA, USA),
70 with diaminobenzidine (Dako A/S, Glostrup, Denmark) as chromogen. Positive and negative
71 controls were included in each run. According to French recommendations, cases were
72 considered positive for ER and PR if at least 10% of tumor nuclei were stained [12]. Tumors
73 were considered hormone receptor (HR)-positive when positive for either ER or PR, and HR-
74 negative when negative for both ER and PR.

75 Concerning Ki-67 assessment, tissue sections were incubated for one hour with the anti-Ki67
76 monoclonal antibody (Clone MIB1, Dako A/S, Glostrup, Denmark) at 1/100 dilution. The
77 revelation of the staining was performed using the Vectastain Elite ABC peroxidase mouse
78 IgG kit (Vector Burlingame, CA, USA) and diaminobenzidine (Dako A/S) as chromogen. The
79 semiquantitative assessment was performed by estimating at x200 magnification the percentage
80 of positive neoplastic nuclei within the area of highest positivity chosen after scanning the
81 entire tumor surface at low power (x10 objective). All nuclei with homogeneous staining, even
82 with a light staining or only a nucleolar staining, were interpreted as positive.

83 *HER2* expression was determined by immunohistochemistry using a monoclonal anti-*HER2*
84 antibody (CB11, Novocastra, New-Castle, UK; 1/800). Scoring was performed according to
85 American Society of Clinical Oncology (ASCO)/College of American Pathologists (CAP)
86 guidelines [13]. Scores 3+ were reported as positive, scores 1+/0 as negative. Tumors with
87 scores 2+ were tested by FISH. FISH was performed using a *HER2*-gene-specific probe and a
88 centromeric probe for chromosome 17 (PathVysion *HER-2* DNA Probe kit, Vysis-Abbott,
89 Abbott Park, IL, USA) according to manufacturers' instructions. *HER2* gene amplification was
90 defined according to ASCO/CAP guidelines [13]. An average of 40 tumor cells per sample

91 was evaluated and mean *HER2* signals per nuclei were calculated. A *HER2*/CEN17 ratio ≥ 2
92 was considered positive, and a ratio < 2 was considered negative [13].

93 Presence or absence of a DCIS component was retrospectively retrieved in pathological reports
94 of pretreatment core needle biopsy and surgical specimens. Presence of DCIS component was
95 considered as binary (yes/no), and all tumor samples that contained DCIS were included,
96 regardless of subtype.

97

98 **Study endpoints**

99 ypTN stage was defined according to the American Joint Committee on Cancer/Union for
100 International Cancer Control staging. Pathological complete response (pCR) was defined as the
101 absence of invasive residual tumor in the breast and axillary nodes (ypT0/is+ ypN0).

102

103 **Statistical analysis**

104 The study population was described in terms of frequencies for qualitative variables, or
105 medians, means and associated ranges for quantitative variables. Comparisons of proportion of
106 samples with a DCIS component before and after NAC were investigated by Mac Nemar tests.

107 Factors predictive of pCR were introduced into a univariate logistic regression model. A
108 multivariate logistic model was then implemented. Covariates selected for multivariate analysis
109 were those with a *p*-value likelihood ratio test below 0.05 in univariate analysis.

110 Significance threshold was of 5%. Analyses were performed with R software, version 3.1.2

111 [14], with the Epi, dplyr, cowplot and ggplot2 libraries.

112 **Results**

113 **Patients and tumor characteristics**

114 A total of 1148 patients were included in the cohort. These patients were mostly premenopausal
115 (63%, n= 713), and 13% (n=146) were obese (BMI>30). Clinically, most patients had stage T2
116 tumors (67%, n=764) and node-positive breast cancers (56%, n=644). 44% of tumors were
117 luminal (n=508), 31% were TNBC (n=359) and 24% were HER2-positive BC (n=281),
118 including 134 HER2/ER- and 147 HER2/ER+. Most tumors were grade 3 (59.1%, n=659).
119 DCIS component was present before NAC in 19.6% of samples (n=225), (Table 1).

120

121 Table 1. Patients and tumor characteristics

		n	
Menopausal status	postmenopausal	426	(37.4%)
	premenopausal	713	(62.6%)
BMI	BMI < 19	68	(6%)
	BMI: 19 to 25	644	(56.4%)
	BMI: 25 to 30	284	(24.9%)
	BMI>30	146	(12.8%)
Tumor size	T1	65	(5.7%)
	T2	764	(66.6%)
	T3	318	(27.7%)
Nodal status	N0	503	(43.9%)
	N1-N2-N3	644	(56.1%)
Mitotic index	<=22	684	(64.7%)
	>22	374	(35.3%)
Histology	other	116	(10.2%)
	(NST)	1022	(89.8%)
Subtype	luminal	508	(44.2%)
	TNBC	359	(31.3%)
	HER2	281	(24.5%)
Grade	Grade I-II	457	(40.9%)
	Grade III	659	(59.1%)
Ki-67	<20	168	(30.3%)
	≥ 20	387	(69.7%)
DCIS component	no	923	(80.4%)
	yes	225	(19.6%)

Abbreviations: Body Mass Index (BMI); Ductal Carcinoma in situ (DCIS); No Specific Type (NST)

Missing data: Menopausal status: 9; BMI: 6; tumor size: 1; nodal status: 1; mitotic index: 90; histology: 10; subtype: 0; grade: 32; ki-67: 593; DCIS: 0

122 Pre-NAC DCIS

123 Pre-NAC, DCIS was found in 225 samples (19.6%). Presence of a DCIS component was
124 associated with BC subtype ($p<0.001$). The percentage of pre-NAC samples with a DCIS
125 component was higher in *HER2*-positive BC (29.5%), compared to luminal BC (21.3%) or
126 TNBC (9.5%) (Fig 1).

127

128 Fig 1. Presence of a DCIS component on pre-NAC breast cancer biopsies

129

130 Presence of a DCIS component on pre-NAC biopsy was associated to menopausal status, BMI,
131 mitotic index and grade (Table 2). Concerning samples with pre-NAC DCIS, most patients
132 were premenopausal (73.2%) with a $BMI \leq 30$ (92.9%); tumors were mostly Grade I-II (51.4%),
133 with lower mitotic index (75.1%).

134 **Table 2. Association between patients and tumor characteristics and presence of pre-NAC**
 135 **DCIS**

		No DCIS pre-NAC	DCIS pre-NAC	<i>p</i>
Menopausal status	premenopausal	549 (60%)	164 (73.2%)	< 0.001
	postmenopausal	366 (40%)	60 (26.8%)	
BMI	BMI<19	502 (54.6%)	142 (63.4%)	0.007
	BMI: 19 to 25	52 (5.7%)	16 (7.1%)	
	BMI: 25 to 30	234 (25.5%)	50 (22.3%)	
	BMI>30	130 (14.2%)	16 (7.1%)	
Tumor size	T1	52 (5.6%)	13 (5.8%)	0.79
	T2	619 (67.1%)	145 (64.7%)	
	T3	252 (27.3%)	66 (29.5%)	
Nodal status	N0	407 (44.1%)	96 (42.7%)	0.75
	N+	515 (55.9%)	129 (57.3%)	
Mitotic Index	≤22	530 (62.1%)	154 (75.1%)	0.001
	>22	323 (37.9%)	51 (24.9%)	
Subtype	luminal	400 (43.3%)	108 (48%)	< 0.001
	TNBC	325 (35.2%)	34 (15.1%)	
	HER2	198 (21.5%)	83 (36.9%)	
Grade	Grade I-II	346 (38.4%)	111 (51.4%)	0.001
	Grade III	554 (61.6%)	105 (48.6%)	
Ki-67	ki-67<20	120 (28.6%)	48 (35.3%)	0.17
	ki-67≥ 20	299 (71.4%)	88 (64.7%)	

Abbreviations: Body Mass Index (BMI), Ductal Carcinoma in situ (DCIS)

Missing data: Menopausal status: 9, BMI: 6, tumor size: 1, nodal status: 1, mitotic index: 90, subtype: 0, grade: 32, ki-67: 593

136 **Post-NAC DCIS**

137 In surgical specimens after NAC, the rate of samples with DCIS differed by BC subtype:

138 54.4% in *HER2*-positive BC; 53.3% in luminal BC; 24% in TNBC, $p<0.001$, (Fig 2).

Fig 2. Presence of a DCIS component on post-NAC breast cancer surgical specimen.

139 **Evolution of DCIS component pre and post NAC**

140 Paired pre and post-NAC data on the presence of DCIS were available in 1148 patients (508
141 luminal, 359 TNBC and 281 *HER2*-positive BC).

142 DCIS was present both in microbiopsy and in the surgical specimen for 143 patients. No DCIS
143 pre and post-NAC was found for 556 cases (Fig 3, 3A, 3B and 3C). 367 (32%) paired samples
144 had no DCIS pre-NAC but had DCIS in the surgical specimen after NAC. For 82 patients,
145 DCIS was present on the pre-NAC sample but not on the surgical specimen.

Fig 3. Presence pre and post NAC DCIS in global population (1148 patients).

Fig 3A. Presence of pre and post NAC DCIS in Luminal BC (508 patients).

Fig 3B. Presence of pre and post NAC DCIS in TNBC (359 patients).

Fig 3C. Presence of pre and post NAC DCIS in *HER2*-positive BC (281 patients).

146 In global population, presence of DCIS was significantly higher after NAC compared to before
147 NAC (44.4% *versus* 19.6%). Similar results were observed for the different BC subtypes
148 (*HER2*-positive BC: 54.4% *versus* 29.5% ($p=0.002$), luminal: 53.3% *versus* 21.3% ($p<0.0001$),
149 TNBC: 24% *versus* 9.5% ($p<0.0001$)), (Table 3).

150 **Table 3. Evolution of DCIS component pre and post NAC**

		pre-NAC	post-NAC
Whole population	no DCIS	923 (80.4%)	638 (55.6%)
	DCIS	225 (19.6%)	510 (44.4%)
Luminal	no DCIS	400 (78.7%)	237 (46.7%)
	DCIS	108 (21.3%)	271 (53.3%)
TNBC	no DCIS	325 (90.5%)	273 (76%)
	DCIS	34 (9.5%)	86 (24%)
HER2	no DCIS	198 (70.5%)	128 (45.6%)
	DCIS	83 (29.5%)	153 (54.4%)

In the whole population, percentage of samples with DCIS was of 19.6% before NAC and of 44.4% after NAC ($p<0.0001$).

Luminal: 21.3% of samples with DCIS before NAC and 53.3% after NAC ($p<0.0001$)

TNBC: 9.5% of samples with DCIS before NAC and 24.0% after NAC ($p<0.0001$)

HER2: 29.5% of samples with DCIS before NAC and 54.4% after NAC ($p=0.002$)

151 Among patients who achieved pCR, 46 (16.3%) had DCIS on pre-NAC biopsy, distributed as
 152 follows: 7 luminal BC (21.9%), 10 TNBC (7.1%), and 29 *HER2*-positive BC (26.4%).
 153 Concerning patients with no pCR, 179 (20.7%) had a DCIS component on pre-NAC sample:
 154 101 luminal (21.2%), 24 TNBC (11%) and 54 *HER2*-positive BC (31.6%).

155

156 **Baseline clinicopathological parameters associated with pCR**

157 Overall, 283 patients achieved pCR (24.7%) after NAC. After univariate analysis, baseline
 158 clinical and pathological factors significantly associated with higher pCR rates were: TNBC or
 159 *HER2*-positive BC subtypes, high grade, high mitotic index and Ki-67 ≥ 20 . Tumor size $\geq T2$
 160 was associated with lower pCR rates (Table 4).

161 In a multivariable regression logistic analysis including subtype, tumor size, grade, mitotic
 162 index and Ki-67 index, only subtype and Ki-67 were significantly associated with pCR.

163 Presence or absence of DCIS in biopsy was not significantly associated with response to NAC.

164 Indeed, pCR rate was of 20.4% in case of pre-NAC DCIS in biopsy vs. 25.7% for samples with
 165 no DCIS (OR=0.74 (95% CI 0.52-1.06), $p=0.1$).

166 **Table 4. Association of baseline clinicopathological factors and pCR**

					Univariate analysis			Multivariate analysis		
Variable		<i>n</i>	pCR	%	OR	95% CI (OR)	<i>p</i>	OR	95% CI (OR)	<i>p</i>
Menopausal status	post	426	113	26.5	1					
	pre	713	167	23.4	0.85	0.64-1.12	0,2			
BMI	19-25	644	164	25.5	1					
	<19	68	13	19.1	0.69	0.35-1.26	0.25			
	>25	430	103	24	0.92	0.69-1.22	0.57			
Tumor size	T1	65	32	49.2	1					
	T2	764	186	24.3	0.33	0.20-0.56	<0.0001			
	T3	318	65	20.4	0.26	0.15-0.46	<0.0001			
Nodal status	N0	503	120	23.9	1					
	N1-N2-N3	644	163	25.3	1.08	0.82-1.42	0.57			
Mitotic index	≤22	684	127	18.6	1					
	>22	374	133	35.6	2.42	1.82-3.22	<0.0001			
Histology	other	116	17	14.7	1					
	NST	1022	265	25.9	2.04	1.2-3.47	0,01			
Grade	I-II	457	55	12	1					
	III	659	221	33.5	3.69	2.66-5.1	<0.0001			
Ki-67	<20	168	11	6.5	1			1		
	≥20	387	105	27.1	5.3	2.77-10.2	<0.0001	3	1.31-7.75	0.01
DCIS	no	923	237	25.7	1					
	yes	225	46	20.4	0.74	0.52-1.06	0,1			
Subtype	luminal	508	32	6.3	1			1		
	TNBC	359	141	39.3	9.62	6.43-14.8	<0.0001	5.4	2.68-11.3	<0.0001
	HER2	281	110	39.1	9.57	6.29-14.9	<0.0001	8.7	4.39-18.3	<0.0001

Abbreviations: neoadjuvant chemotherapy (NAC); Body Mass Index (BMI); Ductal Carcinoma in situ (DCIS), pathological complete response (pCR); No Specific Type (NST)

167

168 **Discussion**

169 In this study, no significant association between the presence of DCIS on pre-NAC biopsy and
170 histological response to NAC was observed.

171 The proportion of samples with an *in situ* component associated to an invasive one (19.6%) was
172 lower than reported in other cohorts. According to different studies, adjacent *in situ* component
173 rates vary from 33% [16] to 53% [10] of invasive BC. This could result from a lower rate of *in*
174 *situ* disease in more advanced BC treated with NAC. Furthermore, because of the small amount
175 of tissue collected in biopsies before treatment, we might have underestimated the real extent of
176 pre-NAC DCIS.

177

178 Concerning pre-NAC samples, the percentage of *in situ* disease in biopsy was higher in *HER2*-
179 positive BC (29.5%), compared to luminal BC (21.3%) or TNBC (9.5%), ($p < 0.001$). To our
180 knowledge, no other study has yet assessed the presence of DCIS in biopsy before treatment
181 according to BC subtypes.

182 On post-NAC surgical specimen, the rate of samples with a DCIS component was higher in
183 *HER2*-positive BC (54.4%), compared to luminal BC (53.3%) or TNBC (24%). Similar post-
184 operative results were found by Wong *et al.* in a study on 1159 no specific type (NST) breast
185 cancer patients treated by upfront surgery [15]. In that study, DCIS was associated with NST
186 carcinoma in 63.2% of *HER2*-positive BC, compared to 53.3% for luminal BC and 33.3% for
187 TNBC.

188

189 In our study, DCIS was present in 82 patients on pre-NAC samples but not on surgical
190 specimens. An explanation could be that the biopsy contained the entire *in situ* component, or
191 that chemotherapy has an effect on DCIS. As we probably underestimated the real extent of
192 adjacent DCIS in pre-NAC biopsy, we probably underestimated the frequency of complete

193 eradication of DCIS as well. Response to NAC of adjacent DCIS has been reported in few
194 studies. Goldberg *et al.* [16] investigated the impact of neoadjuvant chemotherapy on DCIS in a
195 cohort of 92 patients with locally advanced BC and found that NAC +/- trastuzumab may
196 completely eradicate *in situ* component. Indeed, both invasive and non invasive components
197 had disappeared in 33% of the patients in that trial. Matsuo *et al.* [17] reported a strong
198 correlation in pathological response between invasive and non invasive components in a series
199 of 100 primary BC treated with NAC. Von Minckwitz *et al.* [18] found a response to NAC
200 combined with trastuzumab in a cohort of 158 *HER2*-positive breast cancer patients. 50.8% of
201 the samples with DCIS associated with NST carcinoma showed complete eradication of
202 adjacent DCIS after NAC.

203 Conversely, in a study evaluating 25 patients with locally advanced BC after NAC, Wu *et al.*
204 [11] showed that *in situ* component was poorly responsive to NAC. When assessing the
205 proportion of pre-NAC DCIS, they found that although chemotherapy had a favorable effect on
206 tumor reduction, its effectiveness varied with the proportion of pre-NAC *in situ* component.
207 Cases with high rates of DCIS had a lower response to NAC, and an important proportion of
208 cancer cells remained in mammary ducts and maintained proliferative activity.

209

210 For 367 paired samples (32% of the pairs), there was no DCIS pre-NAC but presence of an *in*
211 *situ* component in the surgical specimen after NAC. These paired samples could be considered
212 as “false negative” due to of the lack of representativeness of the pre-NAC biopsy.

213

214 pCR was achieved for 283 patients (24.7%). No significant association between presence of a
215 pre-NAC DCIS component and pCR was observed. Conversely, Von Minckwitz *et al.* [18]
216 found that the presence of DCIS associated to *HER2*-positive breast cancer was an independent
217 negative predictor of pCR after NAC (OR=0.42 (95% CI 0.2-0.9), $p=0.0027$).

218 In conclusion, the presence of a DCIS component on pre-NAC biopsy is not associated with
219 response to NAC in our study. Further studies are expected to validate pre-NAC biomarkers
220 that could potentially improve prediction of response to neoadjuvant treatment.
221

222 **Acknowledgements**

223 Not applicable.

224

225 **References**

- 226 1. Powles TJ, Hickish TF, Makris A, Ashley SE, O'Brien ME, Tidy VA, et al.
227 Randomized trial of chemoendocrine therapy started before or after surgery for treatment of
228 primary breast cancer. *J Clin Oncol Off J Am Soc Clin Oncol*. 1995 Mar;13(3):547–52.
- 229 2. Makris A, Powles TJ, Ashley SE, Chang J, Hickish T, Tidy VA, et al. A reduction in
230 the requirements for mastectomy in a randomized trial of neoadjuvant chemoendocrine therapy
231 in primary breast cancer. *Ann Oncol Off J Eur Soc Med Oncol*. 1998 Nov;9(11):1179–84.
- 232 3. Rastogi P, Anderson SJ, Bear HD, Geyer CE, Kahlenberg MS, Robidoux A, et al.
233 Preoperative chemotherapy: updates of National Surgical Adjuvant Breast and Bowel Project
234 Protocols B-18 and B-27. *J Clin Oncol Off J Am Soc Clin Oncol*. 2008 Feb 10;26(5):778–85.
- 235 4. Luangdilok S, Samarthai N, Korphaisarn K. Association between Pathological
236 Complete Response and Outcome Following Neoadjuvant Chemotherapy in Locally Advanced
237 Breast Cancer Patients. *J Breast Cancer*. 2014 Dec;17(4):376–85.
- 238 5. Loibl S, von Minckwitz G, Untch M, Denkert C, German Breast Group. Predictive
239 factors for response to neoadjuvant therapy in breast cancer. *Oncol Res Treat*.
240 2014;37(10):563–8.
- 241 6. Huober J, von Minckwitz G, Denkert C, Tesch H, Weiss E, Zahm DM, et al. Effect of
242 neoadjuvant anthracycline-taxane-based chemotherapy in different biological breast cancer
243 phenotypes: overall results from the GeparTrio study. *Breast Cancer Res Treat*. 2010
244 Nov;124(1):133–40.
- 245 7. Karatas F, Erdem GU, Sahin S, Aytakin A, Yuce D, Sever AR, et al. Obesity is an
246 independent prognostic factor of decreased pathological complete response to neoadjuvant
247 chemotherapy in breast cancer patients. *Breast Edinb Scotl*. 2017 Apr;32:237–44.
- 248 8. Fasching PA, Heusinger K, Haeberle L, Niklos M, Hein A, Bayer CM, et al. Ki67,
249 chemotherapy response, and prognosis in breast cancer patients receiving neoadjuvant
250 treatment. *BMC Cancer*. 2011 Nov 14;11:486.
- 251 9. Mao Y, Qu Q, Zhang Y, Liu J, Chen X, Shen K. The value of tumor infiltrating
252 lymphocytes (TILs) for predicting response to neoadjuvant chemotherapy in breast cancer: a
253 systematic review and meta-analysis. *PloS One*. 2014;9(12):e115103.
- 254 10. Wong H, Lau S, Yau T, Cheung P, Epstein RJ. Presence of an in situ component is
255 associated with reduced biological aggressiveness of size-matched invasive breast cancer. *Br J*
256 *Cancer*. 2010 Apr 27;102(9):1391–6.
- 257 11. Wu W, Kamma H, Ueno E, Fujiwara M, Satoh H, Hara H, et al. The intraductal
258 component of breast cancer is poorly responsive to neo-adjuvant chemotherapy. *Oncol Rep*.
259 2002 Oct;9(5):1027–31.
- 260 12. Harvey JM, Clark GM, Osborne CK, Allred DC. Estrogen receptor status by
261 immunohistochemistry is superior to the ligand-binding assay for predicting response to
262 adjuvant endocrine therapy in breast cancer. *J Clin Oncol Off J Am Soc Clin Oncol*. 1999
263 May;17(5):1474–81.
- 264 13. Wolff AC, Hammond MEH, Schwartz JN, Hagerty KL, Allred DC, Cote RJ, et al.
265 American Society of Clinical Oncology/College of American Pathologists guideline

- 266 recommendations for human epidermal growth factor receptor 2 testing in breast cancer. *J Clin*
267 *Oncol Off J Am Soc Clin Oncol*. 2007 Jan 1;25(1):118–45.
- 268 14. R Development Core Team. R: A Language and Environment for Statistical Computing.
269 R Foundation for Statistical Computing. 2011; Available from: URL [http://www.R-](http://www.R-project.org/)
270 [project.org/](http://www.R-project.org/).
- 271 15. Wong H, Lau S, Leung R, Chiu J, Cheung P, Wong TT, et al. Coexisting ductal
272 carcinoma in situ independently predicts lower tumor aggressiveness in node-positive luminal
273 breast cancer. *Med Oncol Northwood Lond Engl*. 2012 Sep;29(3):1536–42.
- 274 16. Goldberg H, Zandbank J, Kent V, Leonov-Polak M, Livoff A, Chernihovsky A, et al.
275 Chemotherapy may eradicate ductal carcinoma in situ (DCIS) but not the associated
276 microcalcifications. *Eur J Surg Oncol J Eur Soc Surg Oncol Br Assoc Surg Oncol*. 2017 May 4.
- 277 17. Matsuo K, Fukutomi T, Watanabe T, Hasegawa T, Tsuda H, Akashi-Tanaka S.
278 Concordance in pathological response to neoadjuvant chemotherapy between invasive and
279 noninvasive components of primary breast carcinomas. *Breast Cancer Tokyo Jpn*.
280 2002;9(1):75–81.
- 281 18. von Minckwitz G, Darb-Esfahani S, Loibl S, Huober J, Tesch H, Solbach C, et al.
282 Responsiveness of adjacent ductal carcinoma in situ and changes in HER2 status after
283 neoadjuvant chemotherapy/trastuzumab treatment in early breast cancer--results from the
284 GeparQuattro study (GBG 40). *Breast Cancer Res Treat*. 2012 Apr;132(3):863–70.
285

Fig1

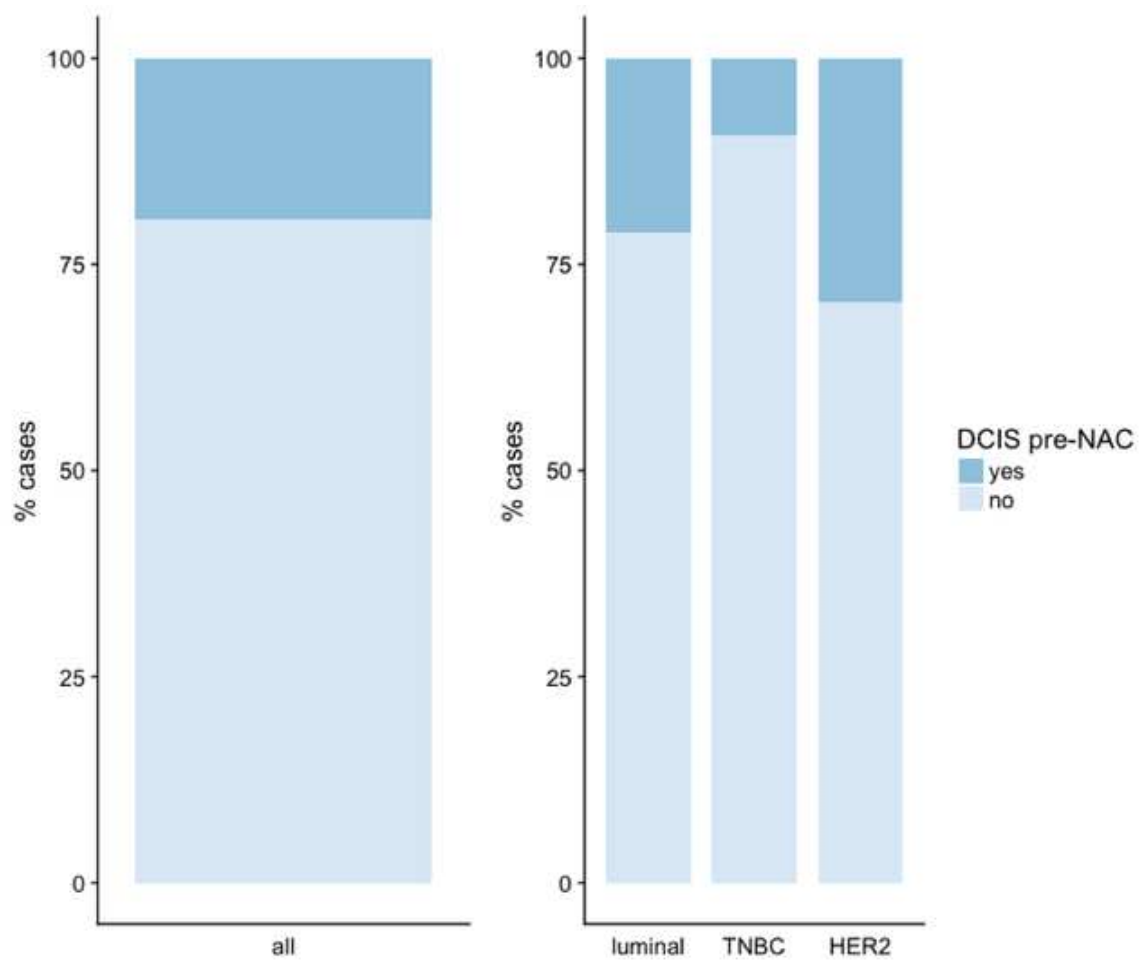
[Click here to access/download;Figure;Fig1.tif](#)

Fig2

[Click here to access/download;Figure;Fig2.tif](#)

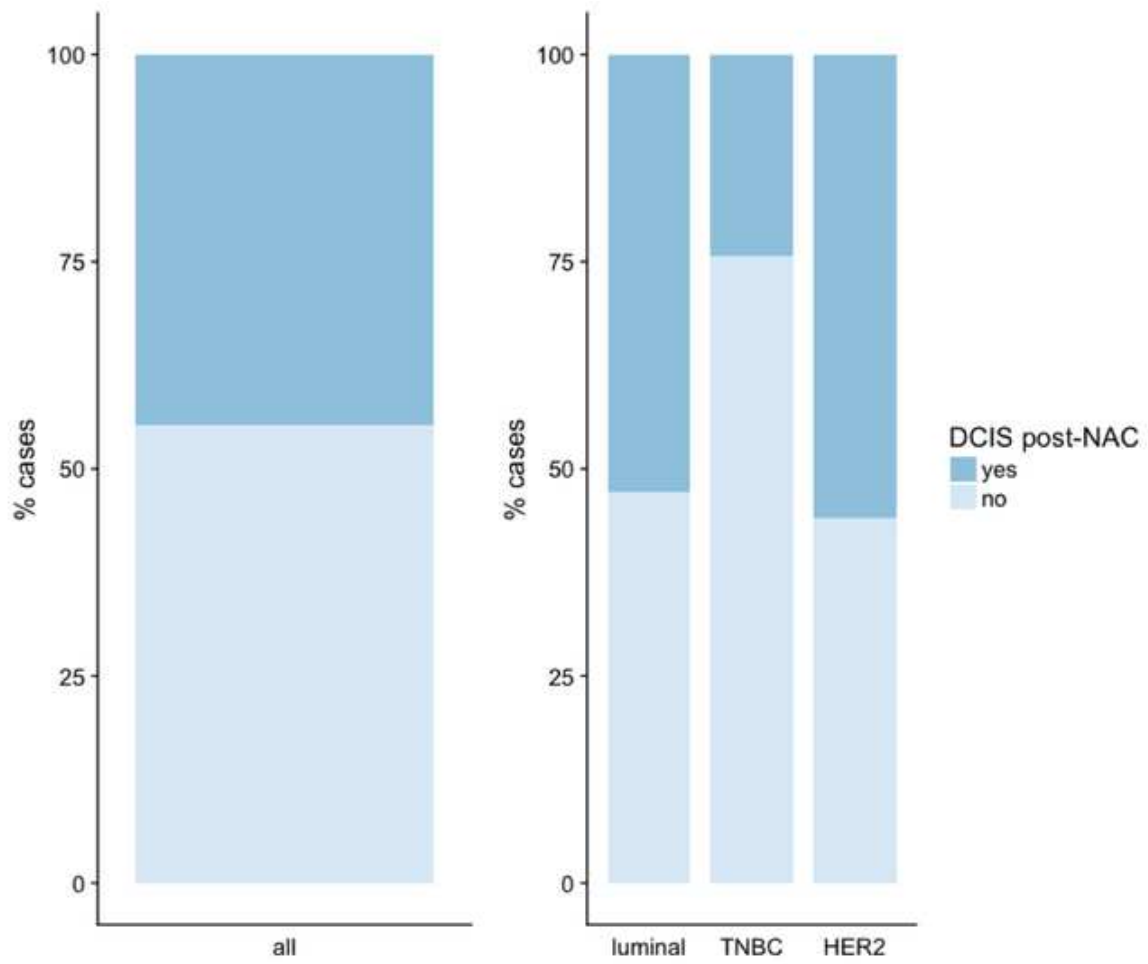


Fig3

[Click here to access/download;Figure;Fig3.tif](#)

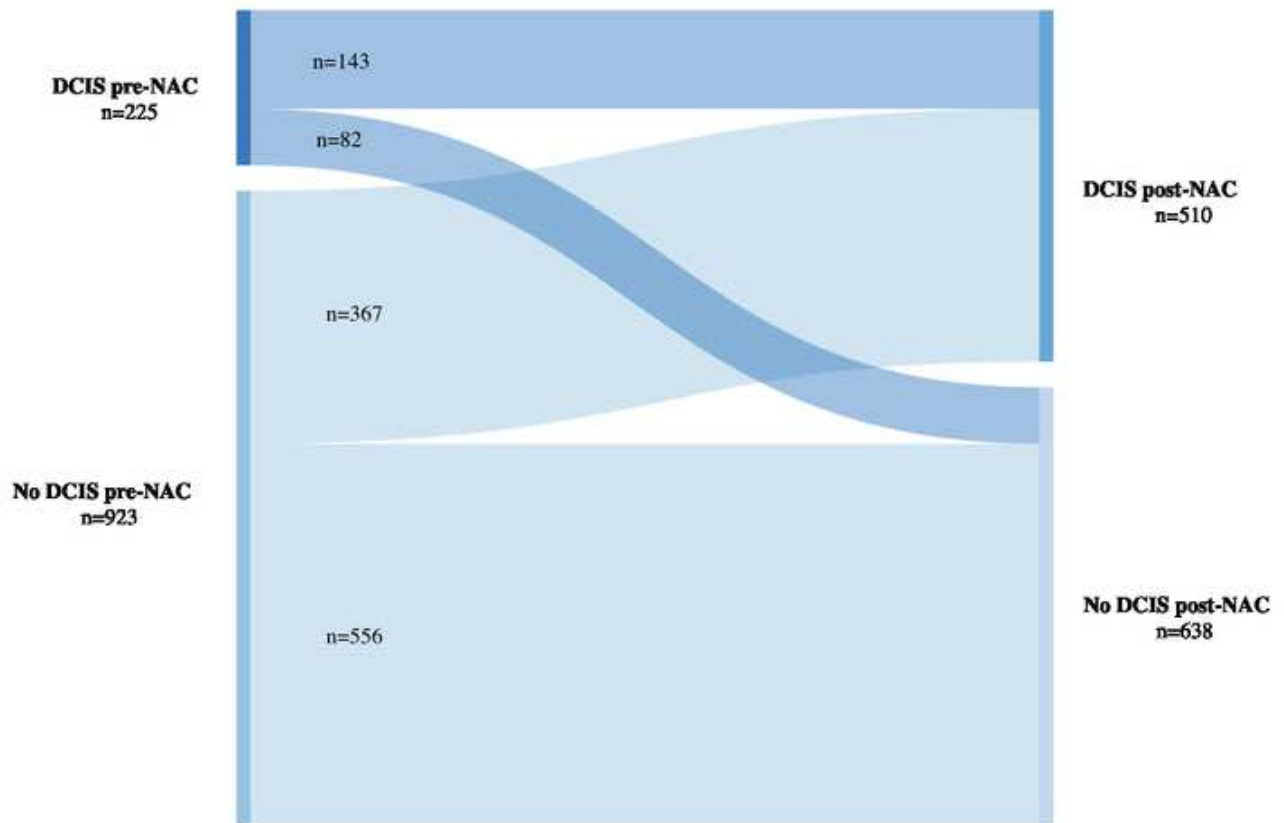


Fig3A

[Click here to access/download;Figure;Fig4.tif](#)

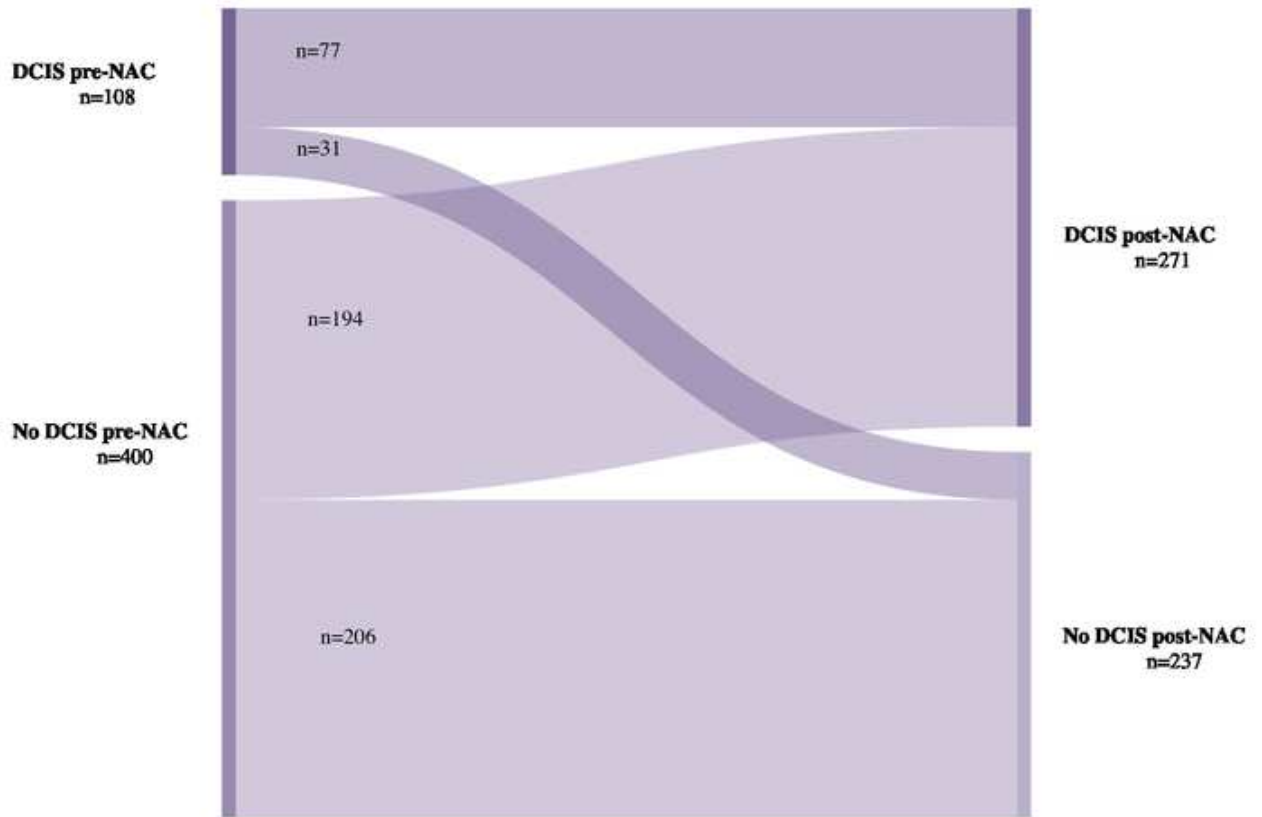


Fig3B

[Click here to access/download;Figure;Fig5.tif](#)

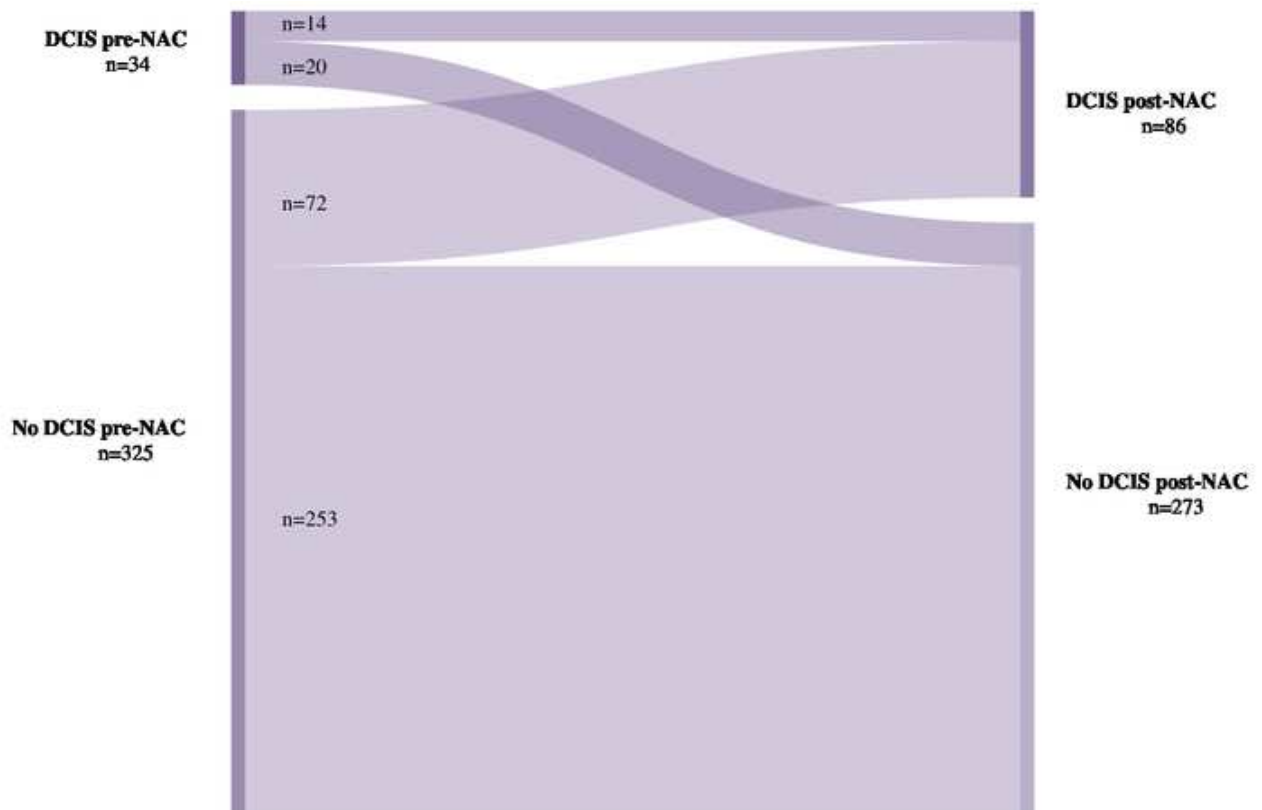
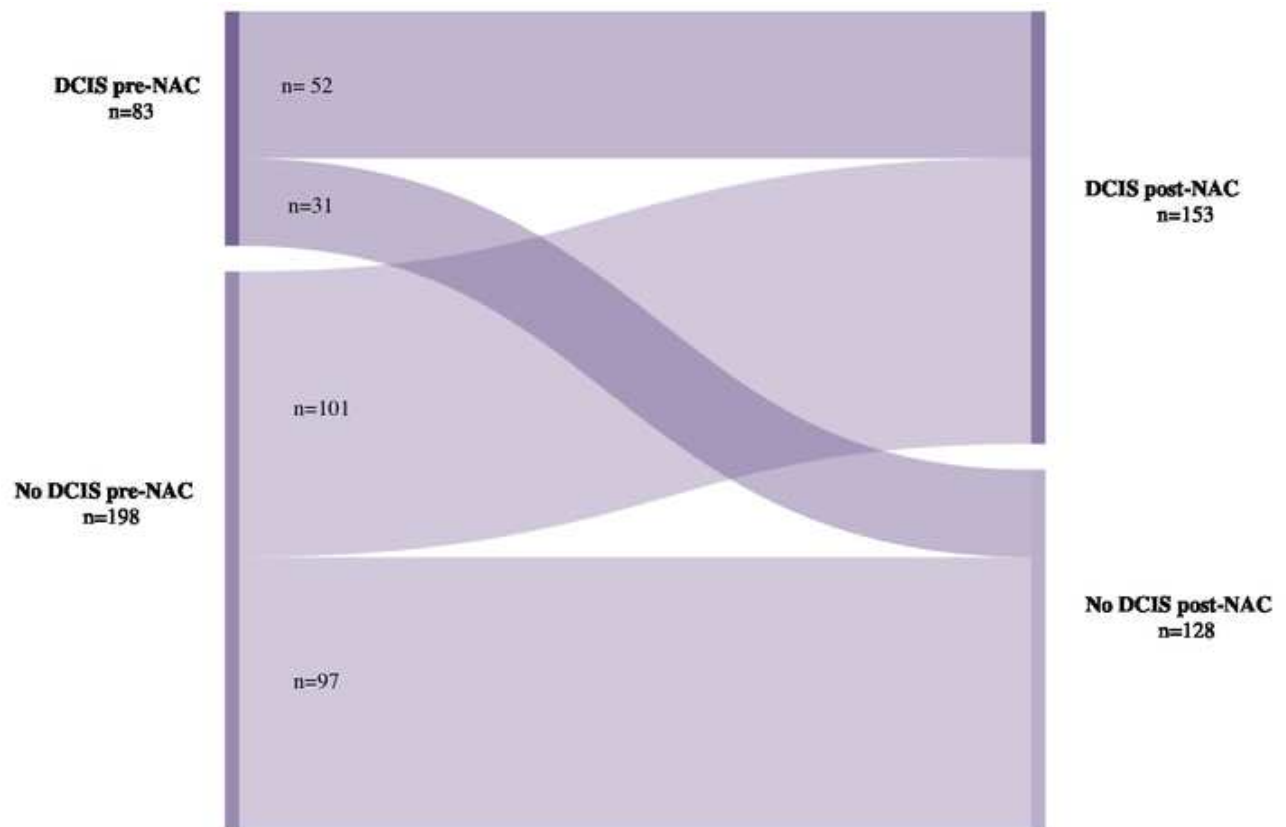


Fig3C

[Click here to access/download;Figure;Fig6.tif](#)

Chemosensitivity, tumor infiltrating lymphocytes (TILs), and survival of postpartum PABC patients treated by neoadjuvant chemotherapy

Julie Labrosse¹, Inès Abdennebi¹, Lucie Thibault², Enora Laas¹, Hilde Merckelbagh³, Charlotte Morel¹, Thanh Lam⁴, Marick Lae², Fabien Rey^{1,5}, Anne-Sophie Hamy⁵

1. Department of Surgery, Institut Curie, 26 rue d'Ulm, 75005 Paris, France
2. Department of Pathology, Centre René Huguenin- Institut Curie, 35 Rue Dailly, 92210 St Cloud, France
3. Port-Royal Maternity Unit, 123 Boulevard de Port Royal, 75014 Paris, France
4. Department of Gynecology and Obstetrics, Geneva University Hospitals, Rue Gabrielle-Perret-Gentil 4, 1205 Geneva, Switzerland.
5. Residual Tumor & Response to Treatment Laboratory, RT2Lab, Translational Research Department, PSL Research University, INSERM, U932 Immunity and Cancer, Institut Curie, 26 rue d'Ulm, Paris, France

- **Corresponding author:**

Pr. Fabien REYAL
Institut Curie, 26 rue d'Ulm, 75005 Paris, France
+33 144324660
fabienreyal@gmail.com

- **Contact of co-authors :**

Julie Labrosse: julie.labrosse3@gmail.com
Inès Abdennebi : ines.abdennebi@gmail.com
Lucie Thibault : lucie.thibault@curie.fr
Enora Laas: enora.laas@curie.fr
Hilde Merckelbagh : hildemerckelbagh@yahoo.fr
Charlotte Morel : charlotte-morel@live.fr
Thanh Lam: lamgiangthanh@gmail.com
Marick Lae: marick.lae@curie.fr
Anne-Sophie Hamy: anne-sophie.hamy-petit@curie.fr

Abstract

Background: Pregnancy-associated breast cancer (PABC) refers to breast cancers (BC) diagnosed during pregnancy or shortly after birth. Although the inflammatory environment of post-partum PABC cases (designed as PP-PABC) may be deleterious, so far PP-PABC have scarcely been distinguished from breast cancers diagnosed during pregnancy. Furthermore, whether PP-PABC cases have an enhanced immune infiltration remains unknown. We investigated chemosensitivity, immune infiltration and survival of PP-PABC patients treated by neoadjuvant chemotherapy (NAC) compared to non-PABC matched BC patients.

Materials and methods: We identified PP-PABC cases among a cohort of 1199 invasive BC patients treated with NAC between 2002 and 2012. Each PP-PABC case was matched with 3 non-PABC controls, according to age and **pathological breast cancer subtypes**. Microbiopsy specimens and paired surgical samples were evaluated for stromal lymphocyte infiltration. The association of clinical and pathological factors with pathological complete response (pCR) and disease-free survival (DFS) was assessed by univariate and multivariate analyses.

Results: Our final population study was composed of 116 patients (29 PP-PABC cases and 87 non-PABC controls). Median follow-up was of 32.4 months. After NAC, pCR rates ($p=0.64$), post-NAC immune infiltration (stromal TILs: $p=0.67$; intratumoral TILs: $p=0.14$), and DFS rates ($p=0.17$) were comparable between PP-PABC and non-PABC patients in global population. Similar results were found after stratification by **pathological** subtype.

Conclusion: We observed similar patterns between postpartum PABC and control tumors in terms of chemosensitivity, immune infiltration, and prognostic. Our results enhance the idea

that PP-PABC should receive the same standard of care treatment as other patients, including neoadjuvant chemotherapy.

Keywords

Pregnancy associated breast cancer; PABC; post-partum breast cancer; chemosensitivity; prognosis; tumor infiltrating lymphocytes; TILs

Abbreviations:

PABC: pregnancy-associated breast cancer

PREG-PABC: breast cancers diagnosed during pregnancy

PP-PABC: post-partum pregnancy-associated breast cancer (breast cancers diagnosed during the first post-partum year)

DFS: disease-free survival

OS: overall survival

BC: breast cancer

TILs: tumor infiltrating lymphocytes

pCR: pathological response rate

NAC: neo-adjuvant chemotherapy

ER: estrogen receptor

PR: progesterone receptor

HER2: human epidermal growth factor receptor 2

TNBC: triple-negative breast cancer

RCB: residual cancer burden

HR: hazard ratio

CI: confidence interval

IT: intratumoral

Tables and Figures

Table 1: Patient characteristics for PP-PABC patients and non-PABC controls

Table 2: Post-NAC characteristics for PP-PABC patients and non-PABC controls, in whole population and by **pathological breast cancer subtype**

Figure 1: Pre-NAC stromal TILs levels in PP-PABC patients and non-PABC controls, in global population and by **pathological breast cancer subtype**

Figure 2: Pathological complete response rates in PP-PABC patients and non-PABC controls, in global population and by **pathological breast cancer subtype**

Figure 3: Post-NAC stromal TILs levels in PP-PABC patients and non-PABC controls, in global population and by **pathological breast cancer subtype**

Figure 4: Disease-free survival curves of PP-PABC patients and non-PABC controls, in whole population and by **pathological breast cancer subtype**

1 **Introduction**

2 **Pregnancy-associated breast cancer (PABC)** refers to breast cancers diagnosed during
3 pregnancy or shortly after birth. Although there is no consensus on the exact time after
4 delivery, PABC is usually defined as breast cancers diagnosed during pregnancy or in the first
5 postpartum year [1]. It represents 8% of breast cancer cases occurring in women younger than
6 45 years old, and up to 15.6% in women younger than 35 years old [2]. The incidence of
7 PABC increases among breast cancer cases in the global population. Since women tend to
8 delay childbearing, it is expected to be more and more frequent [3], [4].

9
10 PABC's pathogenic pathway is probably different from that of non-PABC [2], [5], [6], [7]. It
11 is usually associated with pejorative prognostic factors such as young age, locally advanced
12 tumors, or hormone receptor-negative tumors [4], [8], [9]. However, so far, studies on PABC
13 have scarcely distinguished breast cancers diagnosed during pregnancy (PREG-PABC) from
14 those diagnosed during the first post-partum year (post-partum PABC; designed as PP-
15 PABC).

16
17 Controlling for age, tumor characteristics and adjuvant treatment, the largest observational
18 study lead on PREG-PABC patients did not show any difference in terms of disease-free
19 survival (DFS) nor overall survival (OS) between PREG-PABC and non-pregnant BC
20 patients [10]. However, PP-PABC may have a different prognostic impact [11], as a
21 deleterious role of the inflammatory breast microenvironment during breast-feeding and
22 breast involution favoring metastatic spreading has been evoked [12], [13]. **Indeed, breast
23 cancer cells exposed to the involuting mammary microenvironment may acquire
24 prolymphangiogenic properties that could contribute to peritumor lymphatic expansion, tumor**

1 size, invasion, and distant metastases [14].

2

3 **Tumor-infiltrating lymphocytes'** (TILs) role in breast cancer has been extensively studied
4 over the last decade. High TILs levels have been associated with high pathological complete
5 response (pCR) rates in the neoadjuvant setting and with better outcomes in the adjuvant
6 setting [15], [16]. In PREG-PABC patients, lower mean TILs levels were observed in patients
7 developing a DFS event compared to those that did not experience an event [17]. Despite
8 growing interest in the field of immunity and oncology, no study has provided data on TILs in
9 PP-PABC so far [18], [19]. Hence, whether the inflammatory environment is associated to an
10 enhanced immune infiltration remains unknown.

11

12 **Neoadjuvant chemotherapy (NAC)** is currently administered to patients with locally
13 advanced breast cancers. Beyond increasing breast-conserving surgery rates, it serves as an *in*
14 *vivo* chemosensitivity test and the analysis of residual tumor burden may help understanding
15 resistance to treatments [3]. To our knowledge, only one study with small effectives analyzed
16 PABC's response to NAC and showed that PABC was as chemosensitive as non-PABC
17 tumors [2].

18

19 The objective of the current study is to provide further data on chemosensitivity, immune
20 infiltration, and survival of PP-PABC patients treated by neoadjuvant chemotherapy
21 compared to non-PABC matched BC patients.

22

1 **Materials and methods**

2 ***Patients and tumors***

3 Our retrospective case-control study analyzed a cohort of 1199 female patients with T1-
4 3NxM0 invasive BC (NEOREP Cohort, CNIL declaration number 1547270) treated with
5 NAC at Institut Curie between 2002 and 2012. The cohort included unifocal, unilateral, non-
6 recurrent, non-metastatic tumors, excluding T4 tumors (inflammatory, chest wall or skin
7 invasion). Approved by the Breast Cancer Study Group of Institut Curie, the study was
8 conducted according to institutional and ethical rules concerning research on tissue specimens
9 and patients. Informed consent from patients was not required.

10 Information on clinical characteristics (age, body mass index) and tumor characteristics
11 (tumor size and grade, ER, PR, HER2 status, lymph node involvement, number of mitosis,
12 ki67) were retrieved from electronic health records.

13 Histological grade was described according to the *Elston-Ellis* modification of the *Scarff-*
14 *Bloom-Richardson* grading system [20]. Hormone-receptor expression was analyzed by
15 immunohistochemistry. Tumors were considered positive for estrogen receptor (ER) or
16 progesterone receptor (PR) if 10% of carcinomatous cells displayed positive staining, as
17 recommended by European guidelines [21]. HER2 status was determined according to
18 American Society of Clinical Oncology (ASCO) recommendations [22]. Based on
19 immunohistochemistry surrogates, pathological breast cancer subtypes were defined as
20 follows: tumors positive for either ER or PR and negative for HER2 were classified as
21 luminal; tumors positive for HER2 were considered HER2-positive BC; tumors negative for
22 ER, PR, and HER2 were considered triple negative BC (TNBC).

1 ***Treatment Protocol***

2 Patients were treated according to national guidelines. Every patient included in our study
3 received NAC. NAC regimens changed over time (anthracycline-based regimen or
4 sequential anthracycline–taxane regimen), with trastuzumab used in an adjuvant and/or
5 neoadjuvant setting for *HER2*-positive tumors since the middle of the past decade.
6 Trastuzumab treatments changed over time due to a change of marketing authorization
7 during the study period. Adjuvant hormone therapy (tamoxifen, aromatase inhibitor, or
8 GnRH agonists) was prescribed when indicated. Surgery (breast-conserving or mastectomy)
9 was performed 4 to 6 weeks after NAC. Every patient received adjuvant radiotherapy.
10 Adjuvant chemotherapy (ADJ) was decided after multidisciplinary consultation meeting
11 considering patient characteristics, prognostic factors and response to NAC (residual
12 disease and/or node involvement). Patient follow-up after treatment was of every 3 months
13 during the first 2 years, then every 6 months during 3 years, and once a year starting from the
14 5th year. Follow-up consisted of clinical examination associated to mammography and
15 mammary ultrasound once a year.

16

17 ***Pregnancy associated breast cancer cases***

18 Information on pregnancy at inclusion or the year prior breast cancer diagnosis was extracted
19 from the structured database. Every pregnancy was included irrespective of its outcome (full-
20 term pregnancy, miscarriage, abortion).

1 **Study endpoints and definitions**

2 **pCR** was defined as the absence of residual invasive cancer cells in the breast and axillary
3 lymph nodes (ypT0/is + / ypN0).

4 **Residual cancer burden (RCB)** [23] was assessed for each patient after NAC.

5 **DFS** was defined as the time from surgery to death, loco-regional or distant recurrence, or
6 contralateral cancer, whichever occurred first. Patients for whom none of these events were
7 recorded were censored at the date of their last known contact.

8 **TILs levels** were evaluated **retrospectively, for research purposes**, by two pathologists (ML,
9 LT) **of the Tumor Biology Department of Institut Curie (France)**. **TILs levels were assessed**
10 on pretreatment core needle biopsies and post-NAC surgical specimens for the presence of
11 mononuclear cells infiltrate (including lymphocytes and plasma cells, excluding
12 polymorphonuclear leukocytes), following international TILs Working Group
13 recommendations [24]. They were evaluated in stroma, within tumor scar border, after
14 excluding areas around ductal carcinoma *in situ*, tumor zones with necrosis and artifacts, and
15 were scored continuously as the average percentage of stromal area occupied by mononuclear
16 cells.

17

18 **Statistical analyses**

19 The study population was described in terms of frequencies for qualitative variables, or
20 medians and associated ranges for quantitative variables. Each PP-PABC case was matched
21 with 3 non-PABC controls, according to age and **pathological breast cancer subtypes**. Factors
22 predictive of pCR were introduced in a univariate logistic regression model. A multivariate
23 logistic model was then implemented. Covariates selected for multivariate analysis were those

1 with a p-value no greater than 0.1 after univariate analysis. TILs levels and qualitative
2 variables in classes were compared by ANOVA test with a post hoc Tukey analysis when
3 necessary.

4 Survival probabilities were estimated by Kaplan-Meier method, and survival curves were
5 compared in log-rank tests. Hazard ratios (HR) and their 95% confidence intervals (CI) were
6 calculated with the Cox proportional hazards model. Analyses were performed with R
7 software, version 3.1.2 [25], Ggplot2, MatchIt, Optmatch, Matching and Survival libraries.
8 Significance threshold was of 5%.

9

10 **Results**

11 ***Baseline patient and tumor characteristics***

12 We identified 29 PP-PABC cases in the whole cohort of 1199 BC patients. Each PP-PABC
13 case (n=29) was matched with 3 non-PABC controls (n=87). The final population study was
14 composed of 116 patients. Median age was 35.9 years (range: 26.7-44.3 years). Patient
15 characteristics are described in Table 1. No pattern was significantly different between PP-
16 PABC patients (n=29) and non-PABC controls (n=87) regarding age, body mass index,
17 histology, **pathological breast cancer subtype**, tumor size and grade, or number of mitoses.

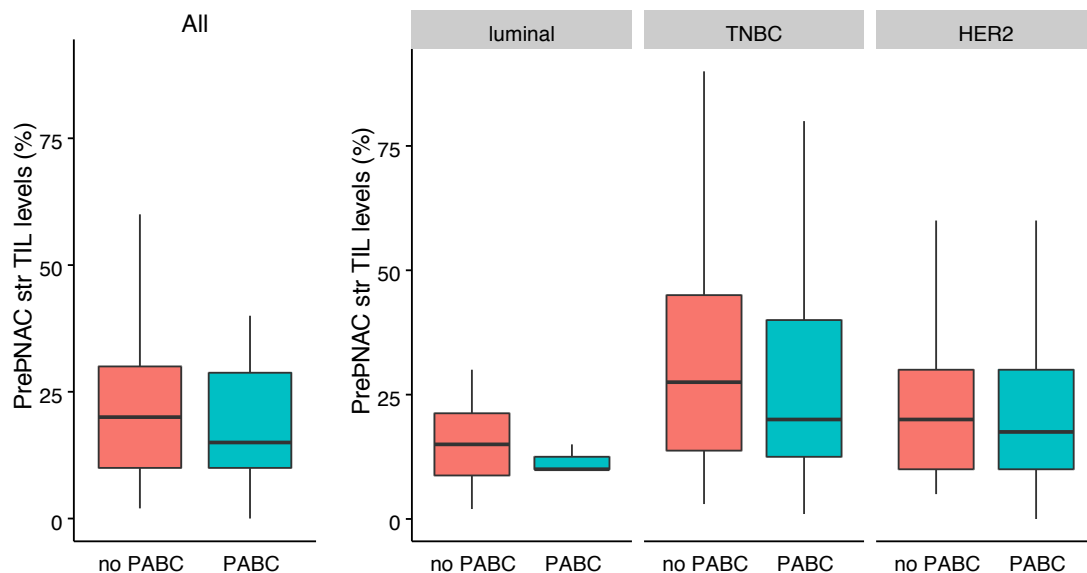
Table 1: Patient characteristics for PP-PABC patients and non-PABC controls

Variables	PP-PABC (n=29)	non-PABC controls (n=87)	p-value
Age (median +/- SD)	35.5 (4.3)	35.9 (4.7)	0.67
Body Mass Index			0.52
<19	1 (3.4%)	7 (8.0%)	
19-25	18 (62.1%)	61 (70.1%)	
25-30	7 (24.1%)	13 (14.9%)	
>30	3 (10.3%)	6 (6.9%)	
Histology			0.99
Ductal	29 (100.0%)	85 (97.7%)	
Other	0 (0.0%)	2 (2.3%)	
Pathological subtypes			0.99
Luminal	7 (24.1%)	21 (24.1%)	
TNBC	9 (31.0%)	27 (31.0%)	
HER2-positive	13 (44.8%)	39 (44.8%)	
Grade Elston Ellis			0.26
I-II	12 (42.9%)	24 (28.9%)	
III	16 (57.1%)	59 (71.1%)	
Number of mitosis			0.99
≤22	15 (57.7%)	46 (59.7%)	
>22	11 (42.3%)	31 (40.3%)	
Tumor size			0.65
T1	2 (6.9%)	9 (10.3%)	
T2	22 (75.9%)	58 (66.7%)	
T3	5 (17.2%)	20 (23.0%)	
Clinical nodal status			0.39
N0	10 (34.5%)	40 (46.0%)	
N1/N2/N3	19 (65.5%)	47 (54.0%)	
Pre-NAC TILs (%)			
Stromal	21.6	25.6	0.39
Intratumoral	8.8	12.3	0.26

Abbreviations: SD: standard deviation

1 Pre-NAC TILs were available for 27 PP-PABC patients and 67 non-PABC patients. Pre-NAC
 2 stromal TILs levels were comparable between PP-PABC and non-PABC patients in global
 3 population (21.6% for PP-PABC vs. 25.6% for non-PABC, respectively, $p=0.39$; Figure 1).
 4 These results were unchanged after stratification by **pathological breast cancer subtype**
 5 (luminal: 13.7% for PP-PABC vs. 15.2% for non-PABC, respectively, $p=0.77$; TNBC: 29.4%
 6 for PP-PABC vs. 32% for non-PABC, respectively, $p=0.82$; *HER2*-positive: 21.7% for PP-
 7 PABC vs. 24.7% for non-PABC, respectively, $p=0.61$; Figure 1). Similar results were found
 8 for intratumoral (IT) TILs.

Figure 1: Pre-NAC stromal TILs levels in PP-PABC patients and non-PABC controls, in whole population and by **pathological breast cancer subtype**



Title Page**Lymphovascular invasion after neoadjuvant chemotherapy is strongly associated with poor prognosis in breast carcinoma****Running title: Lymphovascular invasion after preoperative chemotherapy**

Anne-Sophie Hamy ^{1,2*}, Giang-Thanh Lam ^{3,4*}, Enora Laas ³, Lauren Darrigues ³, Thomas Balezeau ⁵, Julien Guerin ⁵, Alain Livartowski ⁵⁻⁶, Benjamin Sadacca ^{1,2}, Jean-Yves Pierga ⁶, Anne Vincent-Salomon ⁷, Florence Coussy ⁸, Veronique Becette ⁹, H el ene Bonsang-Kitzis ³, Roman Rouzier ^{10,11}, Jean-Guillaume Feron ³, Gabriel Benchimol ³, Marick La e ⁷, Fabien Reyat ^{1,2,3}

Affiliations:

1. Residual Tumor & Response to Treatment Laboratory, RT2Lab, Translational Research Department, Institut Curie, 26, rue d'Ulm, Paris, F-75248, France.
2. U932, Immunity and Cancer, INSERM, Institut Curie, Paris, F-75248, France.
3. Department of Surgery, Institut Curie, 26 rue d'Ulm, Paris, F-75248, France.
4. Department of Gynecology and Obstetrics, Geneva University Hospitals, 30 bd de la Cluse, 1205 Geneva, Switzerland
5. Department of Medical Informatics, Institut Curie, 26 rue d'Ulm, Paris, F-75248, France
6. Department of Medical Oncology, Institut Curie, 26 rue d'Ulm, Paris, F-75248, France
7. Department of Pathology, Institut Curie, 26 rue d'Ulm, Paris, F-75248, France
8. Department of Medical Oncology, H opital Ren e Huguenin, 35, rue Dailly, Saint-Cloud, F-92210, France
9. Department of Pathology, H opital Ren e Huguenin, 35, rue Dailly, Saint-Cloud, F-92210, France
10. Department of Surgery, H opital Ren e Huguenin, 35, rue Dailly, Saint-Cloud, F-92210, France

11. Equipe d'Accueil 7285, Risk and Safety in Clinical Medicine for Women and Perinatal Health, University Versailles-Saint-Quentin, 2 av de la source de la Bièvre, 78180 Montigny-le-Bretonneux, France

*Both authors contributed equally to this work.

Corresponding author:

Dr Fabien REYAL, Institut Curie, Department of Surgery, 26 rue d'Ulm, 75005 Paris
00 33 615271980; fabien.reyal@curie.fr

Word count: 3494

Abstract:

Background: Few studies evaluated the prognostic value of the presence of lymphovascular invasion (LVI) after neoadjuvant chemotherapy (NAC).

Methods: The association between LVI and survival was evaluated in a cohort of BC patients treated by NAC between 2002 and 2011. Five post-NAC prognostic scores (ypAJCC, RCB, CPS, CPS+EG and Neo-Bioscore) were evaluated and compared with or without the addition of LVI.

Results: Out of 1033 tumors, LVI was present on surgical specimens in 29.2 % and absent in 70.8 % of the cases. Post-NAC LVI was associated with impaired disease-free (DFS) (HR, 2.54; 95% CI, 1.96 - 3.31; $P < 0.001$), and the magnitude of this effect depended on BC subtype ($P_{\text{interaction}} = 0.003$), (luminal BC: HR, 1.83; $P = 0.003$; triple negative BC: HR, 3.73; $P < 0.001$; *HER2*-positive BC: HR, 6.21; $P < 0.001$). Post-NAC LVI was an independent predictor of local relapse, metastases and overall survival and increased the accuracy of all 5 post-NAC prognostic scoring systems.

Conclusion: Post-NAC LVI is a strong independent prognostic factor that: (i) should be systematically reported in pathology reports; (ii) should be used as stratification factor after NAC to propose inclusion in second-line trials or adjuvant treatment; (iii) should be included in post-NAC scoring systems.

Key words: Breast carcinoma; lymphovascular invasion; neoadjuvant chemotherapy; prognostic scores

Manuscript text**Introduction**

Lymphovascular invasion (LVI) is defined as the presence of tumor cells in lymphatic or blood vessels in patients with breast carcinoma (BC). Evidence suggest that LVI is a risk factor for axillary and distant metastasis (Lee *et al*, 2006; Rakha *et al*, 2012). However it has been reported that LVI is not an independent factor for overall survival (OS) (Freedman *et al*, 2012). Much controversy remains about the importance of this factor, which is still absent from prediction tools such as Adjuvant on Line! (Ravdin, 1996), cancermath.net (Michaelson *et al*, 2011) or Predict (Wishart *et al*, 2010). LVI is not systematically taken into account in decisions about systemic treatment (not mentioned in the NCCN (NCCN Clinical Practice Guidelines in Oncology, 2016) and Saint Gallen guidelines (Coates *et al*, 2015), and considered only in cases of luminal cancer in the ESMO recommendations (Senkus *et al*, 2015)).

Beyond increasing the rate of breast-conserving surgery, neoadjuvant chemotherapy (NAC) serves as an *in vivo* chemosensitivity test and identifies patients whose tumor reaches a pathological complete response (pCR) as a group with a good prognosis. However, few studies have assessed the prognostic value of LVI in the neoadjuvant setting (Liu *et al*, 2016), and its prognostic value is unknown. The aim of this study was to analyze the prognostic impact on survival of LVI on surgical specimens following NAC in a large cohort of BC patients.

Materials and methods*Patients and tumors*

We analyzed a cohort of 1033 T1-3NxM0 patients with invasive breast carcinoma (NEOREP Cohort) treated with NAC at Institut Curie between 2002 and 2012 with data available on LVI status. Details on this cohort have been published elsewhere (Supplementary material). All

patients received neoadjuvant chemotherapy, and trastuzumab was used in *HER2*-positive tumors in an adjuvant and/or neoadjuvant setting since 2005.

Lymphovascular invasion

LVI was defined as the presence of carcinoma cells within a finite endothelial-lined space (a lymphatic or blood vessel). Presence or absence of LVI was determined by unstained standard formalin-fixed paraffin-embedded examination. Immunostaining with vascular markers was occasionally performed to rule out invasive carcinoma with shrinkage artifact. LVI data were extracted from pathology records by two independent researchers (TL, ASH), and were dichotomized into a binary variable (Post-NAC LVI: yes/no). Patients whose tumor reached pCR were considered as having no LVI unless LVI presence was explicitly mentioned (n=3). Results were crosschecked and a breast expert pathologist (ML) resolved discrepancies.

Study endpoints

A pCR was defined as the absence of invasive residual tumor in breast and axillary nodes (ypT0/is N0). Disease-free survival (DFS) was defined as the time from surgery to death, loco-regional recurrence or distant recurrence, whichever occurred first. Metastasis-free survival (MFS) was defined as the time from surgery to distant metastasis. Recurrence-free survival (RFS) was defined as the time from surgery to recurrence. OS was defined as the time from surgery to death. Patients for whom none of these events were recorded, date of their last known contact was retained. Survival cutoff date analysis was March 13, 2013.

Comparison to prognostic scores

We evaluated five post-NAC staging systems (ypAJCC (Edge *et al*, 2009), RCB (Symmans *et al*, 2007), CPS (Jeruss *et al*, 2008), CPS+EG (Jeruss *et al*, 2008) and Neo-Bioscore (Mittendorf *et al*, 2016)), and we assessed their performance with or without LVI. Prognostic scores were compared using Akaike Information Criterion (AIC). AIC is a method for

selecting the most predictive model from a set of models. It selects the model with the largest likelihood under the constraint of the smallest number of predictors. Lower AIC values represent better models.

Statistical analysis

Survival probabilities were estimated by the Kaplan–Meier method, and survival curves were compared in log-rank tests. Hazard ratios and their 95% confidence intervals were calculated with the Cox proportional hazards model. Variables with a *P*-value for the likelihood ratio test ≤ 0.10 in univariate analysis were selected for inclusion in the multivariate analysis. A forward stepwise selection procedure was used to establish the final multivariate model. The significance threshold was 5%. Analyses were performed with R software, version 3.1.2 (R Development Core Team: R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing., 2011), with the *ggplot2*, *survminer*, *mice* and *survival* libraries.

Results

In total, 1033 patients were included in the analyses. Patient characteristics are summarized in Table 1. Median age at diagnosis was 48.8 years old (range 24–80 years). Tumor distribution by BC subtype was as follows: luminal: *n*= 448 (43.4%); triple negative breast cancer (TNBC): *n*= 330 (31.9%); *HER2*-positive: *n*= 255 (24.7%). Most of the patients received an anthracyclines-taxanes based chemotherapy regimen (*n*=713 [69.0%]).

Characteristics associated with post-NAC LVI

After NAC, 292 patients achieved pCR (28.3%). The rate of pCR differed significantly between BC subtypes (luminal: *n*=36 [8.0%]; TNBC: *n*=143 [43.3%]; *HER2*-positive: *n*=113 [44.3%]) (*P* < 0.001). LVI was present (Supplementary Fig. 1) in 302 surgical specimens

(29.2%), absent in 731 specimens (70.8%). Only three of the 1033 patients had isolated LVI with no invasive residual disease in breast and lymph nodes. The presence of LVI after NAC was significantly different by BC subtype (luminal: 42.2%; TNBC: 19.4%; *HER2*-positive: 19.2%, $P < 0.0001$, Fig. 1A), tumor grade (grade I-II: 36%; grade III: 24.3%, $P = 0.0001$, Fig. 1B). Post-NAC LVI was also associated with an increasing RCB index (pCR: 1.4%; RCB-I: 9.1%; RCB-II: 28.8%; RCB-III: 48.5%, $P < 0.0001$, Fig. 1C) and nodal involvement (N-: 14.5%; 1 to 3N+: 44.5%; $\geq 4N+$: 60.5%, $P < 0.0001$, Fig. 1D)(Supplementary Table 1).

Tumors with post-NAC LVI had a lower pre-NAC mitotic index (17.7 versus 22.9, $P < 0.01$, Supplementary Fig. 2A), the decrease of their mitotic index was less marked (-0.5 versus -10.8, $P < 0.001$, Supplementary Fig. 2C), and the post-NAC mitotic index was not different (20.7 versus 14.7, $P = 0.05$, Supplementary Fig. 2B) than tumors without post-NAC LVI (Supplementary Table 1). Pre-NAC tumor cellularity was not different (Supplementary Fig. 2D), but post-NAC cellularity was higher in tumors with post-NAC LVI than those without ($P < 0.01$) (Supplementary Fig. 2E).

DFS analyses

After a median follow-up of 50.7 months, 224 patients had presented a relapse (locoregional, $n=74$, distant metastasis, $n=178$), and 120 had died. In univariate analysis, the presence of LVI was associated with poor DFS (HR, 2.54; 95% CI, 1.96 - 3.31; $P < 0.001$, Fig. 2A) (Table 2). An adverse effect of post-NAC LVI was observed in all three BC subtypes, but the magnitude of this effect differed between subtypes (Fig. 2) ($P_{\text{interaction}} = 0.003$). Post-NAC LVI impact was the smallest for luminal BC (HR, 1.83; 95% CI, 1.23 - 2.73; $P = 0.003$, Fig. 2B); intermediate for TNBC (HR, 3.73; 95% CI, 2.41 - 5.78; $P < 0.001$, Fig. 2C); and the hazard ratio was the greatest for *HER2*-positive BC (HR, 6.21; 95% CI, 3.36 - 11.45, $P < 0.001$, Fig. 2D) (Supplementary Tables 2-4). Among the whole population (Table 2) and BC subtypes

(Supplementary Tables 2-4), LVI remained an independent prognostic factor after multivariate analysis.

Combination of LVI with pCR status and with pathological nodal involvement

When analyzing together pCR status and LVI, both variables had an independent prognostic value in the whole population and in the TNBC population (Supplementary Figs 6 and Supplementary Table 5). Conversely, in the luminal and the *HER2*-positive population, pCR was of no incremental value to LVI status.

The adverse impact of LVI on DFS was detected both in pN- (HR, 2.51; 95% CI, 1.61-3.93; $P < 0.001$, Supplementary Fig. 3A) and pN+ tumors (HR, 1.94; 95% CI, 1.36-2.28; $P < 0.001$, Supplementary Fig. 3B). In pN- tumors (Supplementary Fig 4), LVI was a significant prognostic factor in all but in the luminal BC subtype ($P_{\text{interaction}}=0.003$) where no interaction was seen. No interaction between BC subtype and the prognostic impact of LVI was seen in pN+ tumors ($P=0.44$) (Supplementary Fig 5).

Figure 3 summarizes the prognostic impact of LVI combined with nodal involvement. In luminal BC, the combination of nodal involvement and LVI was associated with an adverse outcome when compared to other subgroups. In TNBC, only the absence of both nodal involvement and LVI was associated with an improved outcome when compared to other subgroups. In *HER2*-positive BC, the HR of relapse of patients with LVI was about 8 times higher than the reference class, irrespective of nodal status.

Combination of Post-NAC LVI with prognostic scores

We analyzed whether post-NAC LVI added prognostic significance to existing staging or scoring systems following NAC. We retrieved AJCC staging ($n=975$), RCB index ($n=640$), CPS score ($n=975$), CPS + EG score ($n=509$) and Neo-Bioscore ($n=509$). For 473 patients, all these data were available together with post-NAC LVI status. When combined with each score, post-NAC LVI retained a prognostic value for DFS in every model (Table 3) and was associated with a lower AIC criterion when compared with the model with each score alone.

RFS, MFS and OS analyses

After univariate analysis, the presence of post-NAC LVI was significantly associated with RFS, MFS and OS in the whole population and in the three BC subtypes (see Supplemental results, Supplementary Figs 7-9 and Supplementary Tables 6-17) and remained an independent prognostic after multivariate analysis.

Discussion

We identified post-NAC LVI as a strong independent factor predictive of poor survival in a large retrospective series of 1033 BC patients treated by NAC and this factor added independent prognostic information to existing post-NAC scoring systems.

This study presents an evaluation of LVI in the post-NAC setting in the largest cohort studied to date. Liu *et al.* (2016) recently published an analysis of 166 patients for whom pathology reports were evaluated for LVI following NAC. The presence of post-NAC LVI was significantly associated with lower progression-free survival (HR, 3.76; 95% CI, 2.07-6.83; $P < 0.01$) and OS (HR, 5.70; 95% CI, 2.08-15.64; $P < 0.01$). Abdel-Fatah *et al.* (2015) recently developed the Nottingham Clinico-Pathological Response Indexes (NPRI), including fibrosis, LVI status, number of positive nodes and planned hormonal therapy. The NPRI was shown to outperform other prognostic factors including RCB index and pCR (Abdel-Fatah *et al.*, 2015). The presence of LVI had previously been identified as an independent predictor of the early failure of NAC in 397 patients receiving NAC for locally advanced BC (Choi *et al.*, 2014). In accordance with these studies, our results further support the prognostic role of post-NAC LVI in the neoadjuvant setting.

In the adjuvant setting, the largest historical study evaluating LVI in operable BC included 16,172 BC patients treated between 1996 and 2002 (Ejlertsen *et al.*, 2009). In this cohort, LVI was associated with a poor prognosis only for patients at high risk (positive lymph nodes,

tumor size >2 cm, high grade, hormone receptor–negative tumor, patient less than 35 years old). Despite its large sample size, the major limitation of this study was the absence of data for *HER2* status. In another cohort of 3,812 patients with BCs of different subtypes (Rakha *et al*, 2012), LVI was an independent prognostic factor for both breast cancer-specific survival (BCSS) and distant metastasis-free survival (DMFS) in the whole cohort and in the various subgroups considered.

Our study provides new insight into the patterns associated with LVI at the end of NAC. In the neoadjuvant setting, Liu *et al*. (2016) did not find association between post-NAC LVI and the baseline characteristics of the tumor or of the patients. In the adjuvant setting, previous studies on BC showed that LVI was associated with young age (Ragage *et al*, 2010) or premenopausal status (Rakha *et al*, 2012), ductal histological type (Ejlertsen *et al*, 2009), high tumor grade (Rakha *et al*, 2012; Ragage *et al*, 2010), proliferation index (Rakha *et al*, 2012), or tumor size (Rakha *et al*, 2012; Ejlertsen *et al*, 2009; Guarnieri *et al*, 2001). Conflicting findings have been reported concerning the relationship between LVI and ER status, with some studies reporting higher rates of LVI in ER positive tumors (Ugras *et al*, 2014) and others reporting lower rates of LVI in these tumors (Rakha *et al*, 2012; Ejlertsen *et al*, 2009; Ragage *et al*, 2010). Similarly, some studies found a higher incidence of LVI in *HER2*-positive BC (Ugras *et al*, 2014), whereas others did not (Ragage *et al*, 2010). Finally, most studies have reported an association between LVI and the involvement of a larger number of nodes (Rakha *et al*, 2012; Ejlertsen *et al*, 2009). In our cohort, post-NAC LVI was most frequently identified in low-grade and luminal tumors. It was also associated with a smaller decrease in mitotic index, a higher RCB index, and a larger number of involved lymph nodes, consistent with patterns of resistance to chemotherapy.

The molecular mechanisms underlying LVI in invasive BC remain poorly understood. LVI is a crucial step in the invasion-metastasis cascade (Talmadge & Fidler, 2010), including as prerequisites (i) cell detachment from the growing tumor mass, (ii) local invasion of the

surrounding tissues by primary tumor cells, and (iii) migration to the vascular walls through the extracellular matrix (ECM). One key process underlying these steps is the epithelial-mesenchymal transition, which renders more motile and invasive epithelial cells, and increases their ability to degrade ECM components. However, no robust gene expression profile, gene signature or proteomic profile has been validated to date, and the molecular mechanisms driving BC tumor cells to invade vascular spaces and to disseminate remain largely unknown (Aleskandarany *et al*, 2015).

This study has several important implications: (i) it calls for the systematic reporting of LVI in pathology records. International experts recently wrote recommendations acknowledging that there were too few data, at that time, to conclude that post-NAC LVI was an independent prognostic factor, but they advised recording the presence or absence of LVI in the specimen obtained after neoadjuvant treatment (Provenzano *et al*, 2015). Our results reinforce these guidelines by providing robust evidence on its strong prognostic value. (ii) LVI seems a major factor for stratifying patients at high risk of relapse following NAC. pCR status clearly identifies a group of patients with an excellent prognosis; by contrast, post-NAC LVI status identified a subgroup at high risk of relapse, particularly in the TNBC subgroup in which post-NAC median DFS was below 20 months. (iii) Third, LVI should be an important item to incorporate in future research on post-NAC scoring and staging systems. (iv) Finally, the adjuvant capecitabine was recently shown to improve both DFS and OS in patients failing to achieve pCR at the completion of NAC (Masuda *et al*, 2017). Our results clearly support the fact that all TNBC with LVI at the end of NAC should be offered this second-line treatment or inclusion in second-line clinical trials.

Acknowledgment section

We thank Roche* France for financial support for construction of the Institut Curie neoadjuvant database (NEOREP). The funding source had no role in data analysis and interpretation neither in writing the manuscript.

AS Hamy was supported by an ITMO-INSERM-AVIESAN cancer translational research grant.

Fundings: This work was supported by the Site de Recherche Intégrée en Cancérologie/Institut National du Cancer [INCa-DGOS-4654]; and Grant ARC Foundation 2013 [SL220130607090].

Conflict of interest statement: There are not conflict of interest

References

- Abdel-Fatah TM, Ball G, Lee AHS, Pinder S, MacMilan RD, Cornford E, Moseley PM, Silverman R, Price J, Latham B, Palmer D, Chan A, Ellis IO, Chan SYT (2015) Nottingham Clinico-Pathological Response Index (NPRI) after neoadjuvant chemotherapy (Neo-ACT) accurately predicts clinical outcome in locally advanced breast cancer. *Clin Cancer Res Off J Am Assoc Cancer Res* **21**: 1052–1062, doi:10.1158/1078-0432.CCR-14-0685.
- Aleskandarany MA, Sonbul SN, Mukherjee A, Rakha EA (2015) Molecular Mechanisms Underlying Lymphovascular Invasion in Invasive Breast Cancer. *Pathobiology* **82**: 113–123, doi:10.1159/000433583.
- Choi MK, Park YH, Kil WH, Lee JE, Nam SJ, Ahn JS, Im Y-H (2014) Clinicopathological features of early failure of neoadjuvant chemotherapy in locally advanced breast cancer. *Cancer Chemother Pharmacol* **74**: 521–529, doi:10.1007/s00280-014-2542-5.
- Coates AS, Winer EP, Goldhirsch A, Gelber RD, Gnant M, Piccart-Gebhart M, Thürlimann B, Senn H-J, Panel Members (2015) Tailoring therapies--improving the management of early breast cancer: St Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2015. *Ann Oncol Off J Eur Soc Med Oncol* **26**: 1533–1546, doi:10.1093/annonc/mdv221.
- Edge S, Byrd D, Compton C, Fritz A, Greene F, Trotti A (2009) American Joint Committee on Cancer (AJCC) Cancer Staging Manual.
- Ejlertsen B, Jensen M-B, Rank F, Rasmussen BB, Christiansen P, Kroman N, Kvistgaard ME, Overgaard M, Toftdahl DB, Mouridsen HT, Danish Breast Cancer Cooperative Group (2009) Population-based study of peritumoral lymphovascular invasion and outcome among patients with operable breast cancer. *J Natl Cancer Inst* **101**: 729–735, doi:10.1093/jnci/djp090.
- Freedman GM, Li T, Polli L V., Anderson PR, Bleicher RJ, Sigurdson E, Swaby R, Dushkin H, Patchefsky A, Goldstein L (2012) Lymphatic Space Invasion is Not an Independent Predictor of Outcomes in Early Stage Breast Cancer Treated by Breast-Conserving Surgery and Radiation. *Breast J* **18**: 415–419, doi:10.1111/j.1524-4741.2012.01271.x.
- Guarnieri A, Neri A, Correale PP, Lottini M, Testa M, Mariani F, Tucci E, Megha T, Cintonino M, Carli A (2001) Prediction of lymph node status by analysis of prognostic factors and possible indications for elective axillary dissection in T1 breast cancers. *Eur J Surg Acta Chir* **167**: 255–259, doi:10.1080/110241501300091381.
- Jeruss JS, Mittendorf EA, Tucker SL, Gonzalez-Angulo AM, Buchholz TA, Sahin AA, Cormier JN, Buzdar AU, Hortobagyi GN, Hunt KK (2008) Combined use of clinical and pathologic staging variables to define outcomes for breast cancer patients treated with neoadjuvant therapy. *J Clin Oncol Off J Am Soc Clin Oncol* **26**: 246–252, doi:10.1200/JCO.2007.11.5352.
- Lee AHS, Pinder SE, Macmillan RD, Mitchell M, Ellis IO, Elston CW, Blamey RW (2006) Prognostic value of lymphovascular invasion in women with lymph node negative invasive breast carcinoma. *Eur J Cancer* **42**: 357–362, doi:10.1016/j.ejca.2005.10.021.
- Liu YL, Saraf A, Lee SM, Zhong X, Hibshoosh H, Kalinsky K, Connolly EP (2016) Lymphovascular invasion is an independent predictor of survival in breast cancer after

neoadjuvant chemotherapy. *Breast Cancer Res Treat* **157**: 555–564, doi:10.1007/s10549-016-3837-5.

Masuda N, Lee S-J, Ohtani S, Im Y-H, Lee E-S, Yokota I, Kuroi K, Im S-A, Park B-W, Kim S-B, Yanagita Y, Ohno S, Takao S, Aogi K, Iwata H, Jeong J, Kim A, Park K-H, Sasano H, Ohashi Y, Toi M (2017) Adjuvant Capecitabine for Breast Cancer after Preoperative Chemotherapy. *N Engl J Med* **376**: 2147–2159, doi:10.1056/NEJMoa1612645.

Michaelson JS, Chen LL, Bush D, Fong A, Smith B, Younger J (2011) Improved web-based calculators for predicting breast carcinoma outcomes. *Breast Cancer Res Treat* **128**: 827–835, doi:10.1007/s10549-011-1366-9.

Mittendorf EA, Vila J, Tucker SL, Chavez-MacGregor M, Smith BD, Symmans WF, Sahin AA, Hortobagyi GN, Hunt KK (2016) The Neo-Bioscore Update for Staging Breast Cancer Treated With Neoadjuvant Chemotherapy: Incorporation of Prognostic Biologic Factors Into Staging After Treatment. *JAMA Oncol* **2**: 929–936, doi:10.1001/jamaoncol.2015.6478.

NCCN Evidence Blocks, and TM (2016). NCCN Clinical Practice Guidelines in Oncology (NCCN Guidelines) - Breast Cancer. (2016).

Provenzano E, Bossuyt V, Viale G, Cameron D, Badve S, Denkert C, MacGrogan G, Penault-Llorca F, Boughey J, Curigliano G, Dixon JM, Esserman L, Fastner G, Kuehn T, Peintinger F, von Minckwitz G, White J, Yang W, Symmans WF, Residual Disease Characterization Working Group of the Breast International Group-North American Breast Cancer Group Collaboration (2015) Standardization of pathologic evaluation and reporting of postneoadjuvant specimens in clinical trials of breast cancer: recommendations from an international working group. *Mod Pathol Off J U S Can Acad Pathol Inc* **28**: 1185–1201, doi:10.1038/modpathol.2015.74.

R Development Core Team: R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing. (2011).

Ragage F, Debled M, MacGrogan G, Brouste V, Desrousseaux M, Soubeyran I, de Lara CT, Mauriac L, de Mascarel I (2010) Is it useful to detect lymphovascular invasion in lymph node-positive patients with primary operable breast cancer? *Cancer* **116**: 3093–3101, doi:10.1002/cncr.25137.

Rakha EA, Martin S, Lee AHS, Morgan D, Pharoah PDP, Hodi Z, MacMillan D, Ellis IO (2012) The prognostic significance of lymphovascular invasion in invasive breast carcinoma. *Cancer* **118**: 3670–3680, doi:10.1002/cncr.26711.

Ravdin PM (1996) A computer program to assist in making breast cancer adjuvant therapy decisions. *Semin Oncol* **23**: 43–50.

Senkus E, Kyriakides S, Ohno S, Penault-Llorca F, Poortmans P, Rutgers E, Zackrisson S, Cardoso F, ESMO Guidelines Committee (2015) Primary breast cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann Oncol* **26**: v8–v30, doi:10.1093/annonc/mdv298.

Symmans WF, Peintinger F, Hatzis C, Rajan R, Kuerer H, Valero V, Assad L, Poniecka A, Hennessy B, Green M, Buzdar AU, Singletary SE, Hortobagyi GN, Pusztai L (2007) Measurement of Residual Breast Cancer Burden to Predict Survival After Neoadjuvant Chemotherapy. *J Clin Oncol* **25**: 4414–4422, doi:10.1200/JCO.2007.10.6823.

Talmadge JE, Fidler IJ (2010) AACR centennial series: the biology of cancer metastasis: historical perspective. *Cancer Res* **70**: 5649–5669, doi:10.1158/0008-5472.CAN-10-1040.

Ugras S, Stempel M, Patil S, Morrow M (2014) Estrogen Receptor, Progesterone Receptor, and HER2 Status Predict Lymphovascular Invasion and Lymph Node Involvement. *Ann Surg Oncol* **21**: 3780–3786, doi:10.1245/s10434-014-3851-y.

Wishart GC, Azzato EM, Greenberg DC, Rashbass J, Kearins O, Lawrence G, Caldas C, Pharoah PDP (2010) PREDICT: a new UK prognostic model that predicts survival following surgery for invasive breast cancer. *Breast Cancer Res BCR* **12**: R1, doi:10.1186/bcr2464.

Tables legends :

Table 1: Characteristics of patients and tumors.

Abbreviations: BMI: body mass index (kg/m²), ER: estrogen receptor, NST: no specific type, PR: progesterone receptor, TNBC: triple negative breast cancer. Missing data: menopausal status: n=7, BMI: n=4, histological type: n=13, grade: n=35, mitotic index: n=96, PR: n=1.

Table 2: Univariate and multivariate analysis for disease-free survival (whole population)

Abbreviations: BMI: body mass index (kg/m²), DFS: disease-free survival, ER: oestrogen receptor, LVI: lymphovascular invasion, NAC: neoadjuvant chemotherapy, NST: no specific type, pCR: pathological Complete Response, PR: progesterone receptor, TNBC: triple negative breast cancer.

Events: locoregional relapse, distant metastasis or death.

Table 3: Comparison of post-NAC staging and scoring systems alone or in combination with post-NAC LVI.

Abbreviations: AIC: Akaike Information Criterion, AJCC: American Joint Committee on Cancer, CPS: Clinical-pathological Scoring, E: negative oestrogen receptor, G: nuclear grade III tumor pathology, LVI: lymphovascular invasion, pCR: pathological Complete Response, pN+: pathological nodal involvement, RCB: Residual Cancer Burden.

*P** corresponds to the *P*-value for the log-rank test for association between the variable and the DFS, and *P* corresponds to the *P*-value for the association of each class versus the reference class (Cox proportional hazard model).

Table 1: Characteristics of patients and tumors	
Variable	NEOREP Cohort n=1033
Age, years	
<45	376 (36.4)
45-55	392 (37.9)
>55	265 (25.7)
Menopausal status	
Premenopausal	634 (61.8)
Postmenopausal	392 (38.2)
BMI class	
19≤BMI≤25	570 (55.4)
BMI<19	59 (5.7)
BMI>25	400 (38.9)
Tumor size	
T1-T2	754 (73.0)
T3	279 (27.0)
Clinical nodal status	
N0	447 (43.3)
N1-N2-N3	586 (56.7)
Histology	
Ductal carcinoma NST	933 (91.5)
other	87 (8.5)
Grade	
Grade I-II	394 (39.5)
Grade III	604 (60.5)
Ki67	
<20%	139 (29.4)
≥20%	333 (70.6)
Mitotic index	
≤22	590 (63.0)
>22	347 (37.0)
DCIS component	
No	512 (84.5)
Yes	94 (15.5)
ER status	
Negative	483 (46.8)
Positive	550 (53.2)
PR status	
Negative	594 (58.7)
Positive	418 (41.3)

HER2 status	
Negative	778 (75.3)
Positive	255 (24.7)
Subtype	
Luminal	448 (43.4)
TNBC	330 (31.9)
HER2	255 (24.7)
Type of NAC	
Anthracyclines based regimens	189 (18.3)
Anthracyclines-taxanes regimens	713 (69.0)
Other	131 (12.7)
Type of surgery	
Lumpectomy	703 (68.1)
Mastectomy	330 (31.9)
Radiotherapy	
Yes	1017 (98.5)
No	16 (1.5)
Hormonotherapy	
Yes	551 (53.3)
No	482 (46.7)

Table 2: Univariate and multivariate analysis for disease-free survival (whole popul

Variable	n	Univariate analysis				Multivariate analysis	
		Events	HR	[95% CI]	P value	HR	[95% CI]
Age, years							
<45	376	89	1		0.27		
45-55	392	84	0.82	[0.61 - 1.1]			
>55	265	51	0.79	[0.56- 1.11]			
Menopausal status							
Postmenopausal	392	82	1		0.53		
Premenopausal	634	139	1.09	[0.83 - 1.43]			
BMI class							
19≤BMI≤25	570	109	1		0.04 ^a		
BMI<19	59	17	1.64	[0.98 - 2.73]			
BMI>25	400	98	1.35	[1.02 - 1.77]			
Tumor size							
T1-T2	754	142	1			1	-
T3	279	82	1.85	[1.41 - 2.43]	<0.001	1.83	[1.37 - 2.46]
Clinical nodal status							
N0	447	89	1				
N1-N2-N3	586	135	1.28	[0.98 - 1.67]	0.07		
Histology							
Ductal carcinoma NST	933	197	1				
other	87	23	1.22	[0.79 - 1.88]	0.37		
Grade							
Grade I-II	394	85	1				
Grade III	604	131	1.23	[0.93 - 1.62]	0.14		
Ki67							
<20%	139	28	1				
≥20%	333	90	1.63	[1.07 - 2.5]	0.02		
Mitotic index							
≤22	590	124	1			1	-
>22	347	80	1.28	[0.97 - 1.7]	0.08	1.38	[1.02 - 1.87]
DCIS component							
No	512	99	1				
Yes	94	25	1.4	[0.9 - 2.18]	0.13		
ER status							
Negative	483	113	1				
Positive	550	111	0.71	[0.55 - 0.93]	0.01		
PR status							

Negative	594	140	1				
Positive	418	77	0.66	[0.5 - 0.87]	0.003		
HER2 status							
Negative	778	182	1				
Positive	255	42	0.77	[0.55 - 1.08]	0.13		
Subtype							
Luminal	448	99	1		0.003 ^b	1	-
TNBC	330	83	1.56	[1.16 - 2.08]		2.5	[1.78 - 3.51]
HER2	255	42	0.92	[0.64 - 1.33]		1.18	[0.78 - 1.8]
Post-NAC LVI							
No	731	107	1			1	-
Yes	302	117	2.54	[1.96 - 3.31]	<0.001	2.11	[1.55 - 2.87]
pCR							
pCR	292	28	1			1	-
No pCR	741	196	2.57	[1.73 - 3.81]	<0.001	2.47	[1.58 - 3.86]

--

a: *P*-value versus reference class (<19 versus 19-25. *P* =0.06; >25 versus 19-25. *P* =0.03)

b: *P*-value versus reference class (TNBC versus luminal. *P* =0.003; HER2 versus luminal. *P* = 0.66)

-
<0.001
0.438
-
<0.001
-
<0.001

Combination with post-NAC LVI
Multivariate analysis
 [95% CI] P value P value* AIC

-	-	<0.001	900.8
[1.85 - 4.45]	<0.001		

-	-	<0.001	881.3
[0.87 - 7.18]	0.088		
[1.44 - 10.18]	0.007		
[2.37 - 18.51]	<0.001		
[2.49 - 19.78]	<0.001		
[4.56 - 50.9]	<0.001		
-	-		
[1.02 - 2.65]	0.04	0.04	

-	-	0.01	888.5
[0.39 - 5.34]	0.59		
[1.22 - 5.5]	0.013		
[2.11 - 9.99]	<0.001		
-	-		
[1.15 - 2.95]	0.01	0.01	

-	-	<0.001	895.7
[0.65 - 2.98]	0.398		
[1.14 - 4.98]	0.021		
[1.56 - 13.39]	0.006		
-	-		
[1.39 - 3.52]	0.001	<0.001	

-	-	<0.001	883.1
[0.17 - 3.48]	0.724		
[0.14 - 2.53]	0.478		
[0.19 - 3.41]	0.769		
[0.67 - 12.02]	0.155		
[0 - Inf]	0.996		

-	-		
[1.7 - 4.12]	<0.001	0.001	

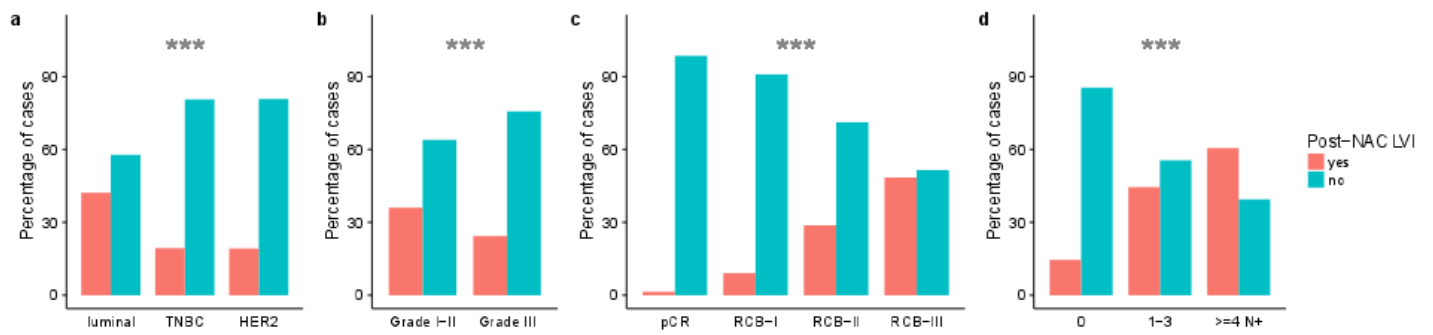
-	-	<0.001	876.8
[0.2 - 1.79]	0.357		
[0.23 - 1.68]	0.353		
[0.48 - 3.19]	0.664		
[1.57 - 11.31]	0.004		
[0 - Inf]	0.996		
-	-	0.002	
[1.68 - 4.09]	<0.001		

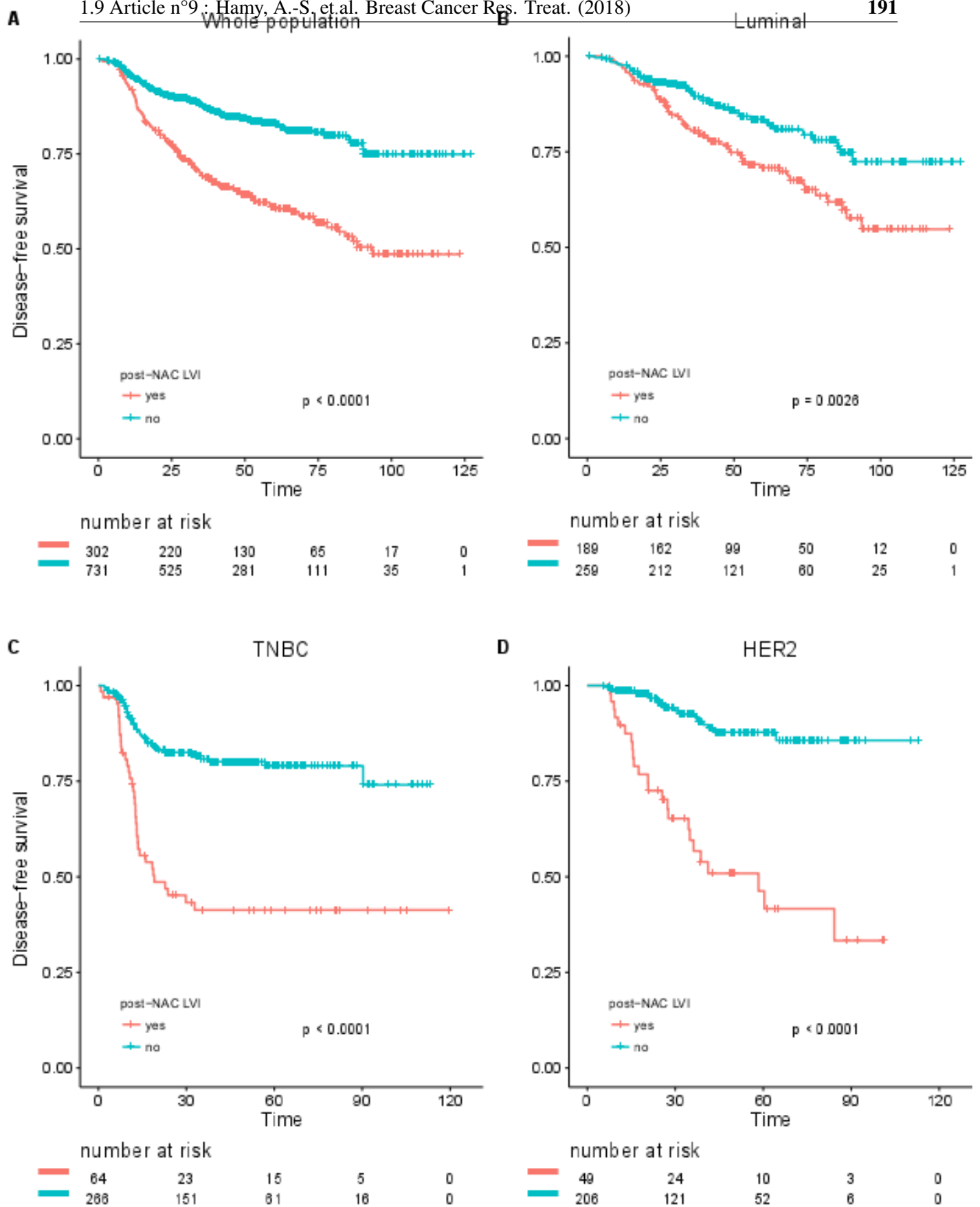
Figure legends:

Figure 1: Association of LVI status with pathological parameters. (A) tumor subtype, (B) tumor grade, (C) RCB index, (D) number of nodes involved. Stars represent *P*-value for *t*-test: *: $0.01 \leq P \leq 0.05$; **: $0.001 \leq P < 0.01$; ***: $P < 0.001$.

Figure 2: Association of LVI status with disease-free survival (DFS). (A) whole population, (B) luminal BC, (C) TNBC, (D) *HER2*-positive BC.

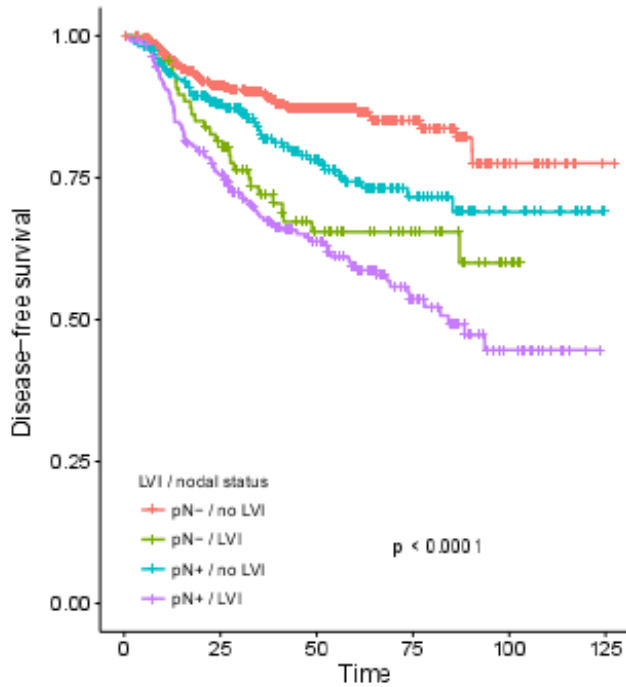
Figure 3: Association of LVI status combined with N status with disease-free survival (DFS).





A 192

Whole population

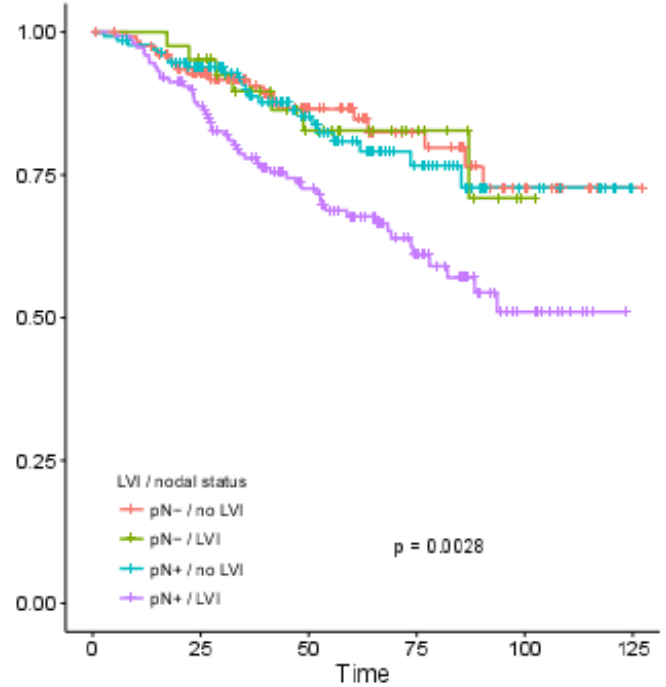


number at risk

517	368	189	71	22	1
88	67	34	18	4	0
214	157	92	40	13	0
214	153	96	47	13	0

B

Neoadjuvant treatment

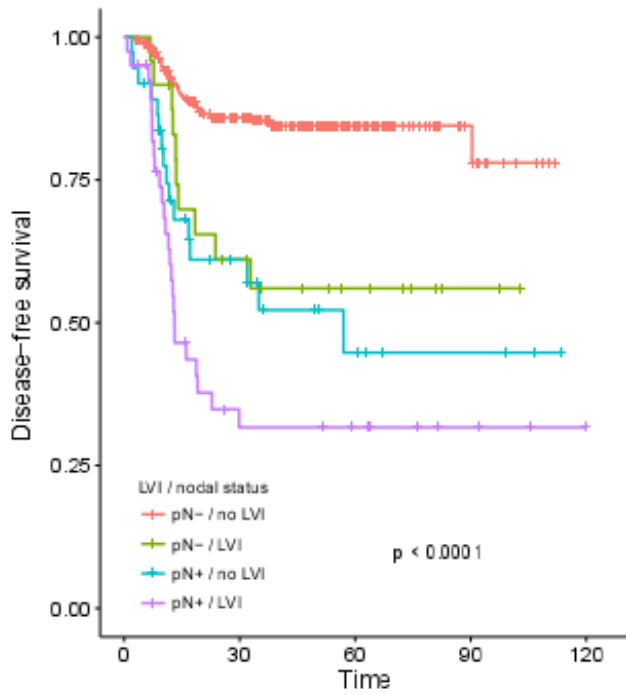


number at risk

126	104	56	30	15	1
41	38	21	11	1	0
133	108	65	30	10	0
148	124	78	39	11	0

C

TNBC

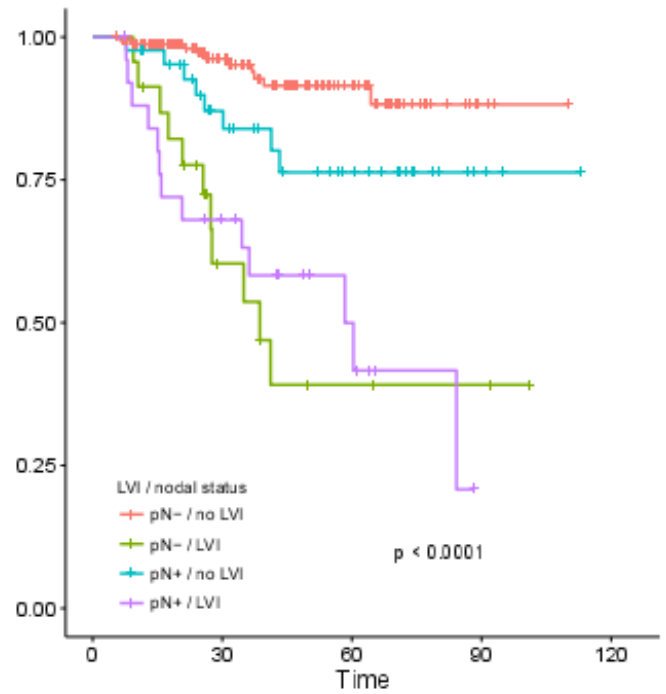


number at risk

229	136	55	13	0
24	13	7	2	0
37	15	6	3	0
40	10	8	3	0

D

HER2



number at risk

182	93	37	3	0
23	9	4	3	0
44	28	15	3	0
26	15	6	0	0

Supplementary Materials

Lymphovascular invasion after neoadjuvant chemotherapy is strongly associated with poor prognosis in breast carcinoma (BC)

Hamy, et al.

Patients and tumors

The NEOREP cohort (CNIL declaration number 1547270) included all consecutive patients with unilateral, non-recurrent, non-inflammatory, non-metastatic BC, and excluded T4 tumors. Patients were treated according to national guidelines. NAC regimens changed over time (anthracycline-based regimen, sequential anthracycline-taxane regimen), with trastuzumab used in an adjuvant and/or neoadjuvant setting since 2005. The surgery was performed four to six weeks after the end of chemotherapy. All but 16 patients received radiotherapy. Endocrine therapy (tamoxifen, aromatase inhibitor, and/or GnRH agonists) was prescribed when indicated. The study was approved by the Breast Cancer Study Group of Institut Curie and was conducted in accordance with institutional and ethical rules regarding research on tissue specimens and patients. Written informed consent from the patients was not required under French regulations.

Tumor samples

Cases were considered estrogen receptor (ER) or progesterone receptor (PR) positive (+) if at least 10% of the tumor cells expressed estrogen and/or progesterone receptors (ER/PR), in accordance with guidelines used in France [1]. *HER2* expression was determined by immunohistochemistry with scoring in accordance with American Society of Clinical Oncology (ASCO)/College of American Pathologists (CAP) guidelines [2]. Scores 3+ were reported as positive, score 1+/0 as negative (-). Tumors with scores 2+ were further tested by FISH. *HER2* gene amplification was defined in accordance with ASCO/CAP guidelines [2]. We evaluated a mean of 40 tumor cells per sample and the mean *HER2* signals per nuclei was calculated. A *HER2*/CEN17 ratio ≥ 2 was considered positive, and a ratio < 2 negative [2]. BC subtypes were defined as follows: tumors positive for either ER or PR, and negative for *HER2* were classified as luminal; tumors positive for *HER2* were considered to be *HER2*-positive BC; tumors negative for ER, PR, and *HER2* were considered to be triple-negative breast cancers (TNBC). Tumor cellularity was defined as the percentage of tumor cells (in situ and invasive) on the specimen (biopsy or surgical specimen). Mitotic index was reported per 10 high power fields (HPF) (1 HPF= 0.301 mm²).

Supplementary results

Recurrence-free survival (RFS) analysis

The presence of LVI was associated with poor RFS (Supplementary Tables S6, 7, 8, 9 and Supplementary Fig. 7) in the whole population (HR, 2.57; 95% CI, 1.63 - 4.06; $P < 0.001$) and in all BC subtypes (luminal BC: HR, 2.58; 95% CI, 1.04 - 6.39; $P = 0.03$; TNBC: HR, 3.75; 95% CI, 1.8 - 7.81; $P < 0.001$; *HER2*-positive BC: HR, 4.22; 95% CI, 1.89 - 9.39; $P < 0.001$). Post-NAC LVI remained an independent factor of poor RFS after multivariate analysis in whole population (HR, 3.66; 95% CI, 2.22 - 6.05; $P < 0.001$), TNBC (HR, 3.55; 95% CI, 1.53 - 8.25, $P = 0.003$) and *HER2* subtypes (HR, 4.22; 95% CI, 1.89 - 9.39, $P < 0.001$).

Metastasis-free survival (MFS) analysis

The presence of LVI was associated with poor MFS (Supplementary Tables S10, 11, 12, 13 and Supplementary Fig. 8) in the whole population (HR, 2.52; 95% CI, 1.88 - 3.38; $P < 0.001$) and in all BC subtypes (luminal BC: HR, 1.89; 95% CI, 1.21 - 2.94; $P = 0.005$; TNBC: HR, 3.26; 95% CI, 2.02 - 5.24; $P < 0.001$; *HER2*-positive BC: HR, 6.96; 95% CI, 3.16 - 15.34; $P < 0.001$). Post-NAC LVI remained an independent factor of poor survival after multivariate analysis in the total population and in BC subtypes.

Overall survival (OS) analysis

Univariate analysis of OS showed that post-NAC LVI was associated with a poor prognosis (Supplementary Tables 14, 15, 16, 17 and Supplementary Fig. 9) in the whole population (HR, 2.5; 95% CI, 1.74 - 3.58; $P < 0.001$) and in BC subtypes. Post-NAC LVI remained an independent factor of poor survival after multivariate analysis in the total population and in BC subtypes.

References

1. Harvey JM, Clark GM, Osborne CK, et al (1999) Estrogen Receptor Status by Immunohistochemistry Is Superior to the Ligand-Binding Assay for Predicting Response to Adjuvant Endocrine Therapy in Breast Cancer. *J. Clin. Oncol.* 17: 1474–1474.
2. Antonio C. Wolff, M. Elizabeth H. Hammond, Jared N. Schwartz, et al (2006) American Society of Clinical Oncology/College of American Pathologists Guideline Recommendations for Human Epidermal Growth Factor Receptor 2 Testing in Breast Cancer. *J. Clin. Oncol.* 25: 118–145.

Supplementary Figure legends:

Supplementary Figure S1: Lymphovascular invasion in a surgical specimen obtained after NAC. H&E (haemotoxylin and eosin) x20, 10 high power fields.

Supplementary Figure S2: Association of LVI status with pathological parameters. (a) Pre-NAC mitotic index; (b) Post-NAC mitotic index; (c) Change in mitotic index; (d) Pre-NAC tumor cellularity; (e) Post-NAC tumor cellularity. Asterisks indicate the *P*-values for *t*-tests: *: $.01 \leq P \leq .05$; **: $.001 \leq P < .01$; ***: $P < .001$.

Supplementary Figure S3: Association of LVI status with disease-free survival (DFS) by pathological node status. (a) DFS in the node-negative cohort, (b) DFS in the node-positive cohort.

Supplementary Figure S4: Association of LVI status with disease-free survival (DFS) in pN- population. (a) DFS in the whole population, (b) DFS in luminal BC, (c) DFS in TNBC, (d) DFS in *HER2*-positive BC.

Supplementary Figure S5: Association of LVI status with disease-free survival (DFS) in pN+ population. (a) DFS in the whole population, (b) DFS in luminal BC, (c) DFS in TNBC, (d) DFS in *HER2*-positive BC.

Supplementary Figure S6: Association of LVI status combined with pCR status with disease-free survival (DFS). (a) DFS in the whole population, (b) DFS in the luminal BC, (c) DFS in the TNBC, (d) DFS in the *HER2*-positive BC.

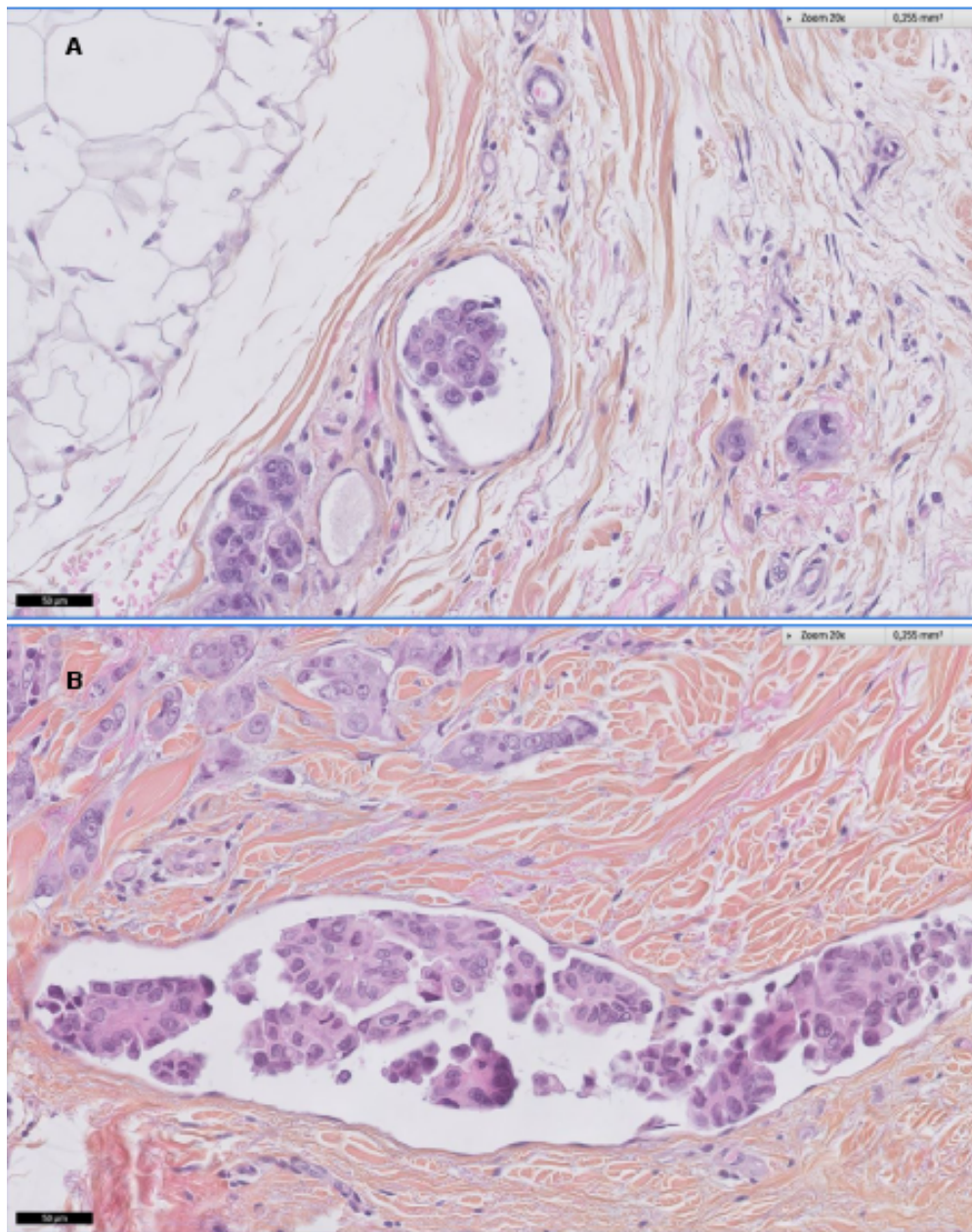
Three patients with LVI in the breast without any residual disease in the breast or in the nodes were removed from the analyses. Hazard ratios are as follows: Whole population: pCR /no LVI, HR=1; no pCR / no LVI, HR= 1.91 [1.23; 2.97]; no pCR / LVI, HR= 3.95 [2.58; 6.05]; luminal BC: pCR /no LVI, HR=1; no pCR / no LVI, HR= 1.75 [0.54; 5.66]; no pCR / LVI, HR= 2.99 [0.94; 9.54]; TNBC: pCR /no LVI, HR=1; no pCR / no LVI, HR= 2.7 [1.48; 4.92]; no pCR / LVI, HR= 6.45 [3.56; 11.67]; *HER2*-positive: pCR /no LVI, HR=1; no pCR / no LVI, HR= 1.67 [0.65; 4.32]; no pCR / LVI, HR= 8.31 [3.56; 19.4].

Supplementary Figure S7: Association of LVI status with recurrence-free survival (RFS). (a) RFS in the whole population, (b) RFS in luminal BC, (c) RFS in TNBC, (d) RFS in *HER2*-positive BC.

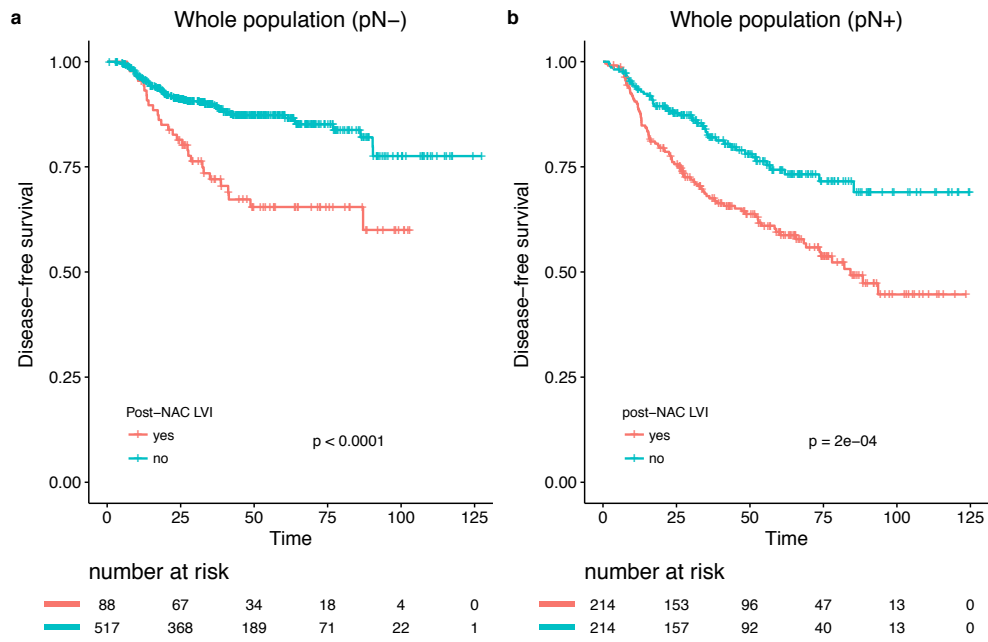
Supplementary Figure S8: Association of LVI status with metastasis-free survival (MFS). (a) MFS in the whole population, (b) MFS in luminal BC, (c) MFS in TNBC, (d) MFS in *HER2*-positive BC.

Supplementary Figure S9: Association of LVI status with overall survival (OS). (a) OS in the whole population, (b) OS in luminal BC, (c) OS in TNBC, (d) OS in *HER2*-positive BC.

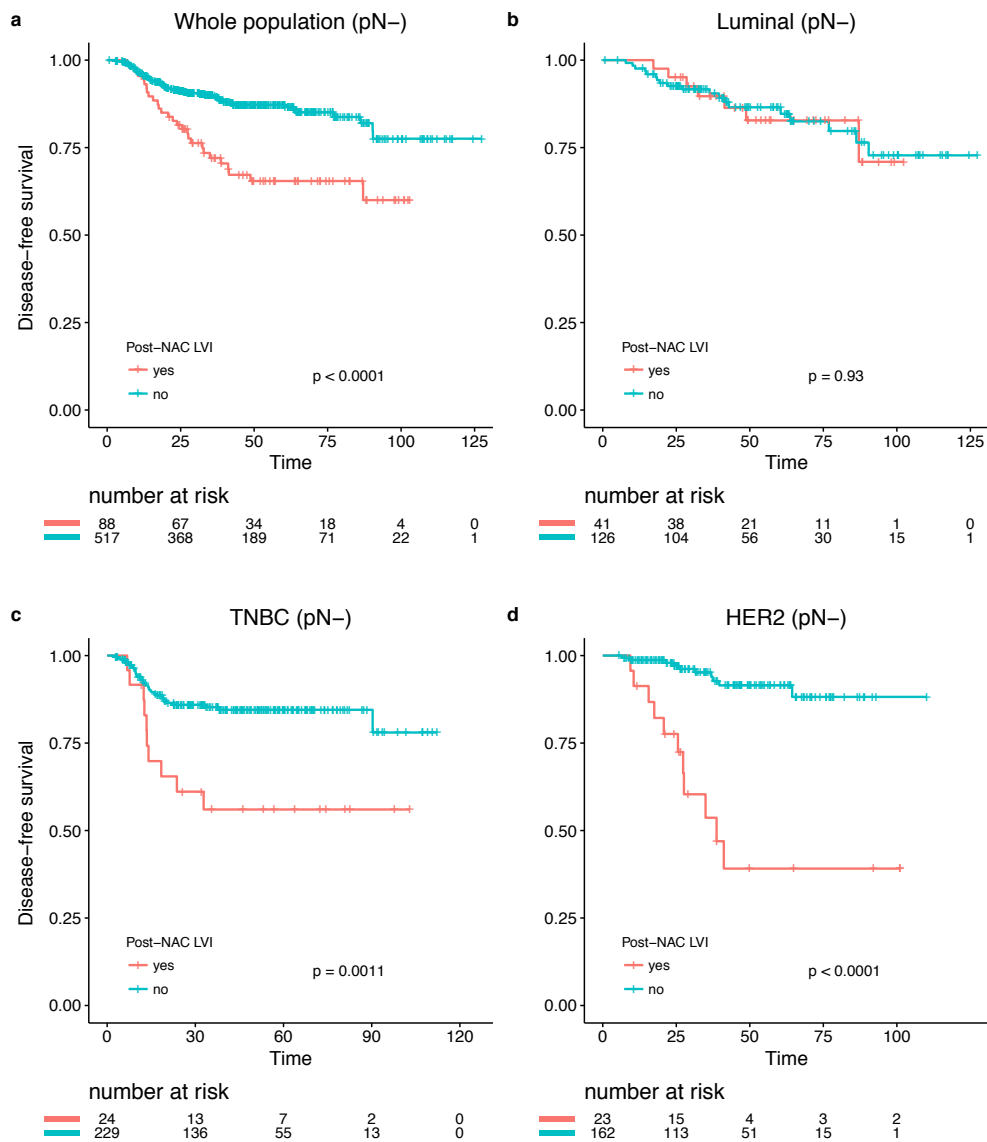
Supplementary Figure S1: Lymphovascular invasion in a surgical specimen obtained after NAC



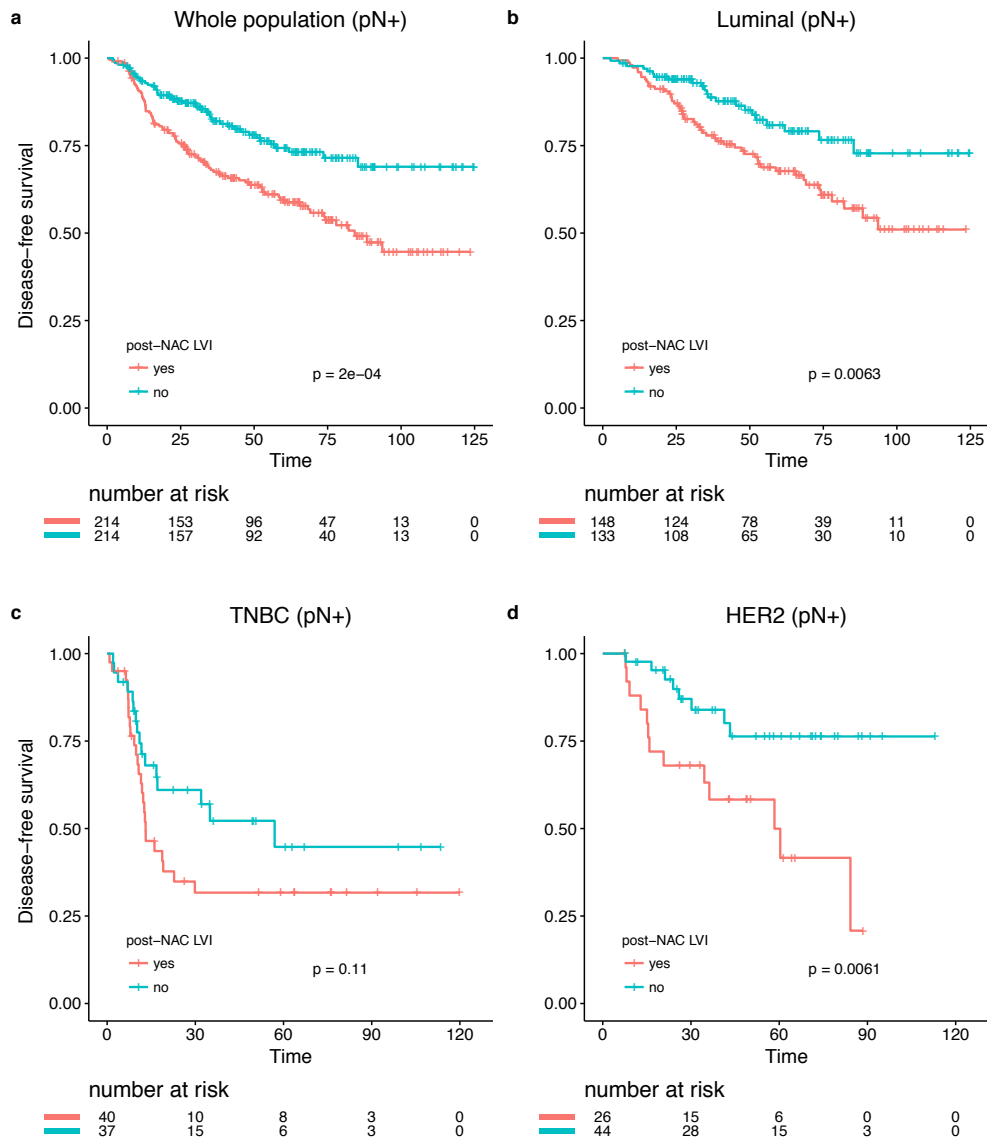
Supplementary Figure S3: Association of LVI status with disease-free survival (DFS) by pathological node status



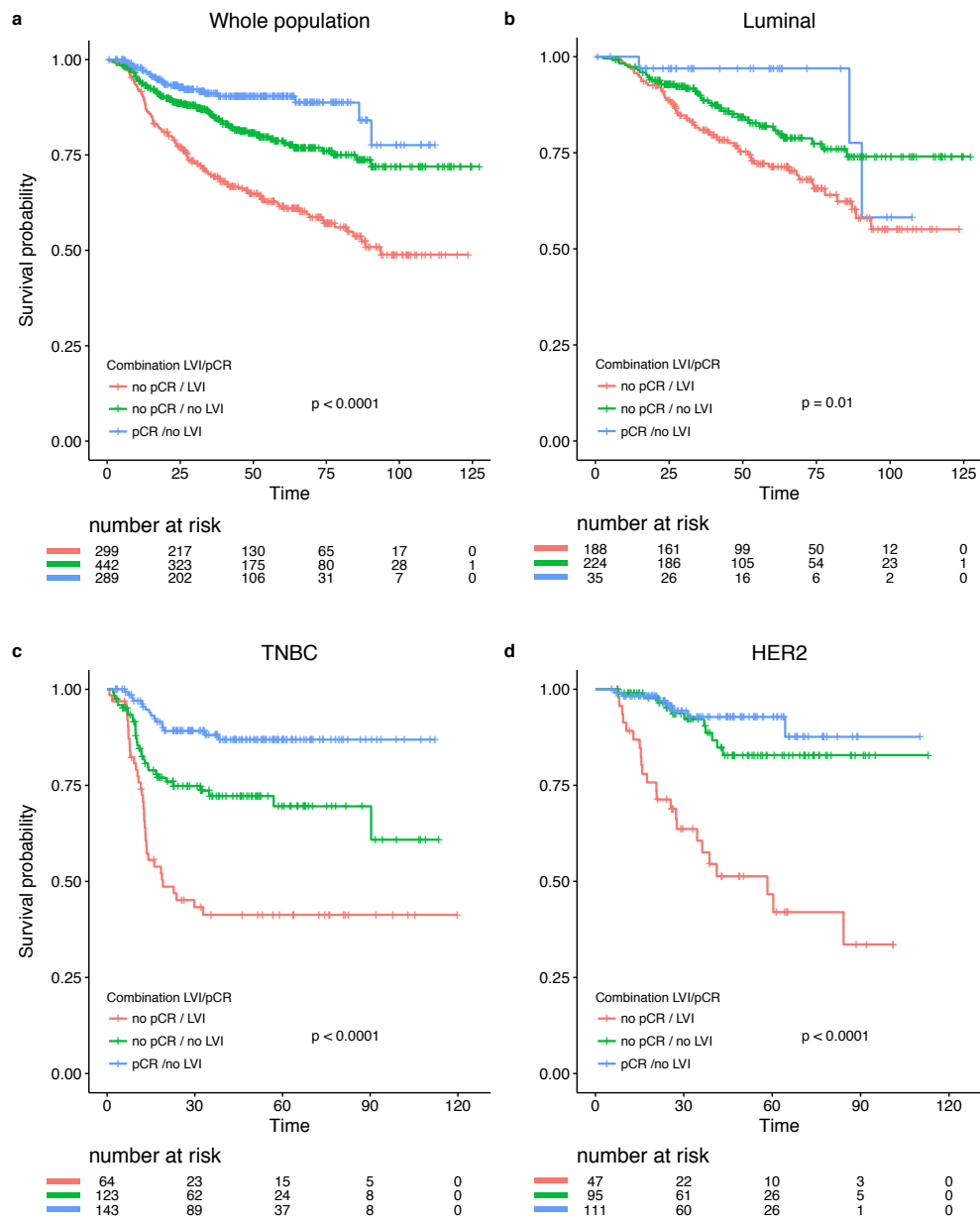
Supplementary Figure S4: Association of LVI status with disease-free survival (DFS) in pN- population



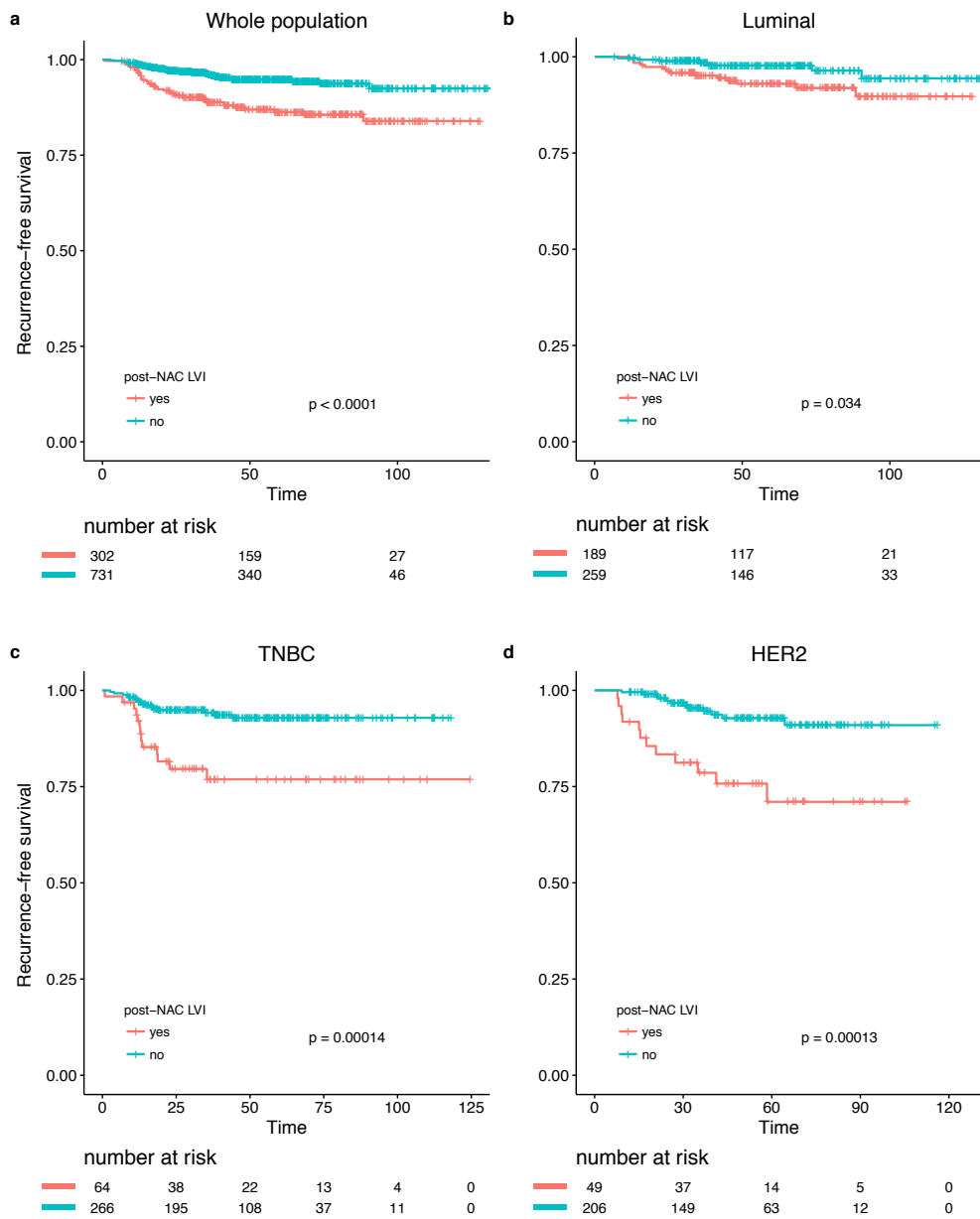
Supplementary Figure S5: Association of LVI status with disease-free survival (DFS) in pN+ population



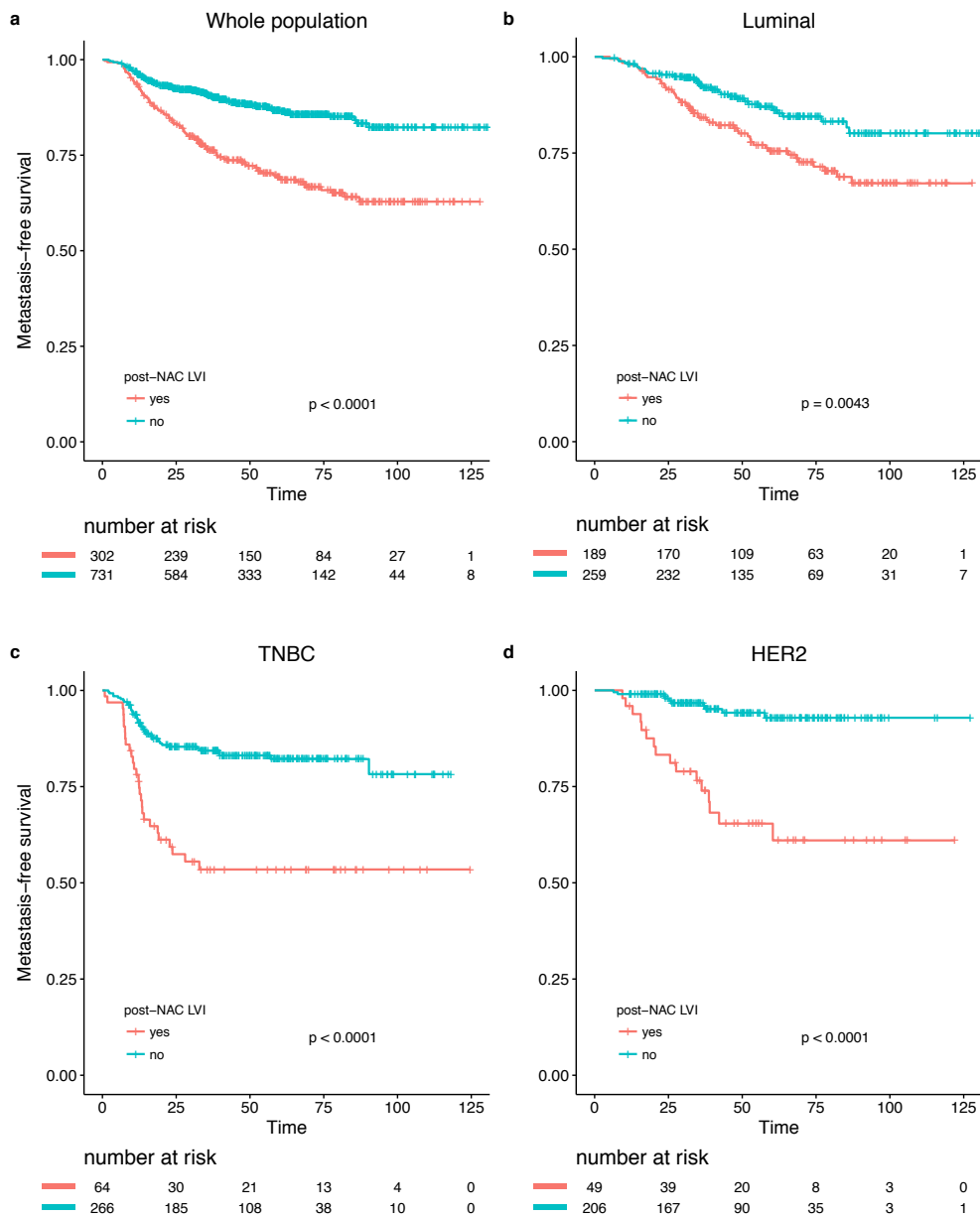
Supplementary Figure S6: Association of LVI status combined with pCR status with disease-free survival (DFS)



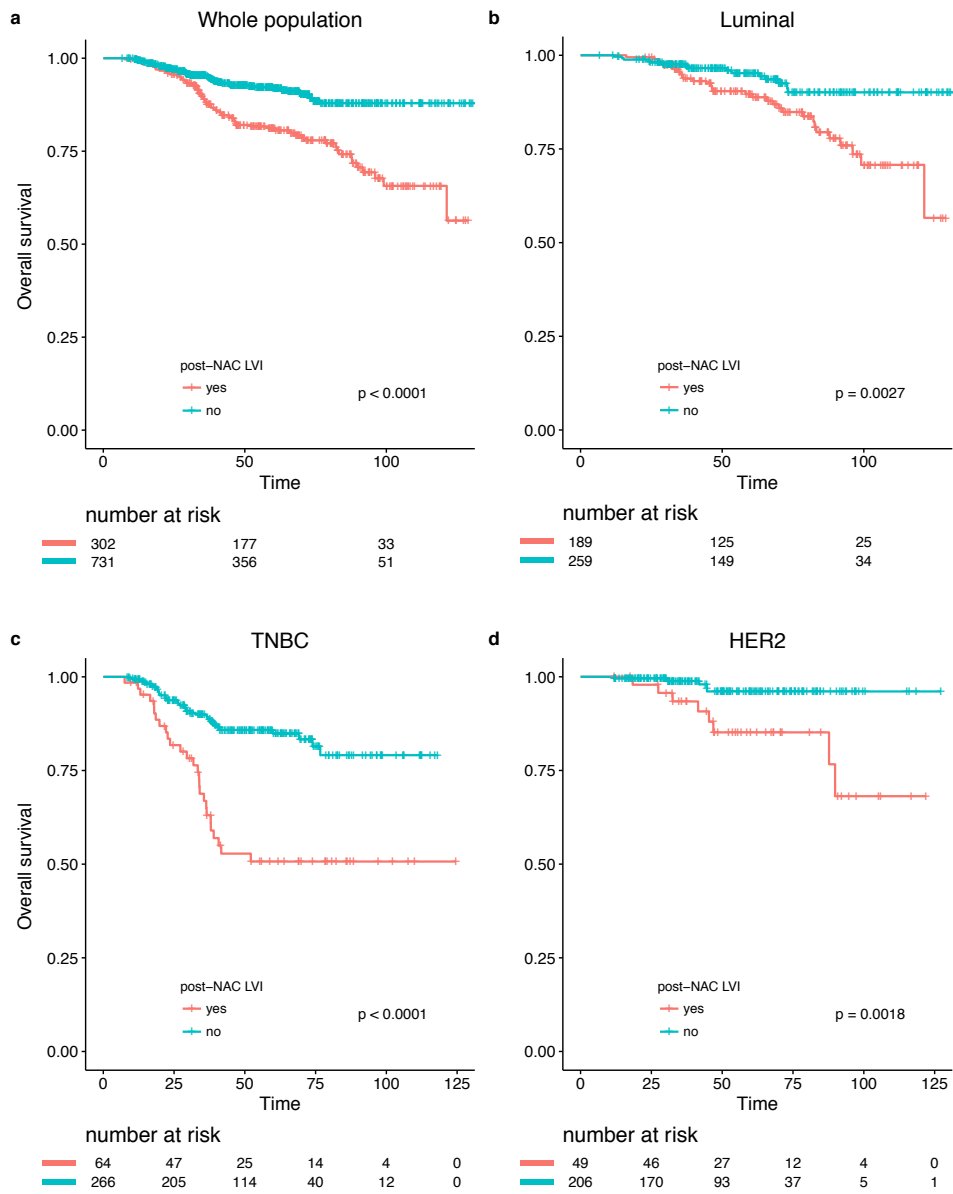
Supplementary Figure S7: Association of LVI status with recurrence-free survival (RFS)



Supplementary Figure S8: Association of LVI status with metastasis-free survival (MFS)



Supplementary Figure S9: Association of LVI status with overall survival (OS)



Supplementary Table legends

Supplementary Table S1: Pre and post-NAC parameters in whole population according to LVI status.

Variable	n	No LVI	LVI	P value
Pre-NAC parameters				
Age, years				
<45	376	270 (71.8)	106 (28.2)	0.49
45-55	392	269 (68.6)	123 (31.4)	
>55	265	192 (72.5)	73 (27.5)	
Menopausal status				
Premenopausal	634	447 (70.5)	187 (29.5)	0.89
Postmenopausal	392	278 (70.9)	114 (29.1)	
BMI class				
19≤BMI≤25	570	408 (71.6)	162 (28.4)	0.78
BMI<19	59	42 (71.2)	17 (28.8)	
BMI>25	400	278 (69.5)	122 (30.5)	
Tumor size				
T1-T2	754	547 (72.5)	207 (27.5)	0.04
T3	279	184 (65.9)	95 (34.1)	
Tumor size				
mean+/- SD, mm		43.92+/-18.8	48.97+/-23.1	<0.01
Clinical nodal status				
N0	447	318 (71.1)	129 (28.9)	0.81
N1-N2-N3	586	413 (70.5)	173 (29.5)	
Histology				
Ductal carcinoma NST	933	663 (71.1)	270 (28.9)	0.68
other	87	60 (69.0)	27 (31.0)	
Grade				
Grade I-II	394	252 (64.0)	142 (36.0)	0.0001
Grade III	604	457 (75.7)	147 (24.3)	
Ki67				
<20	139	80 (57.6)	59 (42.4)	0.17
≥20	333	214 (64.3)	119 (35.7)	
Mitotic index				
≤22	590	394 (66.8)	196 (33.2)	0.0001
>22	347	274 (79.0)	73 (21.0)	
Mean mitotic index				
mean+/- SD		22.9 (20.4)	17.7 (15.8)	<0.01
DCIS component				
No	512	406 (79.3)	106 (20.7)	0.2
Yes	94	69 (73.4)	25 (26.6)	
ER status				
Negative	483	390 (80.7)	93 (19.3)	<0.0001
Positive	550	341 (62.0)	209 (38.0)	
PR status				
Negative	594	455 (76.6)	139 (23.4)	<0.0001
Positive	418	262 (62.7)	156 (37.3)	
HER2 status				
Negative	778	525 (67.5)	253 (32.5)	0.0001
Positive	255	206 (80.8)	49 (19.2)	
Subtype				
Luminal	448	259 (57.8)	189 (42.2)	<0.0001
TNBC	330	266 (80.6)	64 (19.4)	
HER2	255	206 (80.8)	49 (19.2)	
Tumoral cellularity				
mean+/- SD		62.78+/-19.9	65.49+/-19.9	0.15
Post-NAC parameters				
Mitotic index				
mean+/- SD		14.67+/-27.8	20.74+/-32.6	0.05
Mitotic Index variation				
(absolute value)		-10.81	-0.46	<0.001
Tumoral cellularity				
mean+/- SD		21.22+/-26.7	40.71+/-25.3	<0.01
Tumoral cellularity variation				
(absolute value in %)		-41.16	-24.38	<0.001

Supplementary Table S2: Univariate and multivariate analysis for disease-free survival in luminal BC.

Supplementary Table S2: Univariate and multivariate analysis for disease-free survival (luminal BC)

Variable	n	Events	Univariate analysis			Multivariate analysis		
			HR	[95% CI]	P value	HR	[95% CI]	P value
Age, years								
<45	155	43	1		0.06 ^a			
45-55	186	35	0.6	[0.38 - 0.93]				
>55	107	21	0.67	[0.4 - 1.13]				
Menopausal status								
Postmenopausal	162	36	1		0.82			
Premenopausal	283	62	1.05	[0.7 - 1.58]				
BMI class								
19≤BMI≤25	229	47	1		0.04 ^b	1	-	-
BMI<19	36	12	2.22	[1.17 - 4.2]		2.62	[1.37 - 5]	0.003
BMI>25	181	40	1.2	[0.79 - 1.84]		1.26	[0.83 - 1.93]	0.282
Tumor size								
T1-T2	325	67	1		0.04			
T3	123	32	1.55	[1.02 - 2.37]				
Clinical nodal status								
N0	193	37	1		0.04			
N1-N2-N3	255	62	1.54	[1.02 - 2.32]				
Histology								
Ductal carcinoma NST	392	86	1		0.72			
other	53	13	1.11	[0.62 - 2]				
Grade								
Grade I-II	269	57	1		0.14			
Grade III	163	40	1.36	[0.9 - 2.03]				
Ki67								
<20%	101	21	1		0.3			
≥20%	133	33	1.33	[0.77 - 2.3]				
Mitotic index								
≤22	315	71	1		0.8			
>22	86	19	1.07	[0.64 - 1.77]				
DCIS component								
No	142	28	1		0.62			
Yes	28	8	1.22	[0.55 - 2.7]				
ER status								
Negative	24	4	1		0.3			
Positive	424	95	1.69	[0.62 - 4.6]				
PR status								
Negative	98	27	1		0.29			
Positive	334	67	0.79	[0.5 - 1.23]				
Post-NAC LVI								
No	259	41	1		0.003	1	-	-
Yes	189	58	1.83	[1.23 - 2.73]		1.98	[1.32 - 2.97]	0.001
pCR								
pCR	36	4	1		0.26			
No pCR	412	95	1.78	[0.65 - 4.83]				

a: *P*-value versus reference class (45-55 versus <45. *P* =0.02; >55 versus 45-55 *P* =0.14).

b: *P*-value versus reference class (<19 versus 19-25. *P* =0.01; >25 versus 19-25. *P* =0.39).

Supplementary Table S3: Univariate and multivariate analysis for disease-free survival in TNBC.**Supplementary Table S3: Univariate and multivariate analysis for disease-free survival (TNBC)**

Variable	n	Events	Univariate analysis			Multivariate analysis		
			HR	[95% CI]	P value	HR	[95% CI]	P value
Age, years								
<45	121	30	1		0.98			
45-55	123	32	0.98	[0.6 - 1.62]				
>55	86	21	0.96	[0.55 - 1.67]				
Menopausal status								
Postmenopausal	131	33	1		0.92			
Premenopausal	196	48	1.02	[0.66 - 1.6]				
BMI class								
19≤BMI≤25	184	40	1		0.08 ^a			
BMI<19	12	1	0.38	[0.05 - 2.77]				
BMI>25	133	42	1.52	[0.98 - 2.34]				
Tumor size								
T1-T2	245	50	1		<0.001	1	-	-
T3	85	33	2.26	[1.45 - 3.51]		2.57	[1.61 - 4.1]	<0.001
Clinical nodal status								
N0	155	37	1		0.46			
N1-N2-N3	175	46	1.18	[0.76 - 1.81]				
Histology								
Ductal carcinoma NST	300	72	1		0.18			
other	26	9	1.61	[0.8 - 3.21]				
Grade								
Grade I-II	45	11	1		0.63			
Grade III	278	70	1.17	[0.62 - 2.2]				
Ki67								
<20%	24	6	1		0.33			
≥20%	114	36	1.53	[0.64 - 3.63]				
Mitotic index								
≤22	128	27	1		0.08	1	-	-
>22	179	53	1.51	[0.95 - 2.41]		2.18	[1.35 - 3.51]	0.001
DCIS component								
No	242	60	1		0.11			
Yes	30	12	1.65	[0.89 - 3.06]				
Post-NAC LVI								
No	266	48	1		<0.001	1	-	-
Yes	64	35	3.73	[2.41 - 5.78]		2.84	[1.71 - 4.7]	<0.001
pCR								
pCR	143	16	1		<0.001	1	-	-
No pCR	187	67	3.87	[2.24 - 6.68]		2.93	[1.6 - 5.36]	<0.001

a: P-value versus reference class (<19 versus 19-25, $P=0.34$; >25 versus 19-25, $P=0.06$)

Supplementary Table S4: Univariate and multivariate analysis for disease-free survival in *HER2*-positive BC.

Supplementary Table S4: Univariate and multivariate analysis for disease-free survival (HER2-positive BC)

Variable	n	Events	Univariate analysis			Multivariate analysis		
			HR	[95% CI]	P value	HR	(95% CI)	P value
Age, years								
<45	100	16	1		0.46			
45-55	83	17	1.27	[0.64 - 2.52]				
>55	72	9	0.77	[0.34 - 1.74]				
Menopausal status								
Postmenopausal	99	13	1		0.20			
Premenopausal	155	29	1.53	[0.8 - 2.95]				
BMI class								
19≤BMI≤25	157	22	1		0.19			
BMI<19	11	4	2.53	[0.87 - 7.35]				
BMI>25	86	16	1.37	[0.72 - 2.61]				
Tumor size								
T1-T2	184	25	1		0.02			
T3	71	17	2.06	[1.11 - 3.83]				
Clinical nodal status								
N0	99	15	1		0.61			
N1-N2-N3	156	27	1.18	[0.63 - 2.22]				
Histology								
Ductal carcinoma NST	241	39	1		0.65			
other	8	1	0.63	[0.09 - 4.62]				
Grade								
Grade I-II	80	17	1		0.11			
Grade III	163	21	0.6	[0.31 - 1.13]				
Ki67								
<20%	14	1	1		0.15			
≥20%	86	21	3.91	[0.52 - 29.08]				
Mitotic index								
≤22	147	26	1		0.14			
>22	82	8	0.56	[0.25 - 1.23]				
DCIS component								
No	128	11	1		0.23			
Yes	36	5	1.91	[0.66 - 5.49]				
ER status								
Negative	129	26	1		0.22			
Positive	126	16	0.68	[0.36 - 1.27]				
PR status								
Negative	166	30	1		0.21			
Positive	84	10	0.63	[0.31 - 1.3]				
Post-NAC LVI								
No	206	18	1		<0.001	1	-	-
Yes	49	24	6.21	[3.36 - 11.45]		6.21	[3.37 - 11.46]	<0.001
pCR								
pCR	113	8	1		0.002			
No pCR	142	34	3.25	[1.5 - 7.02]				

Supplementary Table S5: Combination of LVI with pCR status and with pathological nodal involvement

Supplementary Table S5: Combination of LVI with pCR status and with pathological nodal involvement

	N	Events	HR	[95% CI]	P value
Combination of LVI with pCR					
Whole population					
pCR /no LVI	289	26	1		<0.001
no pCR / no LVI	442	81	1.91	[1.23 - 2.97]	0.004
no pCR / LVI	299	115	3.95	[2.58 - 6.05]	<0.001
Luminal BC					
pCR /no LVI	35	3	1		0.01
no pCR / no LVI	224	38	1.75	[0.54 - 5.66]	0.35
no pCR / LVI	188	57	2.99	[0.94 - 9.54]	0.06
TNBC					
pCR /no LVI	143	16	1		<0.001
no pCR / no LVI	123	32	2.7	[1.48 - 4.92]	0.001
no pCR / LVI	64	35	6.45	[3.56 - 11.67]	<0.001
HER2-positive BC					
pCR /no LVI	111	7	1		<0.001
no pCR / no LVI	95	11	1.67	[0.65 - 4.32]	0.29
no pCR / LVI	47	23	8.31	[3.56 - 19.4]	<0.001
Combination of LVI with nodal involvement					
Whole population					
pN- / no LVI	517	61	1		<0.001
pN- / LVI	88	28	2.51	[1.6 - 3.92]	<0.001
pN+ / no LVI	214	46	1.72	[1.17 - 2.52]	0.005
pN+ / LVI	214	89	3.36	[2.42 - 4.65]	<0.001
Luminal BC					
pN- / no LVI	126	19	1		0.003
pN- / LVI	41	7	1.03	[0.43 - 2.45]	0.95
pN+ / no LVI	133	22	1.11	[0.6 - 2.05]	0.75
pN+ / LVI	148	51	2.2	[1.3 - 3.72]	0.003
TNBC					
pN- / no LVI	229	32	1		<0.001
pN- / LVI	24	10	3.07	[1.51 - 6.24]	0.002
pN+ / no LVI	37	16	3.98	[2.18 - 7.27]	<0.001
pN+ / LVI	40	25	6.7	[3.96 - 11.34]	<0.001
HER2-positive BC					
pN- / no LVI	162	10	1		<0.001
pN- / LVI	23	11	8.69	[3.68 - 20.53]	<0.001
pN+ / no LVI	44	8	2.65	[1.04 - 6.71]	0.04
pN+ / LVI	26	13	8.5	[3.73 - 19.41]	<0.001

Supplementary Table S6: Univariate and multivariate analysis for recurrence-free survival in whole population.

Supplementary Table S6: Univariate and multivariate analysis for recurrence-free survival (RFS)

Variable	n	Events	Univariate analysis			Multivariate analysis		
			HR	[95% CI]	P value	HR	[95% CI]	P value
Age, years								
<45	376	31	1		0.49			
45-55	392	27	0.79	[0.47 - 1.32]				
>55	265	16	0.72	[0.39 - 1.32]				
Menopausal status								
Postmenopausal	392	23	1					
Premenopausal	634	50	1.38	[0.84 - 2.26]	0.2			
BMI class								
19≤BMI≤25	570	36	1		0.44			
BMI<19	59	5	1.37	[0.54 - 3.5]				
BMI>25	400	33	1.34	[0.84 - 2.15]				
Tumor size								
T1-T2	754	48	1		0.05			
T3	279	26	1.6	[0.99 - 2.59]				
Clinical nodal status								
N0	447	32	1		0.79			
N1-N2-N3	586	42	1.06	[0.67 - 1.68]				
Histology								
Ductal carcinoma NST	933	65	1		0.88			
other	87	6	0.94	[0.41 - 2.16]				
Grade								
Grade I-II	394	20	1		0.02			
Grade III	604	50	1.87	[1.11 - 3.15]				
Ki67								
<20%	139	5	1		0.003			
≥20%	333	43	4.14	[1.64 - 10.47]				
Mitotic index								
≤22	590	34	1		0.011	1	-	-
>22	347	33	1.85	[1.14 - 2.98]		1.66	[1.01 - 2.74]	0.047
DCIS component								
No	512	27	1		0.004			
Yes	94	13	2.63	[1.36 - 5.1]				
ER status								
Negative	483	47	1		<0.001			
Positive	550	27	0.43	[0.27 - 0.7]				
PR status								
Negative	594	57	1		<0.001			
Positive	418	16	0.35	[0.2 - 0.61]				
HER2 status								
Negative	778	50	1		0.07			
Positive	255	24	1.57	[0.97 - 2.56]				
Subtype								
Luminal	448	21	1		0.003 ^a	1	-	-
TNBC	330	29	2.36	[1.35 - 4.16]		3.04	[1.62 - 5.7]	0.001
HER2	255	24	2.37	[1.32 - 4.28]		2.81	[1.47 - 5.39]	0.002
Post-NAC LVI								
No	731	35	1		<0.001	1	-	-
Yes	302	39	2.57	[1.63 - 4.06]		3.66	[2.22 - 6.05]	<0.001
pCR								
pCR	292	12	1		0.04			
No pCR	741	62	1.91	[1.03 - 3.54]				

a: P-value versus reference class (TNBC versus luminal. $P=0.003$; HER2 versus luminal. $P=0.004$)

Supplementary Table S7: Univariate and multivariate analysis for recurrence-free survival in luminal BC.

Supplementary Table S7: Univariate and multivariate analysis for recurrence-free survival (luminal BC)

Variable	n	Events	Univariate analysis			Multivariate analysis		
			HR	[95% CI]	P value	HR	[95% CI]	P value
Age, years								
<45	155	12	1		0.06*			
45-55	186	5	0.32	[0.11 - 0.92]				
>55	107	4	0.47	[0.15 - 1.46]				
Menopausal status								
Postmenopausal	162	17	1		0.41			
Premenopausal	283	15	1.49	[0.58 - 3.83]				
BMI class								
19≤BMI≤25	229	10	1		0.41			
BMI<19	36	3	2.33	[0.64 - 8.51]				
BMI>25	181	8	1.09	[0.43 - 2.77]				
Tumor size								
T1-T2	325	14	1		0.37			
T3	123	7	1.51	[0.61 - 3.74]				
Clinical nodal status								
N0	193	7	1		0.23			
N1-N2-N3	255	14	1.74	[0.7 - 4.31]				
Histology								
Ductal carcinoma NST	392	20	1		0.27			
other	53	1	0.34	[0.05 - 2.54]				
Grade								
Grade I-II	269	9	1		0.07			
Grade III	163	11	2.27	[0.94 - 5.49]				
Ki67								
<20%	101	3	1		0.09			
≥20%	133	11	3.02	[0.84 - 10.82]				
Mitotic index								
≤22	315	14	1		0.51			
>22	86	5	1.4	[0.5 - 3.9]				
DCIS component								
No	142	6	1		0.25			
Yes	28	0	0	[0 - Inf]				
ER status								
Negative	24	0	1		0.23			
Positive	424	21	NA	[0 - Inf]				
PR status								
Negative	98	9	1		0.03			
Positive	334	11	0.38	[0.16 - 0.93]				
Post-NAC LVI								
No	259	7	1		0.03	1	-	-
Yes	189	14	2.58	[1.04 - 6.39]		2.42	[0.97 - 6.07]	0.059
pCR								
pCR	36	2	1					
No pCR	412	19	0.69	[0.16 - 2.96]	0.61			

a: P-value versus reference class (45-55 versus <45. $P=0.03$; >55 versus 45-55 $P=0.19$).

Supplementary Table S8: Univariate and multivariate analysis for recurrence-free survival in TNBC

Variable	n	Events	Univariate analysis			Multivariate analysis		
			HR	[95% CI]	P value	HR	[95% CI]	P value
Age, years								
<45	121	10	1		0.98			
45-55	123	11	1.03	[0.44 - 2.42]				
>55	86	8	1.09	[0.43 - 2.76]				
Menopausal status								
Postmenopausal	131	11	1					
Premenopausal	196	17	1.08	[0.51 - 2.31]	0.84			
BMI class								
19≤BMI≤25	184	14	1		0.64			
BMI<19	12	1	1.14	[0.15 - 8.7]				
BMI>25	133	14	1.43	[0.68 - 2.99]				
Tumor size								
T1-T2	245	16	1		0.01	1	-	-
T3	85	13	2.54	[1.22 - 5.29]		3.53	[1.6 - 7.81]	0.002
Clinical nodal status								
N0	155	15	1		0.71			
N1-N2-N3	175	14	0.87	[0.42 - 1.8]				
Histology								
Ductal carcinoma NST	300	23	1		0.04			
other	26	5	2.69	[1.02 - 7.09]				
Grade								
Grade I-II	45	3	1		0.47			
Grade III	278	26	1.55	[0.47 - 5.11]				
Ki67								
<20%	24	2	1		0.25			
≥20%	114	19	2.3	[0.53 - 9.86]				
Mitotic index								
≤22	128	4	1		0.004	1	-	-
>22	179	24	4.65	[1.61 - 13.41]		7.53	[2.54 - 22.32]	<0.001
DCIS component								
No	242	17	1		<0.001			
Yes	30	9	4.22	[1.88 - 9.47]				
Post-NAC LVI								
No	266	16	1		<0.001	1	-	-
Yes	64	13	3.75	[1.8 - 7.81]		3.55	[1.53 - 8.25]	0.003
pCR								
pCR	143	4	1			1	-	-
No pCR	187	25	5.42	[1.88 - 15.58]	0.002	4.24	[1.34 - 13.4]	0.014

Supplementary Table S9: Univariate and multivariate analysis for recurrence-free survival in *HER2*-positive BC.

Supplementary Table S9: Univariate and multivariate analysis for recurrence-free survival (*HER2*-positive BC)

Variable	n	Events	Univariate analysis			Multivariate analysis		
			HR	[95% CI]	P value	HR	[95% CI]	P value
Age, years								
<45	100	9	1		0.3			
45-55	83	11	1.45	[0.6 - 3.5]				
>55	72	4	0.61	[0.19 - 1.97]				
Menopausal status								
Postmenopausal	99	6	1		0.13			
Premenopausal	155	18	2.02	[0.8 - 5.1]				
BMI class								
19≤BMI≤25	157	12	1		0.43			
BMI<19	11	1	1.06	[0.14 - 8.18]				
BMI>25	86	11	1.7	[0.75 - 3.85]				
Tumor size								
T1-T2	184	18	1		0.83			
T3	71	6	0.9	[0.36 - 2.28]				
Clinical nodal status								
N0	99	10	1		0.82			
N1-N2-N3	156	14	0.91	[0.4 - 2.05]				
Histology								
Ductal carcinoma NST	241	22	1		0.35			
other	8	0	0	[0 - Inf]				
Grade								
Grade I-II	80	8	1		0.64			
Grade III	163	13	0.81	[0.34 - 1.95]				
Ki67								
<20%	14	0	1		0.12			
≥20%	86	13	NA	[0 - Inf]				
Mitotic index								
≤22	147	16	1		0.156			
>22	82	4	0.45	[0.15 - 1.35]				
DCIS component								
No	128	4	1		0.05			
Yes	36	4	4.09	[1.02 - 16.4]				
ER status								
Negative	129	18	1		0.02			
Positive	126	6	0.35	[0.14 - 0.88]				
PR status								
Negative	166	19	1		0.17			
Positive	84	5	0.51	[0.19 - 1.35]				
Post-NAC LVI								
No	206	12	1		<0.001	1	-	-
Yes	49	12	4.22	[1.89 - 9.39]		4.22	[1.89 - 9.39]	<0.001
pCR								
pCR	113	6	1					
No pCR	142	18	2.28	[0.9 - 5.74]	0.08			

Supplementary Table S10: Univariate and multivariate analysis for metastasis-free survival in whole population.

Supplementary Table S10: Univariate and multivariate analysis for metastasis-free survival

Variable	Univariate analysis					Multivariate analysis		
	n	Events	HR	[95% CI]	P value	HR	[95% CI]	P value
Age, years								
<45	376	76	1		0.11			
45-55	392	62	0.72	[0.51 - 1.01]				
>55	265	40	0.74	[0.5 - 1.08]				
Menopausal status								
Postmenopausal	392	62	1					
Premenopausal	634	114	1.16	[0.85 - 1.58]	0.35			
BMI class								
19≤BMI≤25	570	87	1		0.12			
BMI<19	59	13	1.54	[0.86 - 2.76]				
BMI>25	400	78	1.32	[0.97 - 1.8]				
Tumor size								
T1-T2	754	106	1		<0.001	1	-	-
T3	279	72	2.11	[1.56 - 2.84]		2.23	[1.63 - 3.06]	<0.001
Clinical nodal status								
N0	447	71	1		0.16			
N1-N2-N3	586	107	1.24	[0.92 - 1.68]				
Histology								
Ductal carcinoma NST	933	156	1		0.2			
other	87	20	1.35	[0.85 - 2.16]				
Grade								
Grade I-II	394	66	1		0.24			
Grade III	604	105	1.2	[0.88 - 1.64]				
Ki67								
<20%	139	25	1		0.24			
≥20%	333	69	1.31	[0.83 - 2.08]				
Mitotic index								
≤22	590	97	1		0.08	1	-	-
>22	347	66	1.32	[0.96 - 1.8]		1.42	[1.01 - 1.98]	0.042
DCIS component								
No	512	82	1		0.25			
Yes	94	20	1.33	[0.82 - 2.17]				
ER status								
Negative	483	91	1		0.03			
Positive	550	87	0.72	[0.54 - 0.97]				
PR status								
Negative	594	112	1		0.02			
Positive	418	61	0.68	[0.5 - 0.94]				
HER2 status								
Negative	778	152	1		0.003			
Positive	255	26	0.54	[0.35 - 0.81]				
Subtype								
Luminal	448	81	1		<0.001 ^a	1	-	-
TNBC	330	71	1.53	[1.11 - 2.1]		2.46	[1.71 - 3.56]	<0.001
HER2	255	26	0.64	[0.41 - 1]		0.82	[0.5 - 1.34]	0.428
Post-NAC LVI								
No	731	86	1		<0.001	1	-	-
Yes	302	92	2.52	[1.88 - 3.38]		2.04	[1.46 - 2.86]	<0.001
pCR								
pCR	292	21	1			1	-	-
No pCR	741	157	2.83	[1.79 - 4.46]	<0.001	2.66	[1.6 - 4.41]	<0.001

a: P-value versus reference class (TNBC versus luminal. $P=0.009$; HER2 versus luminal. $P=0.04$)

Supplementary Table S11: Univariate and multivariate analysis for metastasis-free survival in luminal BC.

Supplementary Table S11: Univariate and multivariate analysis for metastasis-free survival (luminal BC)

Variable	n	Events	Univariate analysis			Multivariate analysis		
			HR	[95% CI]	P value	HR	[95% CI]	P value
Age, years								
<45	155	38	1		0.02 ^a	1	-	-
45-55	186	25	0.5	[0.3 - 0.83]		0.49	[0.3 - 0.82]	0.006
>55	107	18	0.68	[0.39 - 1.18]		0.63	[0.36 - 1.1]	0.104
Menopausal status								
Postmenopausal	162	29	1					
Premenopausal	283	51	1.03	[0.66 - 1.63]	0.89			
BMI class								
19≤BMI≤25	229	37	1		0.15			
BMI<19	36	9	1.97	[0.95 - 4.09]				
BMI>25	181	35	1.31	[0.83 - 2.09]				
Tumor size								
T1-T2	325	53	1		0.03			
T3	123	28	1.68	[1.06 - 2.65]				
Clinical nodal status								
N0	193	30	1		0.07			
N1-N2-N3	255	51	1.51	[0.96 - 2.38]				
Histology								
Ductal carcinoma NST	392	69	1		0.39			
other	53	12	1.31	[0.71 - 2.41]				
Grade								
Grade I-II	269	45	1		0.13			
Grade III	163	34	1.41	[0.9 - 2.21]				
Ki67								
<20%	101	18	1		0.55			
≥20%	133	26	1.2	[0.66 - 2.19]				
Mitotic index								
≤22	315	56	1		0.49			
>22	86	17	1.21	[0.7 - 2.08]				
DCIS component								
No	142	22	1		0.34			
Yes	28	7	1.51	[0.64 - 3.55]				
ER status								
Negative	24	4	1		0.62			
Positive	424	77	1.29	[0.47 - 3.53]				
PR status								
Negative	98	22	1		0.41			
Positive	334	56	0.81	[0.5 - 1.33]				
Post-NAC LVI								
No	259	33	1		0.005	1	-	-
Yes	189	48	1.89	[1.21 - 2.94]		1.92	[1.23 - 3]	0.004
pCR								
pCR	36	2	1					
No pCR	412	79	3.18	[0.78 - 12.95]	0.11			

a: P-value versus reference class (45-55 versus <45. $P=0.007$; >55 versus 45-55 $P=0.17$).

Supplementary Table S12: Univariate and multivariate analysis for metastasis-free survival in TNBC.

Supplementary Table S12: Univariate and multivariate analysis for metastasis-free survival (TNBC)

Variable	n	Events	Univariate analysis			Multivariate analysis		
			HR	[95% CI]	P value	HR	[95% CI]	P value
Age, years								
<45	121	27	1		0.9			
45-55	123	26	0.88	[0.52 - 1.52]				
>55	86	18	0.92	[0.51 - 1.67]				
Menopausal status								
Postmenopausal	131	26	1					
Premenopausal	196	44	1.19	[0.73 - 1.93]	0.49			
BMI class								
19≤BMI≤25	184	37	1		0.37			
BMI<19	12	1	0.42	[0.06 - 3.03]				
BMI>25	133	33	1.26	[0.79 - 2.01]				
Tumor size								
T1-T2	245	40	1			1	-	-
T3	85	31	2.6	[1.63 - 4.16]	<0.001	2.85	[1.77 - 4.58]	<0.001
Clinical nodal status								
N0	155	31	1		0.4			
N1-N2-N3	175	40	1.23	[0.77 - 1.96]				
Histology								
Ductal carcinoma NST	300	63	1		0.49			
other	26	7	1.32	[0.6 - 2.87]				
Grade								
Grade I-II	45	10	1		0.94			
Grade III	278	59	1.03	[0.52 - 2]				
Ki67								
<20%	24	6	1		0.67			
≥20%	114	31	1.21	[0.5 - 2.9]				
Mitotic index								
≤22	128	26	1		0.41			
>22	179	43	1.23	[0.75 - 2]				
DCIS component								
No	242	52	1		0.18			
Yes	30	10	1.59	[0.81 - 3.12]				
Post-NAC LVI								
No	266	43	1		<0.001	1	-	-
Yes	64	28	3.26	[2.02 - 5.24]		2.49	[1.47 - 4.23]	0.001
pCR								
pCR	143	15	1			1	-	-
No pCR	187	56	3.31	[1.87 - 5.85]	<0.001	2.3	[1.23 - 4.31]	0.009

Supplementary Table S13: Univariate and multivariate analysis for metastasis-free survival in *HER2*-positive BC.

Supplementary Table S13: Univariate and multivariate analysis for metastasis-free survival (*HER2*-positive BC)

Variable	n	Events	Univariate analysis			Multivariate analysis		
			HR	[95% CI]	P value	HR	[95% CI]	P value
Age, years								
<45	100	11	1		0.33			
45-55	83	11	1.17	[0.51 - 2.7]				
>55	72	4	0.51	[0.16 - 1.59]				
Menopausal status								
Postmenopausal	99	7	1					
Premenopausal	155	19	1.77	[0.74 - 4.21]	0.19			
BMI class								
19≤BMI≤25	157	13	1		0.13			
BMI<19	11	3	3.38	[0.96 - 11.86]				
BMI>25	86	10	1.43	[0.62 - 3.26]				
Tumor size								
T1-T2	184	13	1		0.005			
T3	71	13	2.85	[1.32 - 6.15]				
Clinical nodal status								
N0	99	10	1		0.95			
N1-N2-N3	156	16	1.03	[0.47 - 2.27]				
Histology								
Ductal carcinoma NST	241	24	1		0.9			
other	8	1	1.14	[0.15 - 8.42]				
Grade								
Grade I-II	80	11	1		0.12			
Grade III	163	12	0.53	[0.23 - 1.2]				
Ki67								
<20%	14	1	1		0.51			
≥20%	86	12	2	[0.26 - 15.37]				
Mitotic index								
≤22	147	15	1		0.6			
>22	82	6	0.77	[0.3 - 2]				
DCIS component								
No	128	8	1		0.63			
Yes	36	3	1.38	[0.37 - 5.21]				
ER status								
Negative	129	16	1		0.36			
Positive	126	10	0.69	[0.31 - 1.53]				
PR status								
Negative	166	19	1		0.18			
Positive	84	5	0.52	[0.19 - 1.38]				
Post-NAC LVI								
No	206	10	1		<0.001	1	-	-
Yes	49	16	6.96	[3.16 - 15.34]		6.96	[3.16 - 15.34]	<0.001
pCR								
pCR	113	4	1					
No pCR	142	22	4.21	[1.45 - 12.22]	0.008			

Supplementary Table S14: Univariate and multivariate analysis for overall survival in whole population.

Supplementary Table 14: Univariate and multivariate analysis for overall survival (whole population)

Variable	n	Events	Univariate analysis			Multivariate analysis		
			HR	[95% CI]	P value	HR	[95% CI]	P value
Age, years								
<45	376	45	1		0.88			
45-55	392	47	0.92	[0.61 - 1.38]				
>55	265	28	0.9	[0.56 - 1.44]				
Menopausal status								
Postmenopausal	392	44	1		0.77			
Premenopausal	634	75	1.06	[0.73 - 1.53]				
BMI class								
19≤BMI≤25	570	60	1		0.25			
BMI<19	59	10	1.66	[0.85 - 3.24]				
BMI>25	400	50	1.23	[0.85 - 1.8]				
Tumor size								
T1-T2	754	76	1		0.002	1	-	-
T3	279	44	1.8	[1.24 - 2.61]		1.88	[1.26 - 2.81]	0.002
Clinical nodal status								
N0	447	46	1		0.09	1	-	-
N1-N2-N3	586	74	1.38	[0.95 - 1.99]		1.64	[1.1 - 2.45]	0.015
Histology								
Ductal carcinoma NST	933	109	1		0.65			
other	87	10	0.86	[0.45 - 1.65]				
Grade								
Grade I-II	394	35	1		0.001			
Grade III	604	81	1.94	[1.3 - 2.89]				
Ki67								
<20%	139	13	1		0.03			
≥20%	333	50	1.95	[1.06 - 3.6]				
Mitotic index								
≤22	590	59	1		0.001	1	-	-
>22	347	53	1.84	[1.27 - 2.67]		1.85	[1.23 - 2.8]	0.003
DCIS component								
No	512	66	1		0.34			
Yes	94	9	0.71	[0.36 - 1.43]				
ER status								
Negative	483	73	1		<0.001			
Positive	550	47	0.46	[0.32 - 0.66]				
PR status								
Negative	594	81	1		<0.001			
Positive	418	34	0.51	[0.34 - 0.76]				
HER2 status								
Negative	778	107	1		0.003			
Positive	255	13	0.42	[0.23 - 0.74]				
Subtype								
Luminal	448	46	1		<0.001 ^a	1	-	-
TNBC	330	61	2.56	[1.74 - 3.76]		4.1	[2.62 - 6.41]	<0.001
HER2	255	13	0.64	[0.35 - 1.19]		0.54	[0.24 - 1.22]	0.141
Post-NAC LVI								
No	731	54	1		<0.001	1	-	-
Yes	302	66	2.5	[1.74 - 3.58]		2	[1.32 - 3.04]	0.001
pCR								
No pCR	292	11	1			1	-	-
pCR	741	109	3.27	[1.76 - 6.09]	<0.001	4.1	[2.08 - 8.09]	<0.001

a: P-value versus reference class (TNBC versus luminal. <0.001; HER2 versus luminal. P= 0.16)

Supplementary Table S15: Univariate and multivariate analysis for overall survival in luminal BC.**Supplementary Table 15 Univariate and multivariate analysis for overall survival (luminal BC)**

Variable	n	Events	Univariate analysis			Multivariate analysis		
			HR	[95% CI]	P value	HR	[95% CI]	P value
Age, years								
<45	155	15	1		0.97			
45-55	186	20	1.07	[0.55 - 2.1]				
>55	107	11	1.1	[0.5 - 2.39]				
Menopausal status								
Postmenopausal	162	17	1					
Premenopausal	283	28	0.97	[0.53 - 1.78]	0.93			
BMI class								
19≤BMI≤25	229	21	1		0.05 ^a	1	-	-
BMI<19	36	7	2.78	[1.18 - 6.56]		3.36	[1.41 - 8.02]	0.006
BMI>25	181	18	1.24	[0.66 - 2.33]		1.27	[0.68 - 2.39]	0.455
Tumor size								
T1-T2	325	34	1		0.69			
T3	123	12	1.15	[0.59 - 2.21]				
Clinical nodal status								
N0	193	18	1		0.22			
N1-N2-N3	255	28	1.45	[0.8 - 2.63]				
Histology								
Ductal carcinoma NST	392	40	1		0.97			
other	53	6	0.98	[0.42 - 2.33]				
Grade								
Grade I-II	269	26	1		0.17			
Grade III	163	19	1.51	[0.83 - 2.74]				
Ki67								
<20%	101	9	1		0.11			
≥20%	133	19	1.9	[0.86 - 4.22]				
Mitotic index								
≤22	315	33	1		0.44			
>22	86	10	1.32	[0.65 - 2.68]				
DCIS component								
No	142	14	1		0.44			
Yes	28	2	0.56	[0.13 - 2.51]				
ER status								
Negative	24	2	1		0.54			
Positive	424	44	1.55	[0.37 - 6.41]				
PR status								
Negative	98	10	1		0.75			
Positive	334	33	1.12	[0.55 - 2.28]				
Post-NAC LVI								
No	259	15	1		0.003	1	-	-
Yes	189	31	2.49	[1.34 - 4.62]		2.71	[1.45 - 5.07]	0.002
pCR								
pCR	36	0	1		0.07			
No pCR	412	46	NA	[0 - Inf]				

Supplementary Table S16: Univariate and multivariate analysis for overall survival in TNBC.**Supplementary Table 16: Univariate and multivariate analysis for overall survival (TNBC)**

Variable	n	Events	Univariate analysis			Multivariate analysis		
			HR	[95% CI]	P value	HR	[95% CI]	P value
Age, years								
<45	121	26	1		0.44			
45-55	123	22	0.74	[0.42 - 1.3]				
>55	86	13	0.7	[0.36 - 1.36]				
Menopausal status								
Postmenopausal	131	22	1		0.47			
Premenopausal	196	39	1.21	[0.72 - 2.04]				
BMI class								
19≤BMI≤25	184	34	1		0.33			
BMI<19	12	0	0	[0 - Inf]				
BMI>25	133	27	1.08	[0.65 - 1.8]				
Tumor size								
T1-T2	245	36	1		0.002	1	-	-
T3	85	25	2.21	[1.33 - 3.68]		2.76	[1.61 - 4.73]	<0.001
Clinical nodal status								
N0	155	23	1		0.05	1	-	-
N1-N2-N3	175	38	1.66	[0.99 - 2.79]		2.16	[1.27 - 3.65]	0.004
Histology								
Ductal carcinoma NST	300	56	1		0.61			
other	26	4	0.77	[0.28 - 2.12]				
Grade								
Grade I-II	45	6	1		0.25			
Grade III	278	53	1.63	[0.7 - 3.8]				
Ki67								
<20%	24	3	1		0.27			
≥20%	114	24	1.94	[0.58 - 6.44]				
Mitotic index								
≤22	128	19	1		0.07	1	-	-
>22	179	41	1.63	[0.95 - 2.82]		2.72	[1.54 - 4.82]	0.001
DCIS component								
No	242	49	1		0.97			
Yes	30	7	1.02	[0.46 - 2.24]				
Post-NAC LVI								
No	266	34	1		<0.001	1	-	-
Yes	64	27	3.44	[2.07 - 5.71]		2.61	[1.48 - 4.6]	0.001
pCR								
pCR	143	9	1		<0.001	1	-	-
No pCR	187	52	5.04	[2.48 - 10.23]		4.54	[2.11 - 9.8]	<0.001

Supplementary Table S17: Univariate and multivariate analysis for overall survival in *HER2*-positive BC.

Supplementary Table 17: Univariate and multivariate analysis for overall survival (*HER2*-positive BC)

Variable	n	Events	Univariate analysis			Multivariate analysis		
			HR	[95% CI]	P value	HR	[95% CI]	P value
Age, years								
<45	100	4	1		0.81			
45-55	83	5	1.45	[0.39 - 5.42]				
>55	72	4	1.5	[0.38 - 6.01]				
Menopausal status								
Postmenopausal	99	5	1					
Premenopausal	155	8	1.02	[0.33 - 3.13]	0.97			
BMI class								
19≤BMI≤25	157	5	1		0.03 ^a			
BMI<19	11	3	6.04	[1.42 - 25.67]				
BMI>25	86	5	1.86	[0.54 - 6.42]				
Tumor size								
T1-T2	184	6	1		0.02			
T3	71	7	3.42	[1.14 - 10.26]				
Clinical nodal status								
N0	99	5	1		0.98			
N1-N2-N3	156	8	1.01	[0.33 - 3.1]				
Histology								
Ductal carcinoma NST	241	13	1		0.49			
other	8	0	0	[0 - Inf]				
Grade								
Grade I-II	80	3	1		0.55			
Grade III	163	9	1.48	[0.4 - 5.49]				
Ki67								
<20%	14	1	1		0.96			
≥20%	86	7	1.06	[0.13 - 8.63]				
Mitotic index								
≤22	147	7	1		0.41			
>22	82	2	0.52	[0.11 - 2.53]				
DCIS component								
No	128	3	1		0.45			
Yes	36	0	0	[0 - Inf]				
ER status								
Negative	129	10	1		0.1			
Positive	126	3	0.36	[0.1 - 1.31]				
PR status								
Negative	166	10	1		0.1	1	-	-
Positive	84	1	0.21	[0.03 - 1.61]		0.17	[0.02 - 1.37]	0.097
Post-NAC LVI								
No	206	5	1		0.004	1	-	-
Yes	49	8	5.14	[1.67 - 15.78]		4.59	[1.39 - 15.17]	0.013
pCR								
pCR	113	2	1		0.09			
No pCR	142	11	3.42	[0.75 - 15.48]				

a: P-value versus reference class (<19 versus 19-25. *P* =0.01; >25 versus 19-25. *P* =0.33).

See Supplementary Files for Tables.

RCB validation study

Anne-Sophie HAMY-PETIT / Fabien REYAL

1. Patients characteristics

The total number of patients included in the cohort is 718. Patients' main characteristics by subgroup are summarized in Table 1. The median age was 47.5 years old (range 25, 80) and most patients (63%) were premenopausal. Patients repartition by subtype was as follows: luminal (n=224; 31, 3%), TNBC (n=311; 43,4%), HER2-positive (n=181; 25,3%). There were no differences between the groups regarding age, menopausal status, tumor size nor clinical nodal status. TNBC and HER2-positive BC were associated with a higher grade, Ki67 and mitotic index than luminal BC (p<0.001).

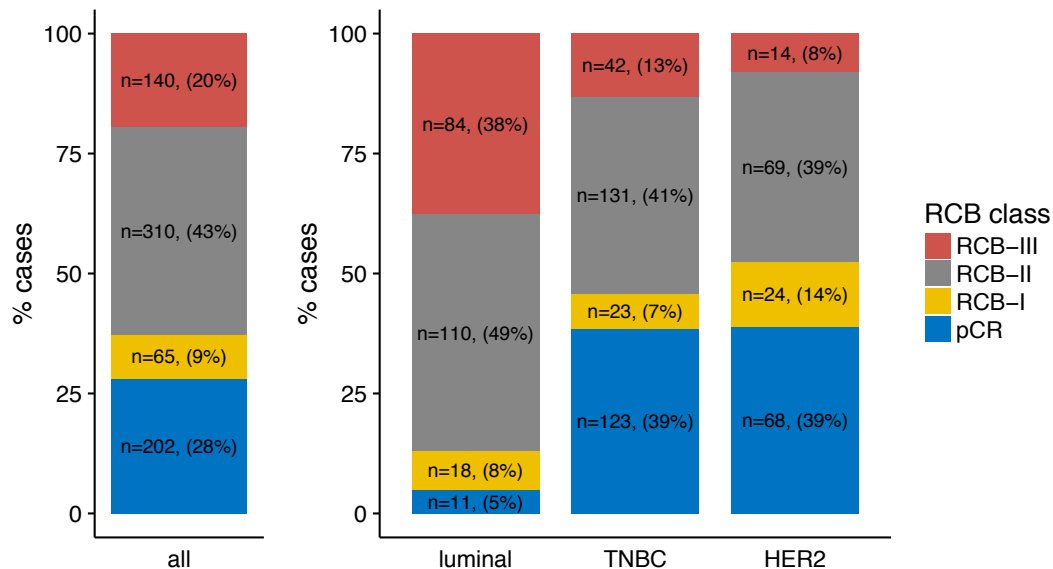
		n (%)
		718
age	<45 y.o	286 (39.8)
	45-55 y.o	254 (35.4)
	>55 y.o	178 (24.8)
menopausal status	postmenopausal	259 (36.4)
	premenopausal	452 (63.6)
BMI class	19<=BMI<=25	414 (57.7)
	BMI<19	41 (5.7)
	BMI>25	262 (36.5)
tumor size	T1	47 (6.5)
	T2	482 (67.1)
	T3	189 (26.3)
clinical nodal status	N0	282 (39.3)
	N1-N2-N3	435 (60.7)
number mitoses	<=22	389 (57.0)
	>22	294 (43.0)
histology	ductal	661 (92.6)
	other	53 (7.4)
grade	Grade I-II	211 (30.1)
	Grade III	491 (69.9)
ki67	ki67<20	33 (18.4)
	ki67>=20	146 (81.6)
ER status	luminal	223 (31.1)
	TNBC	320 (44.6)
	HER2	175 (24.4)
PR status	ER negative	397 (55.3)
	ER positive	321 (44.7)
HER2 status	PR negative	474 (68.2)
	PR positive	221 (31.8)
NAC regimen	HER2 negative	543 (75.6)
	HER2 positive	175 (24.4)
subtype	Anthracyclines based regimens	61 (8.5)
	Anthracyclines-taxanes regimens	577 (80.4)

		n (%)
nodal involvement	Others	80 (11.1)
	0	445 (62.0)
	1-3	188 (26.2)
	>=4 N+	85 (11.8)
RCB class	pCR	202 (28.2)
	RCB-I	65 (9.1)
	RCB-II	310 (43.2)
	RCB-III	140 (19.5)
Post-NAC LVI	no	501 (77.2)
	yes	148 (22.8)

1.1 RCB repartition following NAC

At NAC completion, pCR (RCB 0) was observed in 209 patients (29,2%). Among 499 patients with residual disease, RCB index repartition was as follows RCB-I: n= 64 (8.9%), RCB-II: n=299 (41.6%), and RCB-III: n=136 (19%), (Table 2).

RCB index distribution was significantly different by BC subtypes ($p<0.001$) (Figures 3 /table 1): luminal tumors were more likely to be RCB-II (49.1%) or III (36.9%), whereas HER2-positive BC or TNBC were more likely to be RCB 0 or 1 (52.6% and 48.2% respectively) ($p<0,001$). Only a small subset of tumor was classified as RCB-III in these 2 subtypes (HER2-positive : 8% and TNBC :12,9 % respectively).



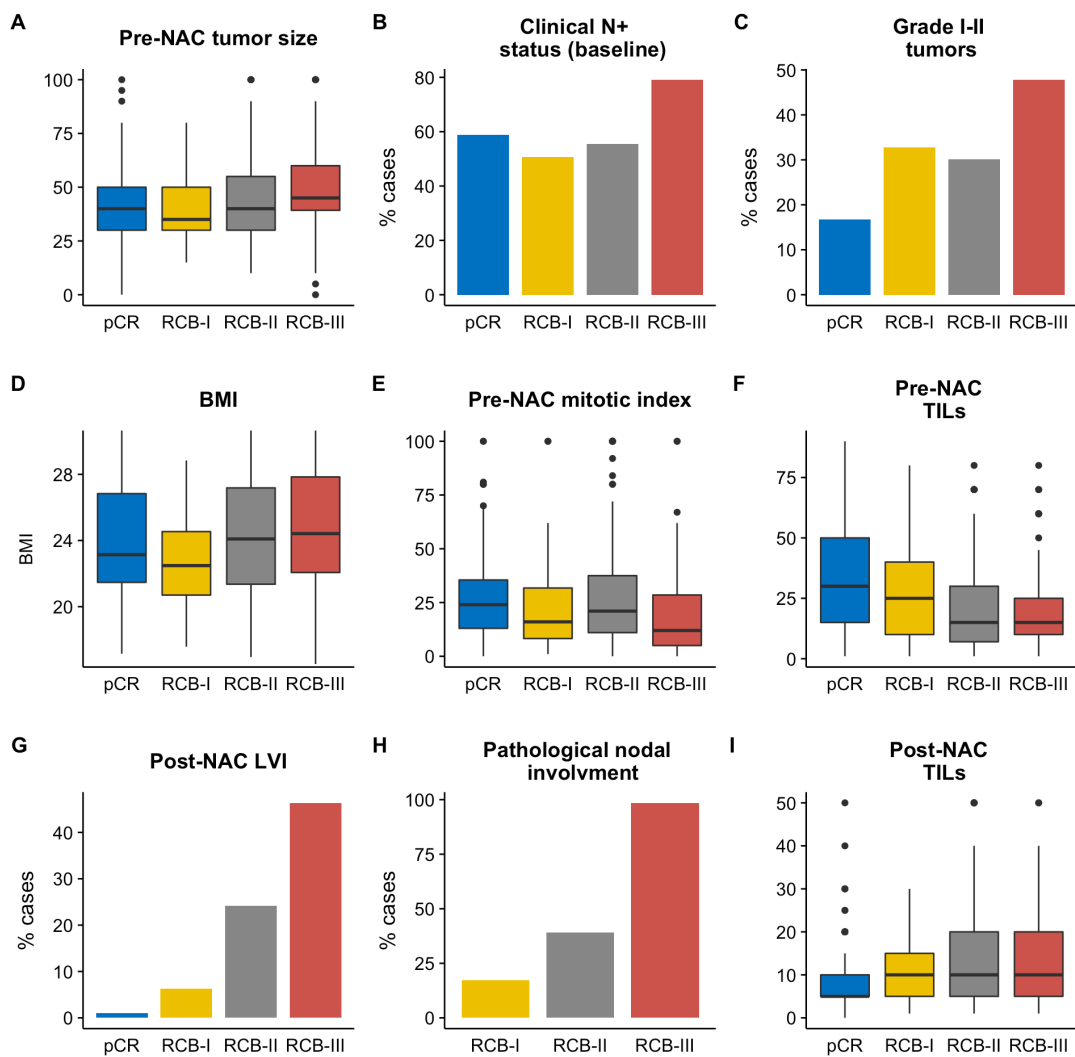
1.2 Patients characteristics by RCB class

Among pre-NAC parameters, RCB class was significantly different by BMI, initial clinical tumor size, Pre-NAC mitotic index, clinical nodal status, histological type, pathological grade, BC subtype. Pre-NAC TILs were inversely associated with RCB. Among post-NAC parameters, RCB class was significantly different by LVI presence, nodal involvement, post-NAC mitotic index. Post-NAC TILs were positively associated with RCB.

		pCR	RCB-I	RCB-II	RCB-III	p
		202	65	310	140	
age		48.64 (10.57)	45.83 (10.21)	47.76 (10.28)	49.35 (10.06)	0.109
bmi		24.53 (4.42)	23.30 (4.22)	24.86 (4.82)	25.22 (4.87)	0.042
clinical tumor size		42.06 (21.83)	39.09 (14.82)	45.57 (19.25)	50.84 (22.33)	<0.001
Pre-NAC mitotic index		27.05 (19.59)	22.81 (21.09)	27.09 (24.11)	18.75 (18.52)	0.001
Pre-NAC TILs		33.99 (23.90)	26.06 (20.32)	19.71 (16.23)	18.86 (16.04)	<0.001
age	<45 y.o	76 (37.6)	31 (47.7)	131 (42.3)	47 (33.6)	0.118
	45-55 y.o	66 (32.7)	25 (38.5)	108 (34.8)	55 (39.3)	
	>55 y.o	60 (29.7)	9 (13.8)	71 (22.9)	38 (27.1)	
menopausal status	postmenopausal	80 (40.2)	18 (28.1)	106 (34.3)	55 (39.9)	0.219
	premenopausal	119 (59.8)	46 (71.9)	203 (65.7)	83 (60.1)	
BMI class	19<=BMI<=25	125 (62.2)	46 (70.8)	176 (56.8)	67 (47.9)	0.011
	BMI<19	8 (4.0)	6 (9.2)	16 (5.2)	11 (7.9)	
	BMI>25	68 (33.8)	13 (20.0)	118 (38.1)	62 (44.3)	
tumor size	T1	26 (12.9)	3 (4.6)	12 (3.9)	6 (4.3)	<0.001
	T2	129 (63.9)	52 (80.0)	213 (68.7)	87 (62.1)	
	T3	47 (23.3)	10 (15.4)	85 (27.4)	47 (33.6)	
clinical nodal status	N0	83 (41.1)	32 (49.2)	138 (44.5)	29 (20.9)	<0.001
	N1-N2-N3	119 (58.9)	33 (50.8)	172 (55.5)	110 (79.1)	
number mitoses	<=22	89 (47.1)	40 (66.7)	168 (56.4)	92 (68.1)	0.001
	>22	100 (52.9)	20 (33.3)	130 (43.6)	43 (31.9)	
histology	ductal	188 (93.5)	59 (90.8)	293 (95.4)	120 (85.7)	0.003
	other	13 (6.5)	6 (9.2)	14 (4.6)	20 (14.3)	
grade	Grade I-II	33 (16.8)	21 (32.8)	91 (30.1)	66 (47.8)	<0.001
	Grade III	164 (83.2)	43 (67.2)	211 (69.9)	72 (52.2)	
ki67	ki67<20	6 (10.2)	3 (30.0)	17 (20.7)	7 (25.0)	0.198
	ki67>=20	53 (89.8)	7 (70.0)	65 (79.3)	21 (75.0)	
ER status	luminal	11 (5.4)	18 (27.7)	110 (35.5)	84 (60.0)	<0.001
	TNBC	123 (60.9)	23 (35.4)	131 (42.3)	42 (30.0)	
	HER2	68 (33.7)	24 (36.9)	69 (22.3)	14 (10.0)	
PR status	ER negative	163 (80.7)	31 (47.7)	153 (49.4)	49 (35.0)	<0.001
	ER positive	39 (19.3)	34 (52.3)	157 (50.6)	91 (65.0)	
HER2 status	PR negative	183 (91.5)	38 (60.3)	186 (61.4)	66 (51.6)	<0.001
	PR positive	17 (8.5)	25 (39.7)	117 (38.6)	62 (48.4)	
NAC regimen	HER2 negative	134 (66.3)	41 (63.1)	241 (77.7)	126 (90.0)	<0.001
	HER2 positive	68 (33.7)	24 (36.9)	69 (22.3)	14 (10.0)	
subtype	Anthracyclines based regimens	17 (8.4)	3 (4.6)	30 (9.7)	11 (7.9)	0.612
	Anthracyclines-taxanes regimens	158 (78.2)	57 (87.7)	245 (79.0)	116 (82.9)	
	Others	27 (13.4)	5 (7.7)	35 (11.3)	13 (9.3)	
nodal involmnet	0	202 (100.0)	53 (81.5)	188 (60.6)	2 (1.4)	<0.001
	1-3	0 (0.0)	12 (18.5)	102 (32.9)	74 (52.9)	
	>=4 N+	0 (0.0)	0 (0.0)	20 (6.5)	64 (45.7)	

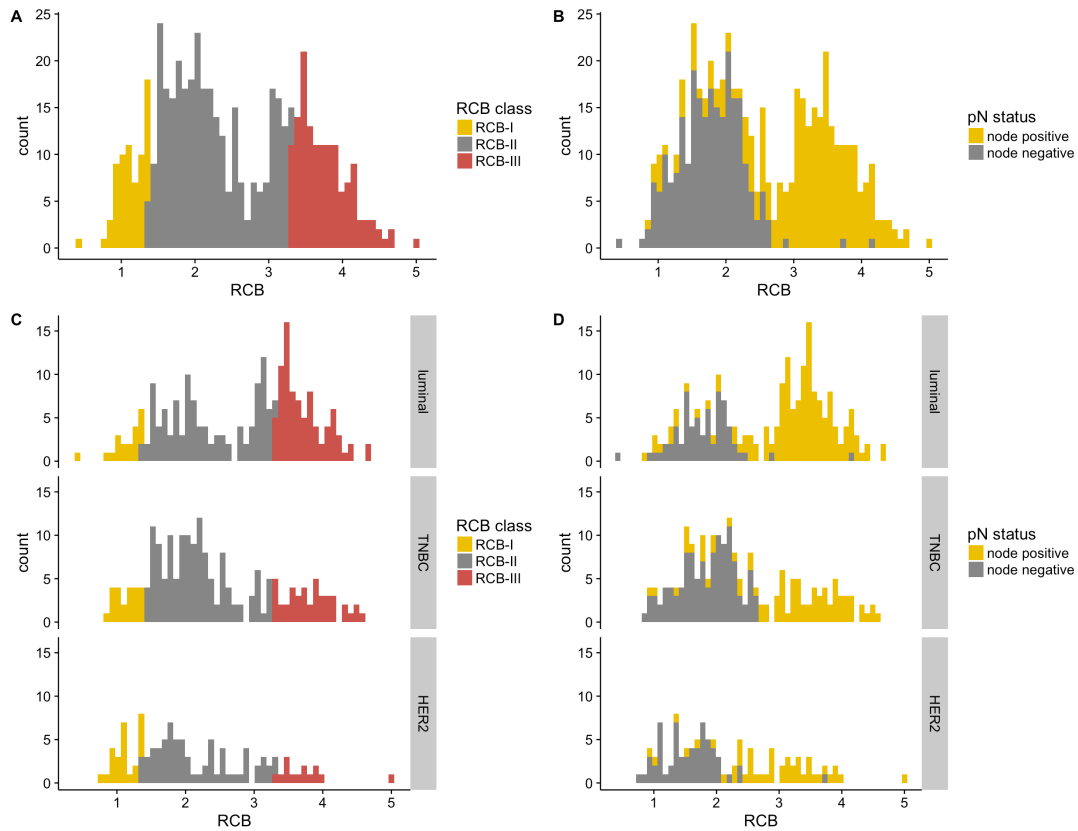
		pCR	RCB-I	RCB-II	RCB-III	p
RCB class	pCR	202 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	<0.001
	RCB-I	0 (0.0)	65 (100.0)	0 (0.0)	0 (0.0)	
	RCB-II	0 (0.0)	0 (0.0)	310 (100.0)	0 (0.0)	
	RCB-III	0 (0.0)	0 (0.0)	0 (0.0)	140 (100.0)	
Post-NAC LVI	no	200 (99.0)	41 (91.1)	191 (71.5)	68 (50.7)	<0.001
	yes	2 (1.0)	4 (8.9)	76 (28.5)	66 (49.3)	
Post-NAC mitotic index		NaN (NA)	0.82 (2.54)	17.83 (28.86)	19.16 (33.60)	<0.001
Post-NAC TILs		8.78 (10.55)	12.84 (14.64)	14.87 (12.47)	15.16 (13.99)	<0.001

1.2.1 association between RCB class and clinico pathological variables



1. 1.3 RCB distribution

RCB distribution was bimodal, with a strong overlap with nodal status.

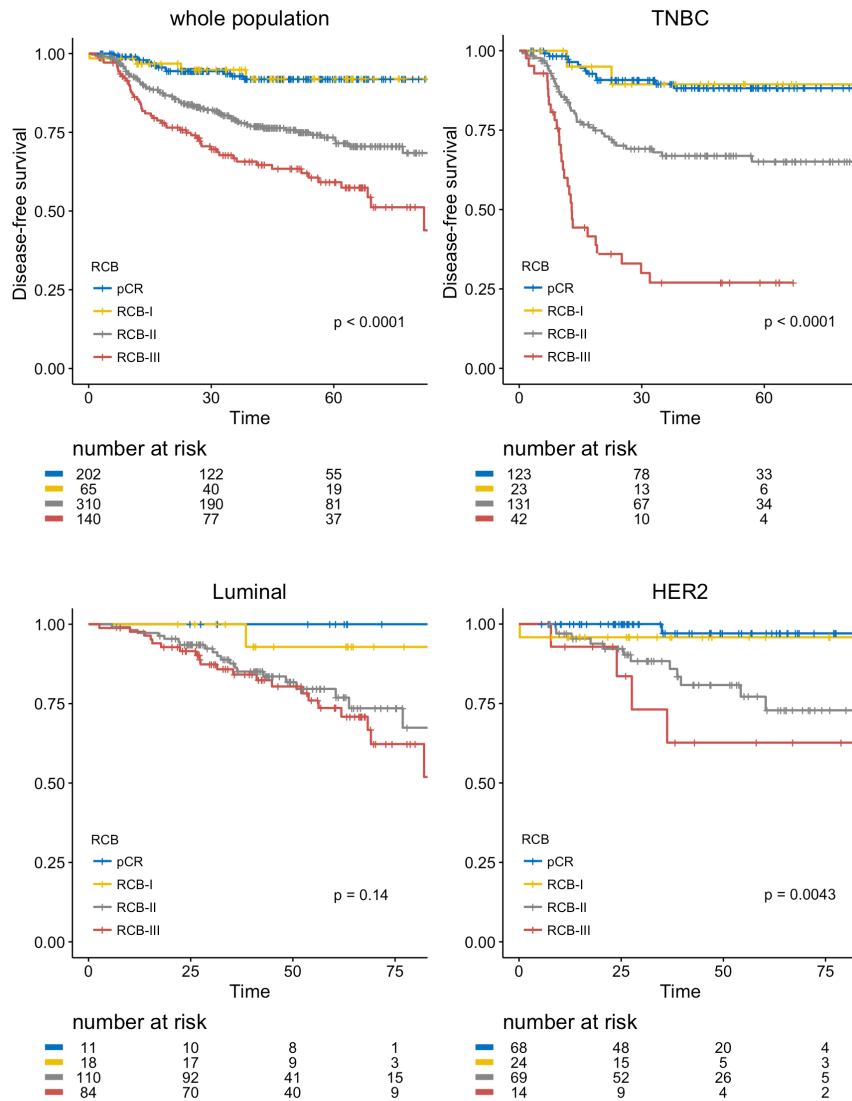


2. 2 Survival analyses

2. 2.1 Disease-free survival

With a median follow-up of 46.4 months, (range[2.8-119.8months]), 145 patients experienced relapse, and 83 deceased .

RCB class was significantly associated with DFS ($p < 0.001$).



2.1.1 Looking for interactions

2.1.1.1 Interactions RCB / BC subtype

The interaction between RCB class and subtype was not significant (P interaction=0.10).

2.1.1.2 Interaction TILs and RCB

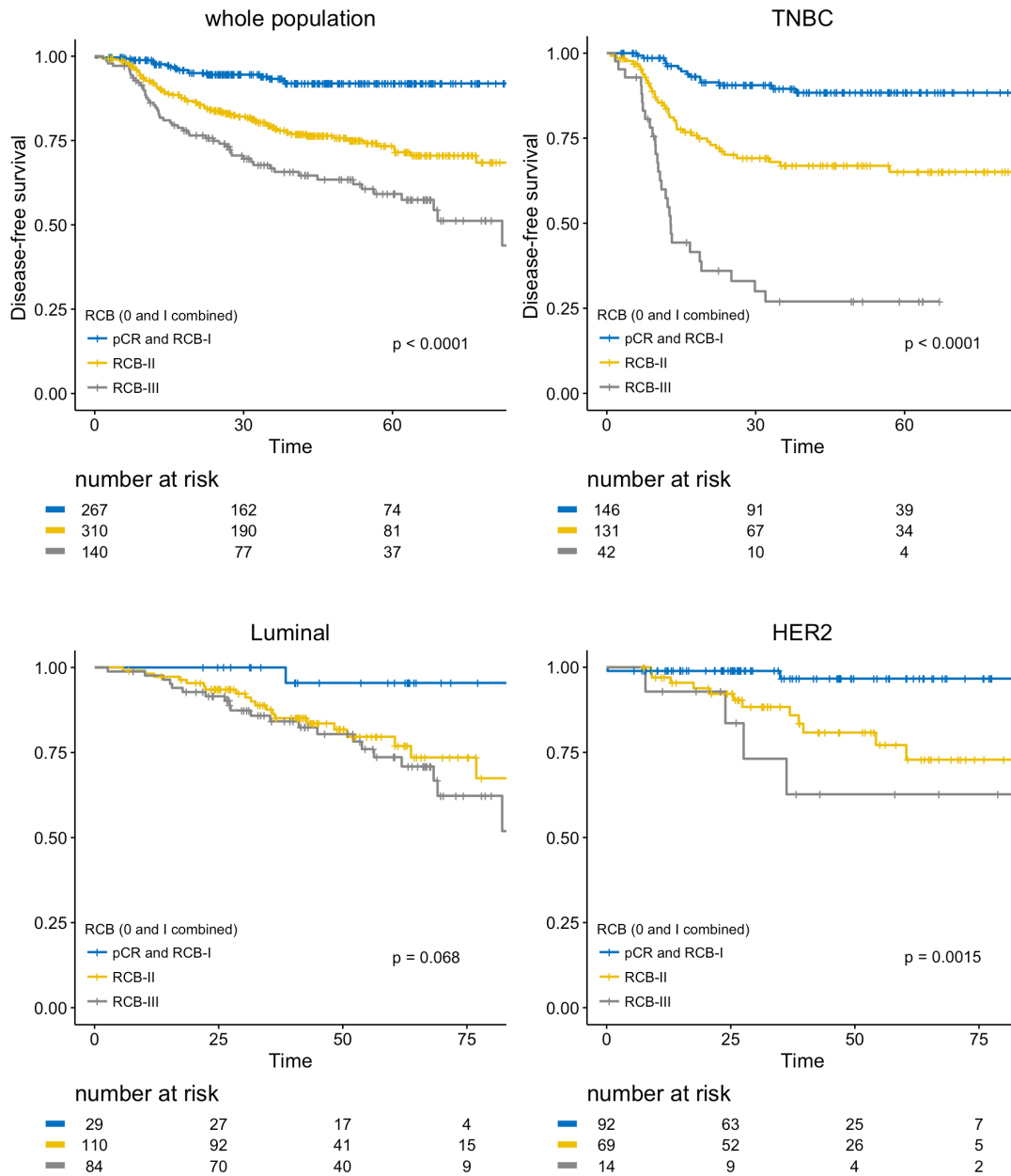
There was a significant interaction between post-NAC TILs and RCB class to predict DFS ($p=0.03$). Post-NAC TILs had no prognostic impact on DFS in pCR, RCB-I and RCB-II subgroups. Conversely, post-NAC TILs had a poor prognostic impact ($HR=1.019$, [1.001-1.037]) in the RCB-III subgroup ($n=140$).

2.1.2 Prognosis by RCB class

The prognosis of RCB-0 and RCB-I patients was not statistically different.

RCB-II and RCB-III had impaired DFS (HR=3.41 [1.92 - 6.04] and HR=6.03 [3.35 - 10.88] respectively).

2.1.3 RCB 0 and I grouped



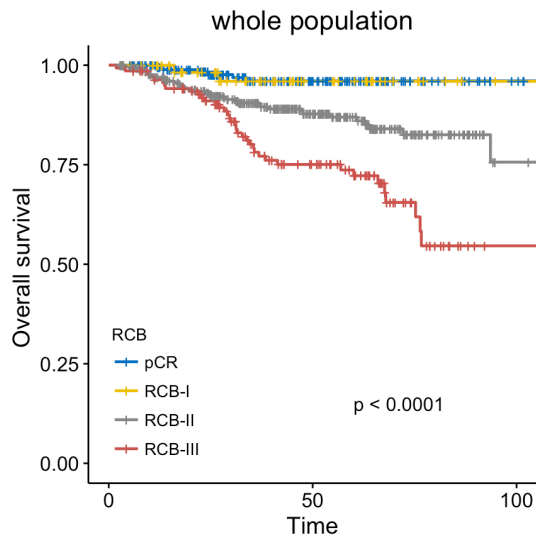
2.1.4 Univariate and multivariate analysis on DFS

				Univariate analysis	Multivariate analysis		
age	<45 y.o	286	60	1	0.67		
	45-55 y.o	254	54	0.98	[0.68 - 1.41]		
	>55 y.o	178	31	0.83	[0.53 - 1.27]		
menopausal status	postmenopausal	259	53	1	0.86		
	premenopausal	452	89	0.97	[0.69 - 1.36]		
BMI class	19<=BMI<=25	414	78	1	0.27		
	BMI<19	41	6	0.74	[0.32 - 1.71]		
	BMI>25	262	61	1.25	[0.89 - 1.75]		
tumor size	T1	47	5	1	<0.01	1	- -
	T2	482	91	1.67	[0.68 - 4.12]	0.26	1.61 [0.58 - 4.48] 0.36
	T3	189	49	2.69	[1.07 - 6.74]	0.04	2.8 [0.98 - 7.99] 0.054
clinical nodal status	N0	282	54	1	0.45		
	N1-N2-N3	435	91	1.14	[0.81 - 1.6]		
number mitoses	<=22	389	71	1	0.03	1	- -
	>22	294	70	1.45	[1.04 - 2.02]	0.03	1.83 [1.23 - 2.7] 0.003
histology	ductal	661	130	1	0.2		
	other	53	14	1.43	[0.82 - 2.48]		
grade	Grade I-II	211	41	1	0.34		
	Grade III	491	101	1.19	[0.83 - 1.71]		
ki67	ki67<20	33	7	1	0.29		
	ki67>=20	146	41	1.54	[0.69 - 3.43]		
ER status	TNBC	320	83	1	<0.01	1	- -
	luminal	223	44	0.61	[0.42 - 0.88]	<0.01	0.33 [0.21 - 0.52] <0.001
	HER2	175	18	0.37	[0.22 - 0.62]	<0.01	0.32 [0.18 - 0.58] <0.001
PR status	ER negative	397	91	1	<0.01		
	ER positive	321	54	0.64	[0.46 - 0.9]	<0.01	
HER2 status	PR negative	474	106	1	<0.01		
	PR positive	221	32	0.57	[0.39 - 0.85]	<0.01	
NAC regimen	HER2 negative	543	127	1	<0.01		
	HER2 positive	175	18	0.45	[0.28 - 0.74]	<0.01	
subtype	Anthracyclines based regimens	61	20	1	0.49		
	Anthracyclines-taxanes regimens	577	111	0.75	[0.46 - 1.22]		
	Others	80	14	0.84	[0.42 - 1.68]		
nodal involvement	0	445	63	1	<0.01		
	1-3	188	45	1.66	[1.13 - 2.43]	<0.01	

		Univariate analysis				Multivariate analysis			
RCB class	>=4 N+	85	37	3.54	[2.36 - 5.32]	<0.01			
	pCR	202	14	1		<0.01	1	-	-
	RCB-I	65	5	1.12	[0.4 - 3.12]	0.82	2.19	[0.71 - 6.76]	0.175
	RCB-II	310	72	3.41	[1.92 - 6.04]	<0.01	3.52	[1.9 - 6.5]	<0.001
	RCB-III	140	53	6.03	[3.35 - 10.88]	<0.01	7.62	[3.84 - 15.14]	<0.001
Post-NAC LVI	no	501	69	1		<0.01	1	-	-
	yes	148	60	3.1	[2.19 - 4.38]	<0.01	1.75	[1.17 - 2.62]	0.006

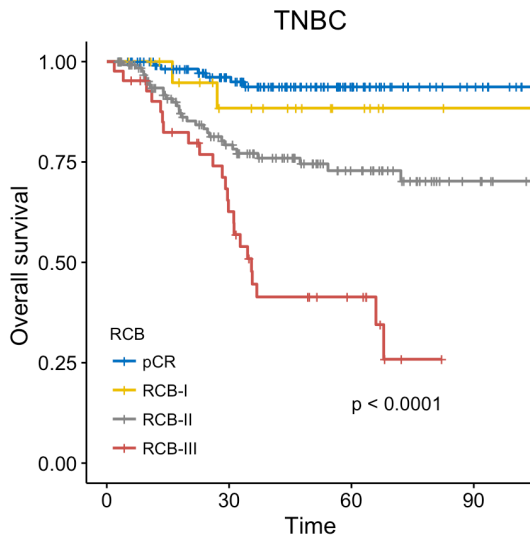
3. 2.2 Overall survival

2.2.1 KM survival curves



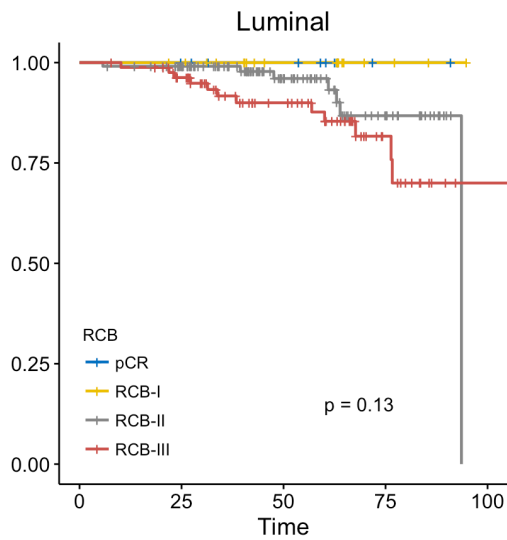
number at risk

202	80	6
65	22	1
310	132	9
140	63	2



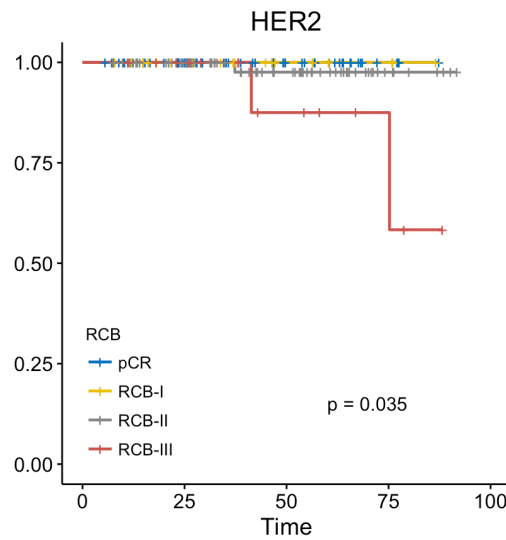
number at risk

123	83	36	11
23	13	6	1
131	76	39	14
42	22	9	0



number at risk

11	10	8	1	0
18	17	9	3	0
110	98	50	17	0
84	74	46	14	2



number at risk

68	48	20	4	0
24	15	5	3	0
69	56	32	7	0
14	11	6	3	0

2.2.2 Univariate and multivariate analysis on OS

				Univariate analysis		Multivariate analysis		
age	<45 y.o	286	60	1		0.67		
	45-55 y.o	254	54	0.98	[0.68 - 1.41]			
	>55 y.o	178	31	0.83	[0.53 - 1.27]			
menopausal status	postmenopausal	259	53	1		0.86		
	premenopausal	452	89	0.97	[0.69 - 1.36]			
BMI class	19<=BMI<=25	414	78	1		0.27		
	BMI<19	41	6	0.74	[0.32 - 1.71]			
	BMI>25	262	61	1.25	[0.89 - 1.75]			
tumor size	T1	47	5	1		<0.01	1	- -
	T2	482	91	1.67	[0.68 - 4.12]	0.26	1.61	[0.58 - 4.48] 0.36
	T3	189	49	2.69	[1.07 - 6.74]	0.04	2.8	[0.98 - 7.99] 0.054
clinical nodal status	N0	282	54	1		0.45		
	N1-N2-N3	435	91	1.14	[0.81 - 1.6]			
number mitoses	<=22	389	71	1		0.03	1	- -
	>22	294	70	1.45	[1.04 - 2.02]	0.03	1.83	[1.23 - 2.7] 0.003
histology	ductal	661	130	1		0.2		
	other	53	14	1.43	[0.82 - 2.48]			
grade	Grade I-II	211	41	1		0.34		
	Grade III	491	101	1.19	[0.83 - 1.71]			
ki67	ki67<20	33	7	1		0.29		
	ki67>=20	146	41	1.54	[0.69 - 3.43]			
ER status	TNBC	320	83	1		<0.01	1	- -
	luminal	223	44	0.61	[0.42 - 0.88]	<0.01	0.33	[0.21 - 0.52] <0.001
	HER2	175	18	0.37	[0.22 - 0.62]	<0.01	0.32	[0.18 - 0.58] <0.001
PR status	ER negative	397	91	1		<0.01		
	ER positive	321	54	0.64	[0.46 - 0.9]	<0.01		
HER2 status	PR negative	474	106	1		<0.01		
	PR positive	221	32	0.57	[0.39 - 0.85]	<0.01		
NAC regimen	HER2 negative	543	127	1		<0.01		
	HER2 positive	175	18	0.45	[0.28 - 0.74]	<0.01		
subtype	Anthracyclines based regimens	61	20	1		0.49		
	Anthracyclines-taxanes regimens	577	111	0.75	[0.46 - 1.22]			
	Others	80	14	0.84	[0.42 - 1.68]			
nodal involvement	0	445	63	1		<0.01		
	1-3	188	45	1.66	[1.13 - 2.43]	<0.01		

		Univariate analysis				Multivariate analysis			
RCB class	>=4 N+	85	37	3.54	[2.36 - 5.32]	<0.01			
	pCR	202	14	1		<0.01	1	-	-
	RCB-I	65	5	1.12	[0.4 - 3.12]	0.82	2.19	[0.71 - 6.76]	0.175
	RCB-II	310	72	3.41	[1.92 - 6.04]	<0.01	3.52	[1.9 - 6.5]	<0.001
	RCB-III	140	53	6.03	[3.35 - 10.88]	<0.01	7.62	[3.84 - 15.14]	<0.001
Post-NAC LVI	no	501	69	1		<0.01	1	-	-
	yes	148	60	3.1	[2.19 - 4.38]	<0.01	1.75	[1.17 - 2.62]	0.006

Chapter 2 Neoadjuvant and Immunity

SCIENTIFIC REPORTS

Corrected: Author Correction

OPEN

New insight for pharmacogenomics studies from the transcriptional analysis of two large-scale cancer cell line panels

Benjamin Sadacca^{1,2,3}, Anne-Sophie Hamy^{1,2}, Cécile Laurent^{1,2}, Pierre Gestraud⁵,
Hélène Bonsang-Kitzis^{1,2,6}, Alice Pinheiro^{1,2}, Judith Abecassis^{1,2,4,5}, Pierre Neuvial^{3,7} &
Fabien Reyat^{1,2,6}

One of the most challenging problems in the development of new anticancer drugs is the very high attrition rate. The so-called "drug repositioning process" propose to find new therapeutic indications to already approved drugs. For this, new analytic methods are required to optimize the information present in large-scale pharmacogenomics datasets. We analyzed data from the Genomics of Drug Sensitivity in Cancer and Cancer Cell Line Encyclopedia studies. We focused on common cell lines (n = 471), considering the molecular information, and the drug sensitivity for common drugs screened (n = 15). We propose a novel classification based on transcriptomic profiles of cell lines, according to a biological network-driven gene selection process. Our robust molecular classification displays greater homogeneity of drug sensitivity than cancer cell line grouped based on tissue of origin. We then identified significant associations between cell line cluster and drug response robustly found between both datasets. We further demonstrate the relevance of our method using two additional external datasets and distinct sensitivity metrics. Some associations were still found robust, despite cell lines and drug responses' variations. This study defines a robust molecular classification of cancer cell lines that could be used to find new therapeutic indications to known compounds.

One of the most challenging problems in the development of new anticancer drugs is the very high attrition rate. Less than 5% of the drugs entering phase I trials eventually obtain marketing authorization¹. Clinical trials are the only real way to assess drug efficacy and toxicity, but this approach is inadequate for testing the hundreds of drugs currently being developed². Scientists need to test hundreds of drugs on numerous tumor models therefore frequently make use of tumor-derived cell lines³⁻⁵. Such studies aim to identify genomic biomarkers for predicting the responses of individual patients to the drug and, ultimately, for identifying the best drug for each patient.

In 2012, the first large-scale pharmacogenomics studies provided an unprecedented wealth to the scientific community. The Broad Institute-Cancer Cell Line Encyclopedia (CCLE) provided a collection of 1,036 human cancer cell lines from 36 tumor types, tested for 24 anticancer drugs. The Genomics of Drug Sensitivity in Cancer (GDSC) assessed the sensitivity of 727 cell lines, from 29 tissue types, to 138 drugs. Both datasets contain genome-wide gene expression and sequencing data for a subset of genes. These studies have provided unprecedented amounts of information about molecular profiles and drug sensitivity and have validated several known genetic biomarkers, such as the BRAF-V600E mutation sensitizing melanomas to vemurafenib⁶ or ERBB2 amplification/overexpression conferring sensitivity to lapatinib⁷.

¹Residual Tumor & Response to Treatment Laboratory (RT2Lab), PSL Research University, Translational Research Department, F-75248, Paris, France. ²U932 Immunity and Cancer; INSERM; Institut Curie, Paris, France. ³Laboratoire de Mathématiques et Modélisation d'Evry, Université d'Evry Val d'Essonne, UMR CNRS 8071, ENSIIE, USC INRA, Evry Val d'Essonne, France. ⁴Mines ParisTech, PSL-Research University, CBIO-Centre for Computational Biology, Mines ParisTech, Fontainebleau, F-77300, France. ⁵Institut Curie, PSL Research University, Mines Paris Tech, Bioinformatics and Computational Systems Biology of Cancer, INSERM U900, F-75005, Paris, France. ⁶Department of Surgery, Institut Curie, Paris, F-75248, France. ⁷Institut de Mathématiques de Toulouse; UMR5219 Université de Toulouse; CNRS UPS IMT, F-31062, Toulouse Cedex 9, France. Correspondence and requests for materials should be addressed to F.R. (email: fabien.reyat@curie.fr)

Received: 23 May 2017

Accepted: 12 October 2017

Published online: 09 November 2017

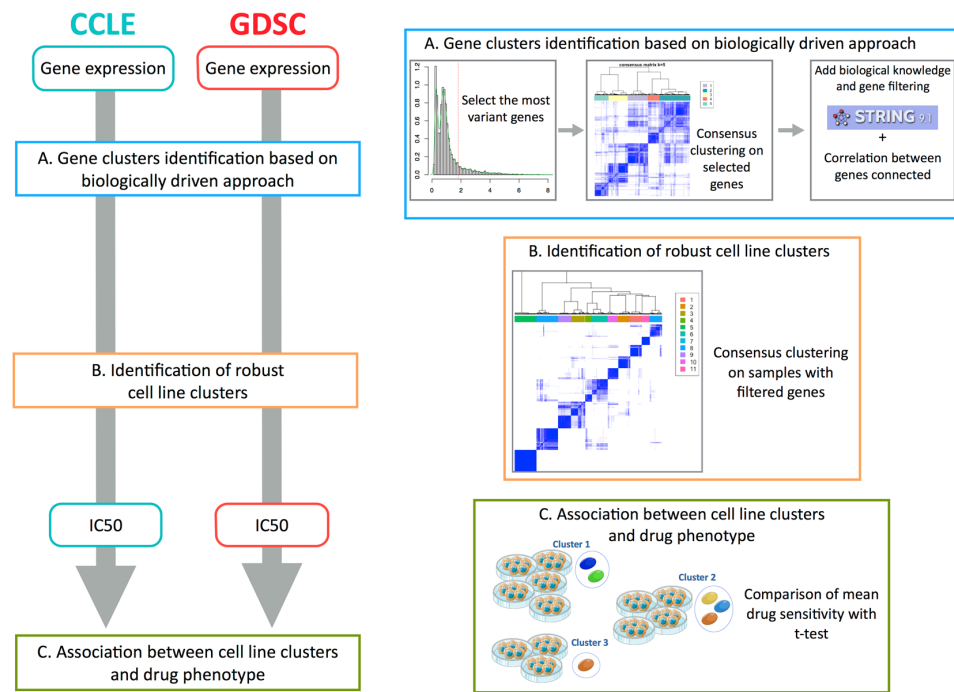


Figure 1. Flow chart of the analysis. We apply the same pipeline of analysis independently to CCLE and GDSC. (a) Biologically driven gene selection was performed to build robust clusters of genes. (b) Robust clusters of cell lines were then built using the selected genes. (c) Cell lines clusters have been associated to distinct drug response.

Previous studies assessed drug sensitivity by pooling all the cell lines or by controlling for tissue source. However, with improvements in our knowledge about tumors, it has become clear that genomic, epigenomic, transcriptional, and proteomic analyses of a given cancer can reveal subtypes differing in pathway activity, progression or treatment response^{8,9}. Conversely, the recent success of basket studies^{10,11} have demonstrated that treatment choices can be based on abnormalities shared by tumors originating from different tissue types.

We present here a comprehensive reanalysis of these two recently published large-scale pharmacogenomics resources. We propose an alternative approach in which cell lines are grouped by transcriptomic profile, based on a biological network-driven gene selection process. This molecular classification of cancer cell lines appeared robust across CCLE and GDSC. We further demonstrated the relevance of this novel classification through the drug response. We validate our approach by robustly found in CCLE and GDSC as in two external dataset the significant associations between cell line clusters and drug responses.

Results

A biologically driven approach identifies four robust gene modules. Gene expression profiles were recovered for 471 cell lines, from 24 different tissues, tested in both CCLE and GDSC. Data were curated and annotated with the pipeline of Haibe-Kains *et al.*¹². We developed a three-step biological network-driven process based on transcriptomic data for identifying robust clusters of genes. This process was applied in parallel for each dataset. We first selected the most variant genes from the set of 12,153 genes common to GDSC and CCLE, by the inflexion point method. We then performed hierarchical consensus clustering¹³ to identify robust gene modules. Finally, we used String@ database software¹⁴ to analyze our gene selection. The goal was to decrease the heterogeneity of each gene cluster. We retained the genes from our initial selection that had (1) high String@ database gene connection indices (greater than 0.7), and (2) similar patterns of expression to other genes within the same biological network (correlation coefficient of at least 0.5) (Fig. 1 step A). This selection process identified four stable clusters in GDSC ($n = 183$ genes) and five in CCLE ($n = 210$ genes), including a subset of 170 genes common to the two datasets. Distinct functional gene ontologies were associated to each gene modules based on a gene ontology analysis: (Supplementary Fig. 1) Gene Cluster - Extracellular Matrix (GC-ECM; $n_{\text{ccle}} = 48$, $n_{\text{GDSC}} = 36$), Gene Cluster - Migration (GC-Migration; $n_{\text{ccle}} = 56$, $n_{\text{GDSC}} = 75$), Gene Cluster - Immunity-Interferon (GC-Immunity; $n_{\text{ccle}} = 22$, $n_{\text{GDSC}} = 14$) and Gene Cluster - Epithelial Phenotype (GC-Epithelial; $n_{\text{ccle}} = 63$, $n_{\text{GDSC}} = 58$). A set of 21 genes enriched in development processes (GC-Development) was found exclusively in the CCLE dataset.

Biologically driven gene selection identifies eleven reproducible cell line clusters. We performed a consensus clustering with the previously selected genes, for each dataset separately, to identify global

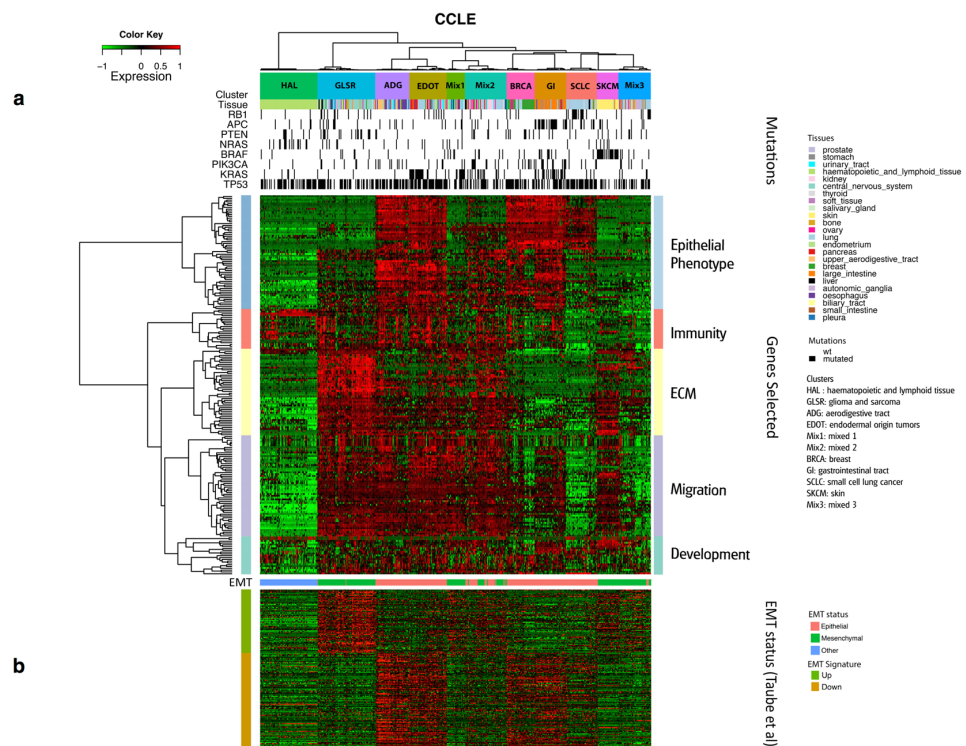


Figure 2. Cell line clustering with CCLE data. (a) Heatmap clustering with 471 cell lines (in columns) and 210 selected genes (in rows) for the CCLE data. (b) EMT status of the cell lines.

differences in gene expression between cancer cell lines (Fig. 1 step B). We obtained eleven stable clusters of cell lines in CCLE and GDSC (Fig. 2a and Supplementary Fig. 2a).

Previous studies reported strong correlations between the expression profiles of identical cell lines¹². We therefore investigated the closeness of the cell line clusters obtained. We defined the similarity between any two cell lines as the number of datasets in which they clustered together (0 = none, 1 = CCLE or GDSC, 2 = CCLE and GDSC). We assessed the consistency between the clustering patterns obtained with CCLE and GDSC data, using a heatmap clustering of the similarity matrix as a visualization tool. The heatmap shows the number of times that two samples are clustered together across datasets (Fig. 3a). Groups of cell lines that frequently cluster with each other are shown in darker shades of blue. The heatmap revealed a well defined 11-block, corresponding to the 11 clusters previously identified. A high degree of consistency between the 11 clusters was observed, with 90% accuracy. As the cell line clusters were highly similar, we use the term “cluster” to denote the same group of cell lines from CCLE and GDSC, unless the dataset is specified.

Tissue-of-origin or transcriptomic features dominate cell line clusters. Our eleven clusters can be organized in three major patterns: (i) four clusters of cell lines were derived mostly from tumors from the same tissue of origin. These cell line clusters were named after the organ or cancer subtypes from which most of the cell lines were derived: hematopoietic and lymphoid tissues (HAL), small cell lung cancer (SCLC), skin (SKCM) and breast (BRCA) clusters; (ii) four clusters of cell lines were derived from tissues from the same organ system or had a common embryonic origin: gastrointestinal tract (GI), aerodigestive tract (ADG), glioma and sarcoma (GLSR) and endodermal origin tumors (EDOT) clusters; (iii) three clusters contained cell lines from different tissues of origin. These clusters were named Mixed 1, Mixed 2 and Mixed 3 (Fig. 3b and c. Details provided in Supplementary data 1 and 2).

Clusters of cell lines with common presumptive tissues of origin. Four cell line clusters appeared very homogeneous in terms of tissue lineage: HAL, SCLC, SKCM and BRCA. These lineages accounted for 84%, on average, of the cells of their respective clusters. The HAL cluster grouped together all the cell lines originating from hematopoietic and lymphoid tissues. This clear clustering pattern can be accounted for by the hematopoietic phenotype of this type of tumor. The SKCM cluster was the second most homogeneous cell line cluster in terms of tissue type (92% of the cell lines in this group originated from melanomas). Breast cancer is a heterogeneous disease with a growing number of recognized biological subtypes, including ER+ Her2-, Her2+ and triple-negative breast cancer (TNBC), which is the most aggressive subtype. BRCA cluster contained all the breast cancer cell lines defined as ER+ Her2- (7/7) and Her2+ (7/7). However, only about half the cell lines defined as triple-negative

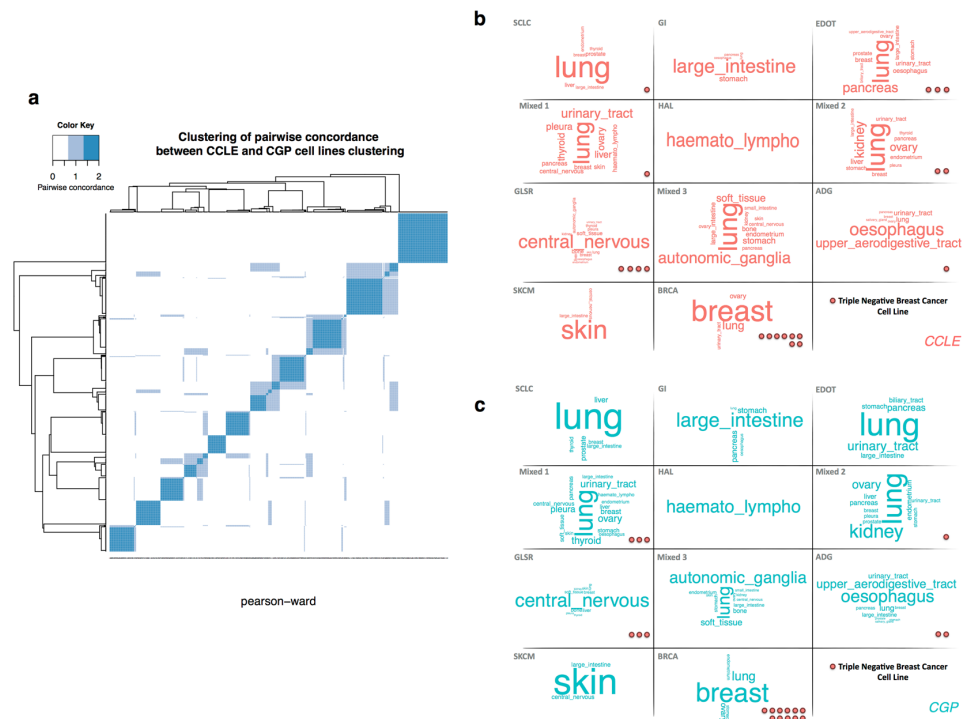


Figure 3. Clustering similarity. (a) Color-coded heatmap for similarity between CCLE and GDSC clustering; Tag Cloud represents the tissue composition of cell lines cluster, in CCLE (b) and GDSC (c). The importance of each tissue is indicated by font size. The TNBC cell lines belonging to each cluster are indicated by red dots.

belonged to this cluster (11/20 in GDSC, 8/20 in CCLE). The remaining triple-negative breast cancer cell lines were found in six different clusters of cell lines (SCLC, EDOT, Mixed 1, Mixed 2, GLSR and ADG) (Fig. 3b,c). SCLC cluster contained 28% of the lung cancer cell lines and 45% of the small-cell lung carcinoma cell lines. We performed a Gene Set Enrichment Analysis¹⁵ (GSEA) based on our previously defined gene modules to characterize the transcriptomic profile of cell line clusters (Supplementary Fig. 3). The immunity gene module was strongly expressed in the cell lines of the HAL cluster. Leukemia affects both the bone marrow and lymphocytes, potentially accounting for the detection of immunity gene expression in cell lines derived from a tumor system with no stromal environment. In the SKCM cell line cluster, the epithelial phenotype gene module was down-regulated. Furthermore, the activation of the ECM and migration gene modules in this cluster is suggestive of aggressive cancer. In the BRCA and SCLC cell line clusters, the epithelial gene module was expressed, whereas the migration and ECM gene modules were not.

Clusters of cell lines from tissues of the same organ system or common embryonic origin. Some clusters could not be defined on the basis of origin from a single tissue type. However, with a more systemic vision, a consistent organization was obtained for four clusters: GI, ADG, GLSR and EDOT. Cell lines derived from tumors of the digestive system belonged to two clusters. The ADG cell line cluster consisted mostly of tumors from the esophagus, upper aerodigestive tract, salivary and also urinary glands, whereas the GI cluster grouped together tumors derived from large intestine, stomach and pancreas cancers. About 70% of the cell lines of the GLSR cluster were derived from tumors of the central nervous system, bone, autonomic ganglia and soft tissue. Finally, the EDOT cell line cluster grouped together cell lines derived from tumors of different tissues (e.g. lung, pancreas, urinary tract) arising from the same germ layer (endoderm). The relevance of the EDOT cluster is supported by studies suggesting that oncogenesis may be initiated by the activation of a common pathway in an endodermal progenitor¹⁶.

The ADG, GI and EDOT clusters all displayed strong expression of the genes of the epithelial phenotype module and weak expression of the ECM gene module. According to GSEA, the migration gene module was less strongly expressed in GI cells. For the EDOT cluster, inconsistencies between the CCLE and GDSC datasets were observed concerning the activation or inhibition of migration gene expression at the transcriptomic level only. The GLSR cluster displayed low levels of expression for the epithelial gene module, and high levels of expression for the ECM and migration modules.

Clusters of cell lines from tumors with heterogeneous tissues of origin. Three clusters displayed no particular prevalence of cell lines corresponding to any particular tissue or organ system. They contained cell lines from tumors

CCLE vs GDSC			CCLE vs GDSC			CCLE vs GSK		
IC50			AUC			IC50		
Drug	Cluster	Response	Drug	Cluster	Response	Drug	Cluster	Response
Erlotinib	ADG	Sensitive	Erlotinib	ADG	Sensitive	Lapatinib	Mixed 1	Resistant
AZD6244	SKCM	Sensitive	AZD6244	SKCM	Sensitive	Lapatinib	SKCM	Resistant
AZD6244	BRCA	Resistant	AZD6244	BRCA	Resistant			
Lapatinib	SCLC	Resistant	Lapatinib	HAL	Resistant			
Lapatinib	ADG	Sensitive	Crizotinib	SKCM	Resistant			
PD0332991	GI	Resistant	AZD0530	SKCM	Resistant			
PD0332991	HAL	Sensitive	PLX4720	SKCM	Sensitive			
PLX4720	SKCM	Sensitive						
PD0325901	GI	Sensitive						
PD0325901	SKCM	Sensitive						
CCLE vs gCSI			GDSC vs gCSI					
IC50			IC50					
Drug	Cluster	Response	Drug	Cluster	Response			
Erlotinib	ADG	Sensitive	PD0325901	SKCM	Sensitive*			
Erlotinib	Mixed 1	Resistant						
Erlotinib	GLSR	Resistant						
Erlotinib	SKCM	Resistant						
Lapatinib	Mixed 1	Resistant						
Lapatinib	ADG	Sensitive						
PD0325901	BRCA	Resistant						
PD0325901	SKCM	Sensitive						
CCLE vs gCSI			GDSC vs gCSI					
Mean Viability			Mean Viability					
Drug	Cluster	Response	Drug	Cluster	Response			
Erlotinib	ADG	Sensitive	PD0325901	SKCM	Sensitive			
Erlotinib	Mixed 1	Resistant						
Erlotinib	GLSR	Resistant						
Erlotinib	SKCM	Resistant						
Erlotinib	HAL	Resistant						
Erlotinib	SCLC	Resistant						
PD0325901	BRCA	Resistant						
PD0325901	SKCM	Sensitive						

Table 1. Significant associations found between CCLE, GDSC, GSK and GCSI. In bold associations found significant in at least three datasets. The association between PD0325901 and SKCM had an adjusted p-values of 0.058 (marked with*).

of 11 to 16 different tissues. We named these clusters Mixed 1, Mixed 2 and Mixed 3. All three of these clusters displayed low levels of epithelial phenotype genes, suggesting that the cell lines they contained were probably mesenchymal. These clusters also displayed an upregulation of ECM genes. Mixed 1 and 2 displayed an upregulation of migration gene expression. These results suggest that some of the cell lines may have been metastatic in origin or subject to drift, from the characteristics of the tissue of origin to a less differentiated state. In this case, transcriptomic profile is more relevant than tissue of origin.

EMT discriminates between cell line clusters. The identification of an epithelial phenotype gene module led us to investigate the epithelial-mesenchymal status of each cell line. A previous study¹⁷ showed that epithelial/mesenchymal transition (EMT)-associated differences in gene expression were a major determinant of the stratification of cancer cell lines based on transcriptomic profiles. Indeed, we found a significant overlap between our gene selections and a published EMT-derived gene signature consisting of 249 genes¹⁸ ($P < 0.0001$, two-tailed Fisher's exact test). We superimposed epithelial/mesenchymal cell line classifications over our gene expression clusters and found a strong association (Fig. 2b and Supplementary Fig. 3b). According to the EMT signature, five cell line clusters (SCLC, GI, EDOT, ADG and BRCA) contained mostly epithelial cell lines, whereas the Mixed 1, Mixed 3, GLSR and SKCM cell line clusters contained mostly mesenchymal cell lines. The Mixed 2 cell line cluster appeared to contain mostly mesenchymal cell lines in GDSC but almost half the cell lines assigned to this cluster in CCLE were epithelial. The HAL cell lines were not concerned by this stratification. Finally, the epithelial/mesenchymal classification was consistent with that obtained with the epithelial phenotype gene module.

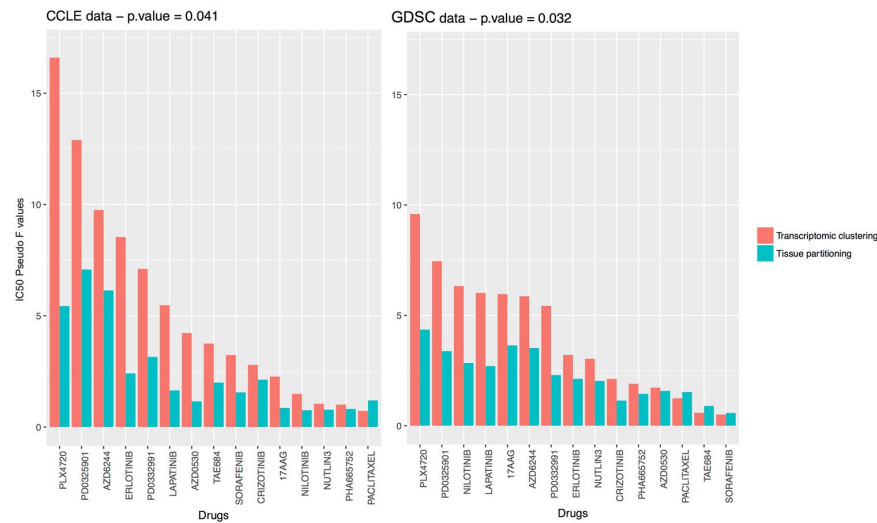


Figure 4. Pseudo F value for the 15 drugs common to CCLE and GDSC. The pseudo F index have been computed from the IC_{50} values for each drug. The pseudo F statistic is the ratio of between-cluster variance to within-cluster variance. Large values of pseudo F indicate well-separated, tight clusters. Drugs are listed in descending order of pseudo F values for clustering.

Cell line clusters are enriched in somatic mutations. We investigated a common set of 64 genes for the presence of mutations in CCLE and GDSC datasets. However, many inconsistencies between both datasets led us to focus on a set of eight genes (TP53, KRAS, NRAS, APC, PIK3CA, BRAF, PTEN and RB1) for which at least 5% of identical cell lines display mutations in both datasets (Supplementary Information). The mutational profile of cell line clusters was then described based on these genes. Mutation profiles clearly distinguished four clusters (Fig. 2a). The SCLC cluster was enriched in RB1 mutations. The GI cluster was rich in APC and KRAS mutations; NRAS mutations were overrepresented in the HAL cluster and the SKCM cluster was enriched in BRAF mutations. Finally, KRAS mutations were particularly abundant in the EDOT clusters. No significant enrichment in mutations was observed for the GLSR, ADG, BRCA and Mixed 3 cell line clusters (Supplementary Tables 1 and 2). These clusters have fewer mean mutation rates than the other clusters (GDSC: 13% vs. 19%, t -test p -value = 0.01; CCLE: 17% vs. 22%, t -test p -value = 0.08).

Transcriptomic clustering is more consistent than clustering on the basis of tissue of origin in terms of drug responses. The large-scale drug screening programs of the Broad and Sanger Institutes have provided to the scientific community an unprecedented wealth of publicly available data. Molecular data have been systematically collected for each cell line, but far less information is available for drug screening (Supplementary Information). Moreover, in many cases (25% in CCLE and 45% in GDSC) it was not possible to extract the IC_{50} from the dose-response curve. In order to overcome these issues, both study also report the AUC (area under the dose response curve) that can always be calculated.

We evaluated whether our clustering was more discriminant than the tissue of origin of the cell lines, in terms of drug response. We calculated a pseudo F -statistic separately for IC_{50} and AUC values for each of the 15 drugs common to CCLE and GDSC. This measurement should capture consistency between the clustering and screening data. It is calculated as the ratio of between-group variance in drug response to the corresponding within-group variance¹⁹. High pseudo F values indicate well-separated, compact clusters. We then compared the pseudo F values calculated with our clustering method with those obtained for ‘tissue partitioning’ for a given drug (i.e. each tissue being to correspond to a cluster of cell lines).

Twelve of the fifteen drugs had a higher ratio in CCLE and GDSC for our clustering than for clustering based on tissue of origin with the IC_{50} (Fig. 4) and ten out of fifteen with the AUC (Supplementary Fig. 4). This trend was confirmed by a t -test comparing the pseudo F values for our clustering with those for ‘tissue partitioning’ (IC_{50} : CCLE t .test p -value = 0.041, GDSC t .test p -value = 0.032, AUC: CCLE t .test p -value = 0.011, GDSC t .test p -value = 0.043). PLX4720 (Raf kinase B inhibitor) and PD0325901 (MEK1 and MEK2 inhibitors) were drugs with the largest pseudo F values in both dataset. Paclitaxel was the only molecule in the panel with a higher pseudo F value for tissue partitioning in CCLE and GDSC. As the drug sensitivity results were not used to determine the clustering of the cell lines, these findings provide independent evidence for a major role of mRNA levels in drug sensitivity.

Robust identification of drug response across datasets. Subgroups of patients or cell lines defined on the basis of transcriptomic data have been shown to be associated with differences in drug sensitivity^{8,9}. We sought to identify associations between clusters of cell lines and ‘sensitive’ or ‘resistant’ drug phenotypes, for the

15 drugs tested in both CCLE and GDSC. For each dataset and each drug separately, we investigated whether the mean IC_{50} of a given cell line cluster differed significantly from those for the other cell line clusters (see Fig. 1 step C and Materials and Methods). Six molecules were found to be significantly associated with six different clusters in both CCLE and GDSC (Table 1 and Supplementary Table 3, Supplementary Fig. 5). The SKCM and GI cell line clusters were both significantly more sensitive than the other cell lines to PD0325901 (MEK 1 and MEK 2 inhibitors) (Fig. 5a). The association of melanoma and PLX4720 (Raf kinase B inhibitor) is already well established and was confirmed by our analysis. Moreover, an inhibitor of MEK 1 and MEK 2, AZD6244, displayed significantly higher levels of activity in cell lines from the SKCM cell line cluster. Both EGFR inhibitors, erlotinib (Fig. 5b) and lapatinib, appeared to be significantly more effective against ADG cell lines than against other cell lines. Hematopoietic and lymphoid tissue cells were sensitive to the CDK4/6 inhibitor PD033991. By contrast, SLCL cell lines appeared to be resistant to lapatinib (EGFR and HER2 inhibitor) and the CDK4/6 inhibitor PD033991 was found inefficient to kill GI cell lines. Finally, AZD6244 (inhibitor of MEK1 and MEK2) appeared ineffective to treat BRCA cell. In addition to variation between drug sensitivity and cell lines, previous studies report variations across the different metrics used to report the drug efficacy^{12,20}. We then performed similar analysis using AUC. More than half of the associations between cell lines clusters and drug sensitivity were found still significant with AUC (Table 1 and Supplementary Information).

We further evaluated the relevance of our clustering regarding the drug sensitivity using two external public datasets. We first study the 118 cell lines tested in common between the CCLE and the GlaxoSmithKline cell line collection (GSK)²¹ on lapatinib and paclitaxel (GDSC was excluded due to small sample size, see Supplementary Information). We found that lapatinib was significantly inactive to kill cells from clusters SKCM and Mixed 1 in both CCLE and GSK (Table 1). Since the set of common cell lines and drugs was small between CCLE, GDSC and GSK (Supplementary Table 4), we consider the Genentech Cell Line Screening Initiative (gCSI)²². A panel of 244 unique cell lines and 5 drugs overlap between CCLE, GDSC and gCSI. Instead of AUC, the gCSI reported the mean viability statistic to measure drug efficacy in addition to the IC_{50} . Eight associations between cell lines clusters and drug sensitivity were found significant using the IC_{50} and nine with the mean viability statistic. Among them, the sensitivity of ADG to erlotinib and lapatinib as well as the efficacy of PD0325901 to kill cells from SKCM cluster were common to CCLE, GDSC and gCSI (Table 1 and Supplementary Information). Our results suggest that our cell line clustering is able to find significant associations with drugs efficacy robustly in four different dataset, despite the large variations across pharmacological data and drug response measures.

Distinct drug profiles were associated with the various cell line clusters. We applied the same procedure to all the drugs tested in the CCLE (24 molecules) and GDSC (129 molecules) studies. For each dataset and each of the 153 drugs separately, we determined whether the mean IC_{50} of a given cell line cluster was significantly different those of the other cell line clusters (Supplementary Tables 5 and 6). Overall, the most striking result was the very small number of drugs associated with a sensitive profile (88 associations, including 71 unique drugs) compared to drugs associated with a resistant profile (163 associations, including 92 unique drugs) (Supplementary Information). It was particularly interesting to observe that Mixed 2 and Mixed 3 clusters were each sensitive to only one drug: respectively midostaurin and vorinostat. Both drugs are targeted agents (PI3K/mTOR inhibitor and HDAC inhibitor). These clusters are made of several cells from different tissue of origin. However, we were able to identify targeted therapies active to kill those cells. These results provide further evidences that our clustering can identify relevant groups of cell sharing unknown features associated to targeted drugs.

Overall, these results suggest that cancer cell lines can be classified, on the basis of their transcriptomic profile, into 11 clusters that may or may not be specific to the tissue of origin. We demonstrated that transcriptomic clustering was more consistent than clustering on the basis of tissue of origin in terms of drug response whatever the drug sensitivity metric considered. We were also able to find several significant associations between clusters of cell lines and “sensitive” or “resistant” drug phenotypes. Many of these associations were robustly found across four different datasets with three different drug response metrics. As the drug sensitivity results were not used to determine the clustering of the cell lines, these findings provide independent evidence about the relevance of this new classification. Furthermore, we show that when we are trying to associate a group of genes from a consistent biological pathway with a group of cell lines, rather than a single gene with a single drug, robust associations can be established across several pharmacologic datasets.

Discussion

Despite the progress in the development of *in vivo* models, cancer cell lines remain a key tool in cancer research. Patients are usually treated with combination therapy. However, it is important to better understand the mechanisms involved with monotherapies before moving forward to study combination therapies. Here, we introduce a new cell line classification constructed from 471 cell lines derived from tumors from 24 different tissues. A biological network analysis for the most variant genes identified 11 clusters of cell lines. These clusters appeared robust in two large-scale cell line panels. This biologically driven gene selection process, which is probably less sensitive to sample fluctuations than other methods, made it possible to capture strong biological signals that might be concealed by the noise present in microarray data. Several studies have reported that the incorporation of network information improves the stability of gene selection and the biological interpretability of biomarker signatures for a given prediction accuracy^{23–25}.

In this new classification, a clear distinction was established between non-epithelial cancer cell lines (GLSR, SKCM, Mixed 3) and epithelial cell lines (EDOT, BRCA, GI). This suggests that EMT-associated differences in gene expression are major determinants of the gene expression-based stratification of cancer cell lines. This new molecular clustering system classified more than 65% of the cell lines differently from the currently used

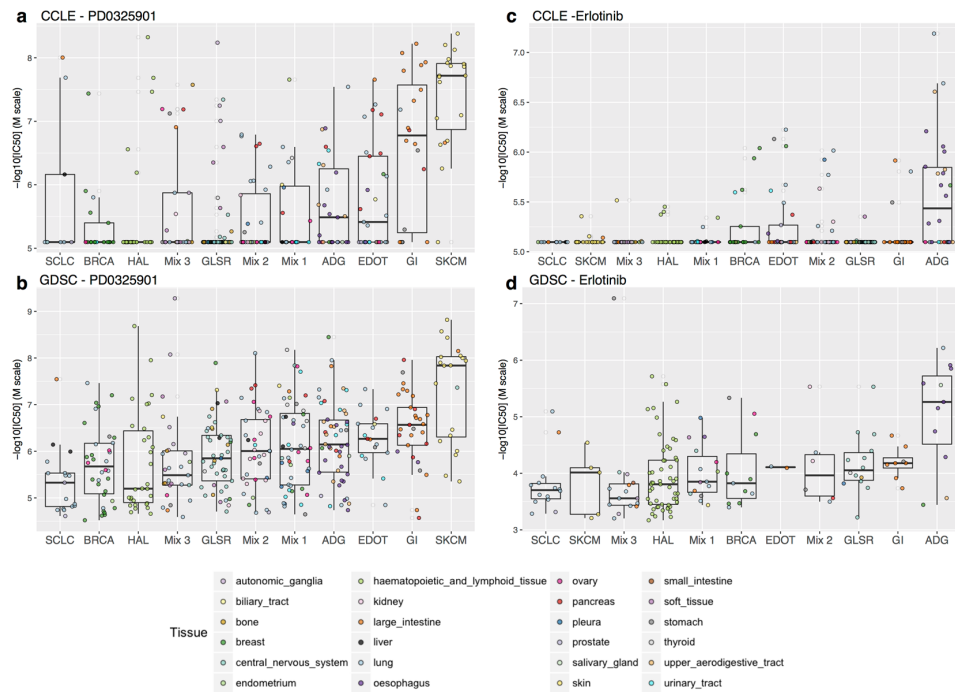


Figure 5. Distribution of IC₅₀ values for each in CCLE and GDSC. Ordered according to mean IC₅₀ for the cluster. From resistant (left) to sensitive (right).

tissue-of-origin cell line classification system. Only four clusters consisted mostly of cell lines originating from a single tissue. Furthermore, three clusters include cells with expression profiles stronger than that of the original tissue (Mixed clusters). Thus, 25% of the cells lines displayed no link to any tissue of origin or related organ system.

One of the most interesting cases was the triple-negative breast cancer (TNBC). We focused on this subtype, as it is the only subtype of breast cancer without any targeted therapy associated. TNBC were found to be highly heterogeneous, falling into six different clusters. This divergence shows the relevance of studying cell lines from various tumor types. Drug response was dependent on cluster membership, with the EDOT cluster sensitive to chemotherapy, whereas the BRCA cluster was resistant. The widely dispersed TNBC cell lines were mostly mesenchymal, whereas the cell lines of the BRCA cluster were exclusively epithelial. TNBC is increasingly emerging as a heterogeneous disease^{26,27}, with tumors differing in histological features, gene expression profiles, clinical behavior, overall prognosis²⁸ and sensitivity to systemic treatment^{9,29,30}. These findings provide strong evidence to suggest that TNBC heterogeneity is reflected at the cell line level. Our results suggest also that particular attention should be paid to the selection of cell lines for studies of particular types or subtypes of cancer.

By analyzing several large-scale public data sets, we demonstrated that drug efficacy is significantly associated to transcriptomic profile. A comparative analysis recently showed that the gene-expression profiles of the 471 cell lines shared by CCLE and GDSC were highly concordant whereas the reported cell-line drug sensitivities for the 15 drugs tested in both studies were highly inconsistent¹². The authors put forward several hypotheses to explain these discrepancies, including differences in experimental protocols, the viability assay and procedures for summarizing dose response and non-observed IC₅₀ (the half maximal inhibitory concentration). Despite discrepancies between the drug sensitivity data retrieved from different databases, we were able to find some robust combinations. Well-known drug associations were found, such as the sensitivity of SKCM lines to vemurafenib. We also found that cancers with BRAF mutations, such as melanoma³¹ and cancers with KRAS/BRAF mutations, such as colorectal cancer³², were more sensitive to MEK inhibitors. Furthermore, CDK4/6 inhibition-induced cell death has been noted in cell lines and xenografts derived from patients with T-cell leukemia³³. SCLC cell lines have been shown to be resistant to lapatinib, but combination with a cytotoxic agent may yield promising results³⁴.

The decline in the number of new treatments approved in recent years is a major challenge for the pharmaceutical industry. One of the reasons for this decline is the lack of systematic evaluation of therapeutic indications for a drug that is either in advanced development phase or has already obtained a marketing authorization. The so-called “drug repositioning process” proposed to find new therapeutic indications to already approved drugs with faster development times and reduced risks. Furthermore, it allows patients to have access to earlier therapeutic advances³⁵. Several robust associations were found. Targeted drugs were found efficient to treat clusters of cell lines constituted of cell from different tissues. These drugs are known to be active in one or several tissues that constitute these clusters. It would be of particular interest to test specifically these drugs on the other tissues

represented in these cell lines clusters. For example, cluster ADG is mostly constituted of upper-aerodigestive, oesophagus and urinary tract cancer cell lines. ADG cluster was particularly sensitivity to the anti EGFR - erlotinib. If EGFR is a validated target for upper-aerodigestive cancer^{36–38} the therapeutic potential of erlotinib has already been highlighted for bladder cancer³⁹ and showed promising results in phase II for oesophagus cancer^{40,41}.

Different types of drugs have been used in the panels. Around 10% of the 153 drugs screened in CCLE and GDSC, and only 1 out of the 15 drugs in common to both studies, are cytotoxic agents. These drugs are expected to be broadly active among the cell line panel since they are not specific molecules. On the contrary, targeted agents are expected to be active only in a subset of cell lines, at least, those carrying the given target. Furthermore, the recent study published by Rees *et al.*⁴² demonstrated that target's expression and drug sensitivity were correlated in only 31% of the cases. Grouping cell lines on the basis of their transcriptomic profiles makes it possible to identify subsets of cells with common off-target features. It is then more relevant to compare the drug sensitivity between cell lines of these groups rather than examined the correlation of response of each cell line to a particular drug reported by one dataset with the response of the same cell line to the same drug reported by another dataset. These results suggest that when robust clusters of cell lines based on biologically network-driven approach are considered, consistency between drug responses can be achieved.

In conclusion, our cell line classification provides novel insight for pharmacogenomics studies. As cell lines remain the most widely used models for the preclinical evaluation of candidate cancer drugs, further investigation should be made to use this classification in the development of cancer treatments with the aim of reducing the attrition rate.

Materials and Methods

Pharmacogenomics data. We collected data from the Broad and Sanger Institutes. The CCLE profiled 24 anticancer drugs on 1,036 cell lines. The GDSC screened 138 drugs on 727 cell lines. Both datasets contain genome-wide gene expression and massive parallel sequencing data. All data were recovered, curated and annotated with the pipeline developed by Haibe-Kains *et al.*¹² (the GDSC was referred to the Cancer Genome Project [CGP] in Haibe-Kains *et al.*). We used this pipeline as described in the original article, but with a different method for the normalization of gene expression. Haibe-Kains *et al.* normalized gene expression data by frozen robust multiarray analysis, fRMA⁴³. This method was designed to combine several datasets and overcome multiple batch issues. This strategy is relevant when trying to ensure assay reproducibility. Even though this approach would be unlikely to have a major effect on gene expression values, we chose to normalize the gene expression data separately with RMA⁴⁴, to ensure that the two datasets were perfectly independent. Our analysis focused on 471 cell lines and 15 drugs for which we have transcriptomic and drug sensitivity data available in both the CCLE and GDSC studies. We also collected two large datasets to validate our classification. Data from the GlaxoSmithKline cell line collection were retrieved from Haibe-Kains *et al.*¹². The Genentech Cell Line Screening Initiative data were available from compareDrugScreens R package published by Haverty *et al.*²².

Gene expression data. Transcriptomic data were restricted to the 12,153 genes common to the two technologies used by GDSC and CCLE (Affymetrix GeneChipHG-U133A and HG-U133PLUS2, respectively). The Jetset method⁴⁵ was used to select a unique probe set for given genes. The same probe set was used in both datasets for 83% of the genes.

Drug sensitivity data. The micromolar concentration (μM) at which the drug inhibited 50% of maximal cell growth was used to assess drug sensitivity as well as the area under the dose response curve (AUC). We also consider the mean viability statistic when comparing with gCSI. These measurements were converted to a common scale ($-\log_{10}(M)$ for IC50, [0,1] for AUC and 1 – mean viability for mean viability), such that high values would be correspond to cell lines sensitive to drugs.

Gene selection by the inflexion point method. We selected the most variant genes, based on the inflexion point of the interquartile range (IQR) distribution for gene expression. This method is more data-driven than a fixed threshold for defining the proportion of genes displaying the highest level of variation. The full procedure is described below. For each gene, we: (1) calculated the IQR for all cell lines, (2) sorted the IQR values of the genes in ascending order, to generate an ordered distribution, (3) estimated the major inflexion point of the IQR curve as the point on the curve furthest away from a line drawn between the start and end points of the distribution, and (4) retained genes with an IQR higher than the inflexion point.

Gene expression-based identification of cell line clusters. We developed a biological network-driven process based on transcriptomic data, to identify robust clusters of genes and cell lines. This process can be broken down into two parts: (A) identification of robust clusters of genes, used for (B) identification of robust clusters of cell lines.

(A) The gene selection process is a three-step procedure. (1) We selected the most variant genes from among the 12,153 genes common to GDSC and CCLE, by the inflexion point method. (2) We performed hierarchical consensus clustering (ConsensusClusterPlus R Package) to identify robust gene modules. The consensus-clustering step, based on Pearson distance and Ward linkage, identified robust clusters of genes. It involved hierarchical clustering by resampling (1,000 iterations) randomly selected genes. (3) We identified known biological networks, for each gene cluster separately, using String[®] database software version 9.1 (<http://string-db.org/>). We then applied a two-step selection process: (1) we selected strong biological networks by retaining only genes for which connection scores of at least 0.7 were obtained with String[®] database software, (2) within each biological network, we selected groups of genes for which expression levels were correlated, with a correlation coefficient of at least 0.5. We used the R package clusterProfiler⁴⁶ for comparing and visualizing gene ontologies profiles among gene modules. (B) We applied a consensus-clustering with hierarchical clustering to the cell line gene expression profiles, using the selected genes to visualize the optimal number of stable cell line clusters.

Characterization of cell line clusters at the transcriptomic and mutational levels. Gene set enrichment analysis (GSEA) was performed on genes modules built in step (A) of the biological network-driven process described above. We identified up-regulated or down-regulated gene modules, associated with each cell line cluster. An analysis was first performed to identify genes differentially expressed between a particular cluster and all the other cell lines, based on a linear model. For a given cluster k , cell lines were partitioned into two groups $j = \{Cluster-k, non-Cluster-k\}$. We then performed a differential analysis by comparing the mean gene expression of each group in a linear model (limma R package⁴⁷). The analysis was performed separately for each dataset. The results were used to rank genes in order of significance and to search for overrepresented gene modules, by pre-ranked gene set enrichment analysis (GSEA).

Genes with significantly higher frequencies of mutation in a given cluster were identified by one-tailed Fisher's exact tests. We compared the occurrence of any given mutation in each cell line clusters with that in all the remaining clusters combined.

Identification of cell line clusters common to different studies. We studied the likeness between the clusterings for CCLE and GDSC, by clustering the cell lines with a similarity matrix (hierarchical clustering with Pearson's metric and the Ward agglomerative method). The similarity matrix contains the number of times two cell lines are clustered together in each dataset (0 = never, 1 = only in one classification, 2 = in both classifications). This similarity matrix constitutes a natural visualization tool for assessing the consistency between two clustering patterns. In particular, if we associate a color gradient to the 0–2 range of real numbers, such that white corresponds to 0, and dark blue corresponds to 2, and if we assume that the matrix is arranged so that items belonging to the same cluster are adjacent to each other (with the same item order used to index both the rows and the columns of the matrix), a matrix corresponding to a perfect consensus will be displayed as a color-coded heatmap characterized by blue blocks along the diagonal, on a white background. The accuracy was calculated as the number of times two cell lines clustered together divided by the number of possible combinations

EMT cell line classification. The “epithelial” or “mesenchymal” status of each cell line was defined with the signature identified by Taube *et al.*¹⁸. This epithelial-to-mesenchymal transition signature consists of 159 down-regulated genes and 90 upregulated genes. We performed a hierarchical clustering of cell lines based on these 249 genes and labeled clusters of cell lines according to the overexpression of known epithelial marker genes, known mesenchymal marker genes or neither.

Definition of breast cancer subtypes. Breast cancer subtypes were defined with a bimodal mixture of two Gaussian distributions for ESR1, PGR and ERBB2 gene expression. Triple-negative (TN) breast cancer cell lines were defined by an absence of estrogen and progesterone receptor expression and a lack of ERBB2 overexpression/amplification ($n = 31$). We subsequently defined breast cancer cell lines overexpressing ESR1 but with a lower level of ERBB2 expression as the ER + Her2- subtype ($n = 7$), with cell lines overexpressing the ERBB2 gene defined as the Her2 + subtype ($n = 7$).

Impact of cell line clustering on drug sensitivity. We investigated the relevance of our clustering for drug sensitivity, by comparing the results obtained for this method with those for ‘tissue partitioning’ (i.e. each tissue of origin being considered to correspond to a cluster of cell lines). We calculated the pseudo F index computed from any drug sensitivity statistic (IC_{50} , AUC, mean viability) for each drug. The pseudo F statistic is the ratio of between-cluster variance to within-cluster variance¹⁹. It is defined as $[Between-cluster\ variance/(N-K)]/[Within-cluster\ variance/(K-1)]$, where N is the number of observations ($N = 471$) and K is the number of clusters ($K = 11$ or $K = 24$). Large values of pseudo F indicate well-separated, tight clusters.

The sensitivity and resistant phenotypes of each cell line for a given drug were defined by comparing the drug sensitivity measure between cell lines from any given cluster and the cell lines in all remaining clusters combined. We focus on IC_{50} for clarity. For a given cluster k , cell lines were partitioned into two groups $j = \{Cluster-k, non-Cluster-k\}$. We then compared the mean IC_{50} values of the two groups in a t test. The sign of the statistical test was used to define the phenotype as sensitive ($t > 0$) or resistant ($t < 0$). We accounted for multiple testing, by calculating the FDR-adjusted p -value for each drug. An FDR-adjusted p -value < 0.05 was considered significant.

Supplementary data. A summary of each cell line cluster, with information regarding tissue composition, molecular profile and drug profile, and other supplementary data for this article can be accessed from the publisher's website.

Consent for publication. All authors read and approved the final manuscript.

Availability of Data and Materials

All data analyzed during this study were retrieved and curated based on the pipeline published by Haibe-Kains *et al.*¹². Data are available from the CCLE website (<http://www.broadinstitute.org>) and GDSC website (<http://www.cancerrxgene.org/downloads/>).

References

1. Moreno, L. & Pearson, A. D. J. How can attrition rates be reduced in cancer drug discovery? *Expert Opin. Drug Discov.* **8**, 363–8 (2013).
2. Tausin, B. More than 900 medicines and vaccines in clinical testing offer new hope in the fight against cancer. *DC Med. Dev. Cancer* **1–2** (2009).
3. Weinstein, J. N. *et al.* An information-intensive approach to the molecular pharmacology of cancer. *Science* **275**, 343–9 (1997).
4. Garnett, M. J. *et al.* Systematic identification of genomic markers of drug sensitivity in cancer cells. *Nature* **483**, 570–575 (2012).

5. Barretina, J., Caponigro, G. & Stransky, N. The Cancer Cell Line Encyclopedia enables predictive modelling of anticancer drug sensitivity. *Nature* **483**, 603–607 (2012).
6. Chapman, P. B. et al. Improved survival with vemurafenib in melanoma with BRAF V600E mutation. *N. Engl. J. Med.* **364**, 2507–16 (2011).
7. Konecny, G. E. et al. Activity of the dual kinase inhibitor lapatinib (GW572016) against HER-2-overexpressing and trastuzumab-treated breast cancer cells. *Cancer Res.* **66**, 1630–9 (2006).
8. Heiser, L. M. et al. Subtype and pathway specific responses to anticancer compounds in breast cancer. *Proc. Natl. Acad. Sci.* **109**, 2724–2729 (2012).
9. von Minckwitz, G. et al. Definition and Impact of Pathologic Complete Response on Prognosis After Neoadjuvant Chemotherapy in Various Intrinsic Breast Cancer Subtypes. *J. Clin. Oncol.* **30**, 1796–1804 (2012).
10. Tsimberidou, A.-M. et al. Personalized Medicine in a Phase I Clinical Trials Program: The MD Anderson Cancer Center Initiative. *Clin. Cancer Res.* **18**, 6373–6383 (2012).
11. Hyman, D. M. et al. Vemurafenib in Multiple Nonmelanoma Cancers with BRAF V600 Mutations. *N. Engl. J. Med.* **373**, 726–36 (2015).
12. Haibe-Kains, B. et al. Inconsistency in large pharmacogenomic studies. *Nature* **504**, 389–93 (2013).
13. Monti, S., Tamayo, P., Mesirov, J. & Golub, T. Consensus Clustering - A resampling-based method for class discovery and visualization of gene expression microarray data Stefano. *Mach. Learn.* **52**, 1–34 (2003).
14. Szklarczyk, D. et al. The STRING database in 2011: functional interaction networks of proteins, globally integrated and scored. *Nucleic Acids Res.* **39**, D561–D568 (2011).
15. Subramanian, A. et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc. Natl. Acad. Sci. USA* **102**, 15545–50 (2005).
16. Quinlan, M. P., Quatela, S. E., Phillips, M. R. & Settleman, J. Activated Kras, but Not Hras or Nras, May Initiate Tumors of Endodermal Origin via Stem Cell Expansion. *Mol. Cell Biol.* **28**, 2659–2674 (2008).
17. Klijin, C. et al. A comprehensive transcriptional portrait of human cancer cell lines. *Nat. Biotechnol.*, doi:<https://doi.org/10.1038/nbt.3080> (2014).
18. Taube, J. H. et al. Core epithelial-to-mesenchymal transition interactome gene-expression signature is associated with claudin-low and metaplastic breast cancer subtypes. *Proc. Natl. Acad. Sci. USA* **107**, 15449–15454 (2010).
19. Calinski, T. & Harabasz, J. A dendrite method for cluster analysis. *Commun. Stat. - Theory Methods* **3**, 1–27 (1974).
20. Fallahi-Sichani, M., Honarnejad, S., Heiser, L. M., Gray, J. W. & Sorger, P. K. Metrics other than potency reveal systematic variation in responses to cancer drugs. *Nat. Chem. Biol.* **9**, 708–14 (2013).
21. Greshock, J. et al. Molecular target class is predictive of *in vitro* response profile. *Cancer Res.* **70**, 3677–3686 (2010).
22. Haverty, P. M. et al. Reproducible pharmacogenomic profiling of cancer cell line panels. *Nature* **533**, 333–337 (2016).
23. Sontrop, H. M., Moerland, P. D., van den Ham, R., Reinders, M. J. & Verhaegh, W. F. A comprehensive sensitivity analysis of microarray breast cancer classification under feature variability. *BMC Bioinformatics* **10**, 389 (2009).
24. Cun, Y. & Fröhlich, H. Prognostic gene signatures for patient stratification in breast cancer - accuracy, stability and interpretability of gene selection approaches using prior knowledge on protein-protein interactions. *BMC Bioinformatics* **13**, 69 (2012).
25. Sanavia, T., Aioli, F., Da San Martino, G., Bisognin, A. & Di Camillo, B. Improving biomarker list stability by integration of biological knowledge in the learning process. *BMC Bioinformatics* **13**, S22 (2012).
26. Bonsang-Kitzis, H. et al. Biological network-driven gene selection identifies a stromal immune module as a key determinant of triple-negative breast carcinoma prognosis. *Oncoimmunology* **37–41** (2015).
27. Lehmann, B. & Bauer, J. Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies. *J. Clin. Investig* (2011).
28. Turner, N. C. & Reis-Filho, J. S. Tackling the Diversity of Triple-Negative Breast Cancer. *Clin. Cancer Res.* **19**, 6380–6388 (2013).
29. Liedtke, C. et al. Response to Neoadjuvant Therapy and Long-Term Survival in Patients With Triple-Negative Breast Cancer. *J. Clin. Oncol.* **26**, 1275–1281 (2008).
30. Cortazar, P. et al. Pathological complete response and long-term clinical benefit in breast cancer: the CTNeoBC pooled analysis. *Lancet* **384**, 164–172 (2014).
31. Wang, D., Boerner, S. A., Winkler, J. D. & LoRusso, P. M. Clinical experience of MEK inhibitors in cancer therapy. *Biochim. Biophys. Acta - Mol. Cell Res.* **1773**, 1248–1255 (2007).
32. Yeh, J. J. et al. KRAS/BRAF mutation status and ERK1/2 activation as biomarkers for MEK1/2 inhibitor therapy in colorectal cancer. *Mol. Cancer Ther.* **8**, 834–843 (2009).
33. Sawai, C. M. et al. Therapeutic Targeting of the Cyclin D3:CDK4/6 Complex in T Cell Leukemia. *Cancer Cell* **22**, 452–465 (2012).
34. Minami, T. et al. HER2 As Therapeutic Target for Overcoming ATP-Binding Cassette Transporter-Mediated Chemoresistance in Small Cell Lung Cancer. *Mol. Cancer Ther.* **11**, 830–841 (2012).
35. Ashburn, T. T. & Thor, K. B. Drug repositioning: identifying and developing new uses for existing drugs. *Nat. Rev. Drug Discov.* **3**, 673–683 (2004).
36. Van Allen, E. M. et al. Genomic Correlate of Exceptional Erlotinib Response in Head and Neck Squamous Cell Carcinoma. *JAMA Oncol.* **1**, 238–44 (2015).
37. Vokes, E. E. et al. A phase I study of erlotinib and bevacizumab for recurrent or metastatic squamous cell carcinoma of the head and neck (HNC). *J. Clin. Oncol.* **23**, 5504–5504 (2005).
38. Cohen, E. E. et al. Erlotinib and bevacizumab in patients with recurrent or metastatic squamous-cell carcinoma of the head and neck: a phase I/II study. *Lancet Oncol.* **10**, 247–257 (2009).
39. Pruthi, R. S. et al. A phase II trial of neoadjuvant erlotinib in patients with muscle-invasive bladder cancer undergoing radical cystectomy: Clinical and pathological results. *BJU Int.* **106**, 349–356 (2010).
40. Li, G. et al. Phase II study of concurrent chemoradiation in combination with erlotinib for locally advanced esophageal carcinoma. *Int. J. Radiat. Oncol. Biol. Phys.* **78**, 1407–1412 (2010).
41. Wainberg, Z. A. et al. Phase II trial of modified FOLFOX6 and erlotinib in patients with metastatic or advanced adenocarcinoma of the oesophagus and gastro-oesophageal junction. *Br. J. Cancer* **105**, 760–5 (2011).
42. Rees, M. G. et al. Correlating chemical sensitivity and basal gene expression reveals mechanism of action. *Nat Chem Biol* **12**, 109–116 (2016).
43. McCall, M. N., Bolstad, B. M. & Irizarry, R. A. Frozen robust multiarray analysis (fRMA). *Biostatistics* **11**, 242–253 (2010).
44. Irizarry, R. A. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Biostatistics* **4**, 249–264 (2003).
45. Li, Q., Birkbak, N. J., Györfy, B., Szallasi, Z. & Eklund, A. C. Jetset: selecting the optimal microarray probe set to represent a gene. *BMC Bioinformatics* **12**, 474 (2011).
46. Yu, G., Wang, L.-G., Han, Y. & He, Q.-Y. clusterProfiler: an R package for comparing biological themes among gene clusters. *OMICS* **16**, 284–7 (2012).
47. Ritchie, M. E. et al. limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res.* **43**, e47–e47 (2015).

Acknowledgements

The authors thank Sergio Roman-Roman for reviewing the study and the manuscript. This work was supported by the Department of Translational Research of the Institut Curie and the *Fondation ARC (Association pour la Recherche contre le Cancer Grant No. DOC20160604097)*. Funding was also obtained from the *Site de Recherche Intégrée en Cancérologie/Institut National du Cancer (Grant No. INCa-DGOS-4654)*.

Author Contributions

Conceptualization: F.R., P.N. Data curation: B.S. Formal analysis: B.S., J.A. Investigation: B.S., C.L., P.N. Methodology: B.S., P.N., C.L., P.G. Validation: A.P. Project administration: F.R., P.N. Supervision: F.R., P.N. Writing – original draft: B.S., A.S.H.P. All authors reviewed the manuscript.

Additional Information

Supplementary information accompanies this paper at <https://doi.org/10.1038/s41598-017-14770-6>.

Competing Interests: The authors declare that they have no competing interests.

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit <http://creativecommons.org/licenses/by/4.0/>.

© The Author(s) 2017



[Click for updates](#)

Oncolmmunology

Publication details, including instructions for authors and subscription information:
<http://www.tandfonline.com/loi/koni20>

Biological network-driven gene selection identifies a stromal immune module as a key determinant of triple-negative breast carcinoma prognosis

H. Bonsang-Kitzis^{abe}, B. Sadacca^{ab}, A S. Hamy-Petit^{ab}, M. Moarii^{cd}, A. Pinheiro^{ab}, C. Laurent^{ab} & F. Reyat^{abe}

^a Residual Tumor & Response to Treatment Laboratory, RT2Lab, Translational Research Department, Institut Curie, Paris, F-75248, France.

^b U932 Immunity and Cancer, INSERM, Institut Curie, Paris, F-75248, France.

^c Mines Paristech, PSL-Research University, CBIO-Centre for Computational Biology, Mines ParisTech, Fontainebleau, F-77300 France.

^d U900, INSERM, Institut Curie, Paris, F-75248, France.

^e Department of Surgery, Institut Curie, Paris, F-75248, France.

Accepted author version posted online: 24 Jun 2015.

To cite this article: H. Bonsang-Kitzis, B. Sadacca, A S. Hamy-Petit, M. Moarii, A. Pinheiro, C. Laurent & F. Reyat (2015): Biological network-driven gene selection identifies a stromal immune module as a key determinant of triple-negative breast carcinoma prognosis, *Oncolmmunology*, DOI: [10.1080/2162402X.2015.1061176](https://doi.org/10.1080/2162402X.2015.1061176)

To link to this article: <http://dx.doi.org/10.1080/2162402X.2015.1061176>

Disclaimer: This is a version of an unedited manuscript that has been accepted for publication. As a service to authors and researchers we are providing this version of the accepted manuscript (AM). Copyediting, typesetting, and review of the resulting proof will be undertaken on this manuscript before final publication of the Version of Record (VoR). During production and pre-press, errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal relate to this version also.

PLEASE SCROLL DOWN FOR ARTICLE

Taylor & Francis makes every effort to ensure the accuracy of all the information (the "Content") contained in the publications on our platform. However, Taylor & Francis, our agents, and our licensors make no representations or warranties whatsoever as to the accuracy, completeness, or suitability for any purpose of the Content. Any opinions and views expressed in this publication are the opinions and views of the authors, and are not the views of or endorsed by Taylor & Francis. The accuracy of the Content should not be relied upon and should be independently verified with primary sources of information. Taylor and Francis shall not be liable for any losses, actions, claims, proceedings, demands, costs, expenses, damages, and other liabilities whatsoever or howsoever caused arising directly or indirectly in connection with, in relation to or arising out of the use of the Content.

This article may be used for research, teaching, and private study purposes. Any substantial or systematic reproduction, redistribution, reselling, loan, sub-licensing, systematic supply, or distribution in any form to anyone is expressly forbidden. Terms & Conditions of access and use can be found at <http://www.tandfonline.com/page/terms-and-conditions>

**Biological network-driven gene selection identifies a stromal immune module
as a key determinant of triple-negative breast carcinoma prognosis**

**H. Bonsang-Kitzis^{1,2,5}, B. Sadacca^{1,2}, A-S. Hamy-Petit^{1,2}, M. Moarii^{3,4},
A. Pinheiro¹⁻², C. Laurent¹⁻², F. Reyal^{1,2,5}**

¹ Residual Tumor & Response to Treatment Laboratory, RT2Lab, Translational Research Department, Institut Curie, Paris, F-75248, France.

² U932 Immunity and Cancer, INSERM, Institut Curie, Paris, F-75248, France.

³ Mines Paristech, PSL-Research University, CBIO-Centre for Computational Biology, Mines ParisTech, Fontainebleau, F-77300 France.

⁴ U900, INSERM, Institut Curie, Paris, F-75248, France.

⁵ Department of Surgery, Institut Curie, Paris, F-75248, France.

Corresponding author:

Dr Fabien REYAL

Institut Curie

Department of Surgery

26 rue d'Ulm

75005 Paris

0033615271980

fabien.reyal@curie.fr

Abstract

Triple-negative breast cancer is a heterogeneous group of aggressive breast cancers for which no targeted treatment is available. Robust tools for TNBC classification are

required, to improve the prediction of prognosis and to develop novel therapeutic interventions.

Methods

We analyzed 3247 primary human breast cancer samples from 21 publicly available datasets, using a five-step method: 1) selection of TNBC samples by bimodal filtering on ER-HER2 and PR, 2) normalization of the selected TNBC samples, 3) selection of the most variant genes, 4) identification of gene clusters and biological gene selection within gene clusters on the basis of String© database connections and gene-expression correlations, 5) summarization of each gene cluster in a metagene. We then assessed the ability of these metagenes to predict prognosis, on an external public dataset (METABRIC).

Results

Our analysis of gene expression in 557 triple-negative breast cancers from 21 public datasets identified a six-metagene signature (167 genes) in which the metagenes were enriched in different gene ontologies. The gene clusters were named as follows: Immunity1, Immunity2, Proliferation/DNA damage, AR-like, Matrix/Invasion1 and Matrix2 clusters respectively. This signature was particularly robust for the identification of TNBC subtypes across many datasets ($n=1125$ samples), despite technology differences (Affymetrix© A, Plus2 and Illumina©). Weak Immunity 2 metagene expression was associated with a poor prognosis (disease-specific survival; HR=2.68 [1.59-4.52], $p=0.0002$).

Conclusion

The six-metagene signature (167 genes) was validated over 1125 TNBC samples. The Immunity 2 metagene had strong prognostic value. These findings open up interesting possibilities for the development of new therapeutic interventions.

Keywords

Triple-negative breast cancer, molecular subtype, predictive value, prognosis impact.

Introduction

Triple-negative breast cancer (TNBC), defined by the absence of estrogen and progesterone receptor expression and a lack of *HER2* overexpression/amplification, is an aggressive disease accounting for 15% to 20% of breast cancers. It differs from other molecular subtypes¹⁻³ in displaying axillary lymph node involvement, local and regional recurrence, differences in the time lag to metastasis (distant metastatic events occurring within five years of diagnosis), high rates of brain, lung and distant nodal metastasis and in its response to neoadjuvant treatment.

TNBC constitutes a major clinical challenge because there has been no substantial improvement in treatment for this subgroup in the recent past. Even if adjuvant chemotherapy has significantly improved outcome, reducing the risk of death by approximately 30%⁴, but these cancers do not respond to endocrine or targeted therapy. TNBC is, thus, currently the breast cancer subgroup with the worst outcome⁵. Moreover, the shape of the survival curve for this subgroup differs from that for other BC subtypes: there is a sharp decrease in survival during the first three to five years after diagnosis, but distant relapses, occurring after this interval, are much less common⁵.

TNBC is a highly heterogeneous group of tumors differing in terms of their histological features, gene expression profiles, clinical behavior, overall prognosis⁶ and sensitivity to systemic treatment⁷⁻⁹.

Robust classifiers are urgently required, to improve our understanding of the molecular basis of TNBC and to define novel therapeutic interventions. Lehmann *et al.* recently published a classification of six molecular subtypes of TNBC¹⁰ and

developed a website (<http://cbc.mc.vanderbilt.edu/tnbc/>)¹¹ for the classification of TNBC samples on the basis of their gene expression profiles. This classification has been shown to be relevant, as it identifies the main biological component and pathways of TNBC. However, the large number of genes defining this TNBC molecular classification (2188 genes) constituted a potential source of instability^{12,13}.

We developed a two-step biological network-driven gene selection process: 1) identification of the most variant genes displaying highly correlated patterns of expression, 2) direct connection of these genes within known biological networks. This method has been reported to be efficient for the construction of molecular signatures^{14,15}. We defined a robust TNBC molecular subtype classification, providing considerable biological insight, with great potential for use in the development of therapeutic interventions. We also identified a stromal immune module gene expression profile strongly correlated with TNBC prognosis.

Results

TNBC gene expression profiles identify 6 main gene clusters

Gene expression (GE) profiles were obtained from 21 publicly available datasets, containing data for 3,247 primary human breast cancer samples. These profiles were processed according to the flow chart in Figure 1. The training set included samples hybridized on HGU-133A Affymetrix© arrays (12 datasets, $n=1,995$), to eliminate cross-platform discrepancies and to ensure robust normalization. The validation set included samples hybridized on HGU-133Plus2 Affymetrix© arrays (9 datasets, $n=1,014$). We filtered out 42 outlier samples from the training set and 17 from the validation set.

We also collected two large datasets, for the validation of our classification: the Ignatiadis set ($n=996$) and the METABRIC set ($n=1992$). The processing of these two datasets has been described elsewhere^{16,17}.

Bimodal filtering on ER-PR and HER2 GE identified 262, 295, 314 and 254 TNBC samples in the training set, the validation set, the Ignatiadis set and the METABRIC set, respectively.

We developed a gene selection process based on biological networks, to decrease the intrinsic instability of molecular classification methods.

We identified the 830 most variant genes ($SD>0.8$) in the training set ($n=262$). A consensus clustering method and hierarchical clustering identified four main gene clusters. Further increases in cluster number yielded no significant increase in the consensus distribution function area (Supplementary Figure S1 and Materials and Methods).

The various gene clusters were associated with different gene ontologies (Supplementary Figure S2). The clusters were thus named as follows (Supplementary Figure S3A): Immunity cluster (145 genes), Proliferation/DNA damage cluster (397 genes), AndrogenReceptor(AR)-like cluster (139 genes) and Matrix/Invasion cluster (149 genes).

The Immunity cluster was the most homogeneous, with strong correlations between the gene expression profiles of most of the genes within this cluster (Supplementary Figure S3B).

We used String© database software to analyze our gene selection, with the aim of decreasing the heterogeneity of each main gene cluster. We retained the genes from our initial selection that 1) had high String© database gene connection indexes (greater than 0.7, Supplementary Figure S4), 2) had similar patterns of

expression to other genes within the same biological network (correlation coefficient of at least 0.5). We selected a final set of 167 genes [Immunity cluster (80), Proliferation/ DNA damage (15), AR-Like(15), Matrix/Invasion (57)] (Supplementary Figure S5).

Following biological network-driven gene selection, it became clear that the original Immunity and Matrix/Invasion clusters were more accurately described by splitting them into two subclusters displaying minor differences [Immunity1 (33), Immunity2 (47), Matrix/Invasion1 (43), Matrix2 (14)] (Supplementary Figure S6A). This approach yielded an increase in the area under the consensus distribution function (CDF) curve (Supplementary Figure S7).

For each of the six gene clusters identified in this way, we defined a metagene. The Immunity1 and Immunity2 metagenes displayed similar patterns of expression, with a Pearson correlation coefficient of 0.58; the Pearson correlation coefficient for the expression patterns of Matrix/Invasion1 and Matrix2 was 0.48. The Proliferation/DNA damage and Matrix metagenes displayed the strongest inverse correlation (coefficients of -0.43 and -0.60 for Matrix/Invasion1 and Matrix2, respectively) (Supplementary Figure S6B).

We validated this six-gene cluster classification, by applying hierarchical clustering based on the 167 genes selected to the validation set ($n=295$). Clustering was highly consistent between the training and validation gene sets (concordance: 93-100%).

The 6 gene clusters identify 6 stable TNBC subgroups

Hierarchical clustering was performed on the four TNBC datasets [training set (262), validation set (295), Ignatiadis (314) and METABRIC (254)]. For Affymetrix© arrays,

we used the 167 selected genes. For the Illumina© platform, we used 153 common genes. We identified six reproducible subgroups of TNBC, for which GE patterns were similar in the training set and in the three validation sets (total of 1125 samples). The corresponding heatmaps are shown in Figure 2. The Pearson correlation coefficients for the relationships between each sample subgroup centroid in the three validation sets and the corresponding subgroup centroid in the training set are shown in Figure 2.

We illustrated the dynamic links between genes within a biological network, as defined by the String© database, by showing GE levels for a “prototype sample” (Supplementary Figure S8).

We compared our sample classification with those reported by Lehmann *et al.* and Curtis *et al.* (Supplementary Figure S9). Our classification appears very different from that of Lehmann at first glance (χ^2 test p value=0.05), but the samples assigned to Centroids 1 and 6 (with high levels of Matrix/Invasion 1 and Matrix 2 gene expression, respectively) tended to be classified as Mesenchymal (M) or Mesenchymal stem-like (MSL), the samples in Centroid 5 (strong expression of Immunity2 genes) tended to be classified as Immunomodulatory (IM), and the samples in Centroid 4 (strong expression of AR-like genes) tended to be classified as of the Luminal androgen receptor (LAR) subtype (Supplementary Figure S10A and Figure S10B). Curtis *et al.* aimed at defining a new classification across all cancer subtypes, not specific to TNBC subtypes. In this classification, the TNBC samples were mostly classified as IntClust10 or IntClust4, with an even distribution.

Prognostic value of the Immunity2 metagene in TNBC

The prognostic value of the 167-gene TNBC signature was assessed with the

METABRIC dataset. The 254 TNBC samples were split into two subgroups: a subgroup treated by chemotherapy (139) and a subgroup not treated by chemotherapy (115). The chemotherapy-naïve (noCT) population and the chemotherapy-treated population were significantly different (Supplementary Table S1). The patients in the noCT population were older (mean age of 61.5 years vs. 50.1 years, $p < 1.2 \times 10^{-11}$), more likely to be postmenopausal (77% vs. 47%, $p = 5.38 \times 10^{-5}$), and their tumors were of lower grade ($p = 0.01$), with less lymph node involvement (81% vs. 17%, $p < 2.2 \times 10^{-16}$), a lower Nottingham Prognostic Index (NPI < 3.4, 17% vs. 2%, $p = 2.57 \times 10^{-5}$), and less cellularity ($p = 0.03$).

Univariate analysis identified three factors significantly correlated with a poor outcome (distant disease-free survival) in the chemotherapy-treated population: NPI > 5.4 (HR = 2.15 [1.28-3.60], $p = 0.003$); *p53* mutation (HR = 2.42 [1.15-5.09], $p = 0.02$); and weak Immunity2 metagene expression (HR = 2.59 [1.54-4.34], $p = 0.0002$) (Table 1A, Figure 4A). We did not include *p53* mutation status in the multivariate model, due to missing data ($n = 79$). A NPI > 5.4 and low levels of Immunity2 metagene expression were retained in the multivariate model and were significantly associated with a poor outcome (HR = 2.30 [1.36-3.89], $p = 0.002$; HR = 2.68 [1.59-4.52], $p = 0.0002$, respectively) (Table 1A). The combined variable, NPI score/Immunity2 metagene expression was found to be of particular interest. In a first model, a NPI score greater than 5.4 was associated with a worse prognosis: HR = 3.98 [2.00-7.92], $p = 8.72 \times 10^{-5}$. For patients with NPI scores of 5.4 or below, Immunity2 metagene expression discriminated between two groups of patients with different outcomes (HR = 2.90 [1.51-5.56], $p = 0.001$). In a second model, NPI3 patients can also be split into two groups on the basis of Immunity2 metagene expression. The NPI3 group with high levels of Immunity2 metagene expression had a prognosis similar to that of the

NPI1/2 group with low levels of Immunity2 metagene expression (Table 1B, Figure 4A).

Univariate analysis identified four factors significantly correlated with poor outcome in the noCT population: tumor size > 20 mm (HR=2.36 [1.01-5.48], $p=0.04$), lymph node-positive status (HR=3.66 [1.65-8.11], $p=0.001$), NPI score >5.4 (HR=10.69 [2.74-41.76], $p=0.001$) and low levels of Immunity2 metagene expression (HR=2.33 [1.09-4.95], $p=0.03$) (Table 2A, Figure 4B). Two of these factors were retained in the multivariate model: NPI score>5.4 (HR=12.03 [3.05-47.50], $p=0.0004$) and low levels of Immunity2 metagene expression (HR=2.42 [1.13-5.16], $p=0.02$) (Table 2A). As in the chemotherapy-treated subpopulation, the combined variable, NPI score/Immunity2 metagene expression discriminated between two groups of patients with different outcomes in this noCT population (Table 2B, Figure 4B). The chemotherapy-naïve group contained only seven patients classified as NPI3. Stratification of this subgroup defined on the basis of treatment was therefore not considered methodologically relevant.

We compared the prognostic value of the Immunity2 metagene with that of eight previously published immune signatures,^{18,19,20,21,22,23,24,25} using the METABRIC dataset.

We generated a heatmap (Supplemental Figure S11) of the gene expression profiles of each of the above prognostic signatures applied to the METABRIC dataset. The samples were ordered according to our classification of low/high Immunity2 metagene expression. Expression patterns were very similar between the Immunity2 gene expression signature and all the other gene expression signatures, with the exception of the Bianchini, Karn and Burstein (BLIS) gene-expression signatures.

We first performed a univariate analysis of the prognostic value of the eight-gene expression signatures, as described in the corresponding original manuscripts. The Rody, Sabatier, Teschendorff, Desmedt, Gu-Trantein Tfh, Gu-Trantien Th1 and Burstein signatures were significantly correlated with the prognosis of TNBC. The Bianchini and Karn gene expression signatures were not correlated with the prognosis of TNBC (Supplemental Figure S12, Supplemental Table S2). We then performed a multivariate analysis. We included NPI score, the Immunity2 metagene and each of the Rody, Sabatier, Teschendorff, Desmedt, Gu-Trantein Tfh, Gu-Trantien Th1, and Burstein signatures, one-by-one, in the model. In all comparisons the only significant variables remaining in the multivariate model were NPI score and the Immunity2 metagene (Supplemental Table S2).

The Immunity2 metagene corresponds to B-cell and T-cell pathways

String database connections between the Immunity1 or Immunity2 genes and the genes of the eight published prognostic immune signatures^{18,19,20,21,22,23,24,25} are provided in Figure 3. The gene intersection was poor, but our immune signature nevertheless appears to be strongly correlated with other published signatures (Supplementary data), suggesting the use of similar immune pathways. The Immunity2 metagene was strongly correlated with the expression metagenes of the above signatures (coefficient greater than 0.8), except for the Bianchini, Karn and BLIS metagenes (Supplemental Figure S13).

We explored the pathways relating to the Immunity metagenes in detail, by analyzing the correlation between the expression of the Immunity1 and Immunity2 metagenes and the metagenes defined by Gatzka *et al.*²⁶ (IFN-alpha, IFN-gamma, STAT3, TGF-beta, TNF-alpha) and Palmer *et al.*²⁷ (LB, LT, CD8, GRANS,

LYMPHS). This analysis was performed on the METABRIC dataset published by Curtis *et al.*¹⁷.

We showed that the Immunity2 metagene was highly correlated with the B-cell, T-cell and CD8 cell metagenes (Pearson correlation scores: 0.93, 0.91, 0.87) (Supplemental Figure S14). The Immunity1 metagene was highly correlated with the interferon alpha and gamma pathways (Pearson correlation scores: 0.97, 0.94).

Furthermore, in cancer cell lines (CCLE and CGP datasets), the Immunity2 metagene displayed very low levels of expression, similar to those of the CD8 metagene (Supplemental Figure S15). This was true for all cell lines and breast cancer cell lines tested.

Moreover, the IFN-alpha, IFN-gamma, STAT3, TGF-beta, TNF-alpha, LB, LT, GRANS metagenes were more strongly expressed in TN breast cancer cell lines than in HER2-positive and luminal breast cancer cell lines (Supplemental Figure S16).

We investigated Immunity2 gene expression in white blood cell populations (Palmer *et al.*²⁷), by performing a consensus clustering of the Immunity2 genes on Palmer's dataset. This analysis identified four stable clusters of the genes of the Immunity2 signature. Some genes were more strongly expressed in B cells (GZMA, GZMB, CCR7, LY96, MS4A1, CD74 for example), others in T cells (CD3D, CCL2, CD14, CD2, LCK, IL7R), and still others in granulocytes (named Pax cells) (CD163, MND4, NCF2, CSF2RB, FGL2) (Supplemental Figure S17). These findings suggest that, even if the 'Immune2' signal is highly homogeneous within tumor samples (the entire set of genes being coordinately either over- or under-expressed), different subpopulations of cells express different subsets of these genes in the periphery.

The Immunity2 metagene is probably expressed by stromal cells

In TNBC cell lines (TNBC_CL), genes from the Immunity2 module displayed very low medians and narrow ranges of expression, suggesting that they were expressed only in the tumor stromal compartment. A similar trend was observed for all breast cancer cell lines (BC_CLs). The Immunity1 module genes had higher median expression levels and a broader range of expression in TNBC_CL and in all BC_CL, suggesting that Immunity1 genes were expressed by the tumor cells (Figure 5A and Figure 5B).

Furthermore, we explored the contributions of stromal and cancer cells to Immunity1 and Immunity2 expression in detail, by comparing our gene lists to the “stromal contribution to global gene expression evaluated in PDX RNAseq data”, as defined by Isella *et al.*²⁸. The Immunity2 metagene had a very high stromal fraction, as for the Matrix/Invasion1 and Matrix2 metagenes. The Immunity1 metagene had a very low stromal fraction, like the ARLike and Proliferation/DNA damage metagenes (Supplemental Figure S18).

The Immunity2 metagene open up interesting new possibilities for therapeutic interventions

To highlight the new opportunities for therapeutic intervention provided by this study, we represented the existing drugs (with or without US Food and Drug Administration approval) for each metagene (Supplemental Figure S19 and Supplementary data). Some are undergoing clinical investigation in patients with TNBC.

We explored the links between PD1, PDL1, CTLA4 (and their respective metagenes) and the Immunity2 metagene. We compared the Immunity2 metagene with the TILs signature defined by Schalper *et al.*²⁹, who showed that PD-L1 mRNA synthesis was associated with increases in the expression of TILs and recurrence-

free survival. This analysis was performed on the METABRIC dataset. The PD1 and CTLA-4 metagenes were constructed from the genes most strongly correlated with the PD1 and CTLA-4 genes, respectively (Pearson correlation score > 0.8). The PDL1 metagene was defined by Sabatier *et al.*³⁰.

The Immunity2 metagene was highly correlated with the PD1, PDL1 and CTLA-4 metagenes (Pearson correlation: 0.90, 0.96, 0.91). The coefficient of correlation between the Immunity2 metagene and the TILs signature was up to 0.90 (Supplemental Figure S20).

In cell lines, the PD1, PDL1, CTLA-4 and TILs metagenes were very weakly expressed, like the Immunity2 metagene (Supplemental Figure S21).

Using the METABRIC dataset, we compared the prognostic value of these metagenes (PD1, PDL1, CTLA4 and TILs) with that of the Immunity2 metagene. In univariate analysis, high levels of PD1, PDL1, CTLA-4 and TILs metagene expression were associated with a good prognosis (Supplemental Figure S22, Supplemental Table S3). In multivariate analysis, we included NPI score, the Immunity2 metagene and each of the PD1, PDL1, CTLA4 and TILs metagenes, one-by-one, in the model. In all comparisons, the only significant variables remaining in the multivariate model were NPI score and the Immunity2 metagene.

Discussion

New tools for classifying TNBCs are urgently required, to improve our understanding of the molecular basis of TNBC and to identify potentially useful novel therapeutic interventions. By analyzing the GE profiles of 1125 triple-negative breast cancers, we identified a six-metagene signature (167 genes) in which the various metagenes were enriched in different gene ontologies: two clusters were enriched in immunity

genes, one in proliferation/DNA damage genes, one in AR pathway genes, and two in matrix/invasion genes. This signature appeared to be particularly robust for identifying TNBC subtypes across different datasets, independently of the genechip technology used to generate the data. Furthermore, one metagene (Immunity2) was found to be of strong prognostic value for TNBC samples.

Lehmann *et al.*¹⁰ recently developed a classification of TNBCs in which a 2188-gene signature was used to classify tumors. They suggested that this classification could also be used to classify xenografts or cell lines. They also developed a website (<http://cbc.mc.vanderbilt.edu/tnbc/>) for the classification of TNBC samples¹¹. This study provided important biological insight into the molecular drivers of TNBC, but it also raised several key concerns. First, the normalization process involved data from different platforms. Several studies have shown that large discrepancies in signature composition and absences of concordance concerning outcome may be due to differences in the array platform and preprocessing method used¹². Second, Lehmann *et al.* used a very large number of genes (2188 genes) to establish their molecular signature, and this may have constituted a source of instability, due to the noise introduced^{12,13}. As shown by Weigelt *et al.*³¹, microarray-based single-sample predictors do not allocate individual samples to a given molecular subtype reproducibly, probably because the use of large numbers of genes leads to instability of the classification when new samples are added. Third, it would be unwise to transpose this classification to various *in vitro* and *in vivo* breast cancer models (primary tumor xenografts, cell lines, cell line-derived xenografts), because the stromal environment and the original tumor are very different^{32,33}. We found that genes from the Immunity compartment (Immunity2 module) were highly relevant for the classification of TNBC samples and that these genes were not expressed in

breast cancer cell lines. The observed lack of reproducibility between classifiers may reflect major differences in the methodology and aims of the studies concerned. Further validation will be required before these models can be used in routine clinical practice.

We developed a strategy for the definition of a gene expression signature based on the analysis of biological networks for the most variant genes. Within these networks, we then analyzed gene expression parameters, to select the genes with the most strongly correlated patterns of expression. The validation process showed that our gene matrix identified similar GE patterns across 1125 TNBC samples. This first step in biological network analysis, which is probably less sensitive to sample fluctuations than other methods, made it possible to capture strong biological signals that might be concealed by the noise present in microarray data. Several studies have reported that the incorporation of network information improves the stability of gene selection and the biological interpretability of biomarker signatures for a given prediction accuracy^{14,15,34}.

The Immunity2 module was identified as a strong prognostic factor for disease-free survival (strong expression of this metagene is correlated with a good outcome), regardless of the characteristics of the tumor (NPI score, tumor size, tumor grade and lymph node status). It clearly suggest the presence of an hemopoietic infiltrate, composed of activated cytotoxic T cells, B cells, myeloid cells, NK cells and neutrophils. This module includes adhesion molecule-associated genes (SELL, ITGB2), and genes encoding proteins involved antigen processing and presentation (CD74 or ligand, HLA-DRA), B-lymphocyte cell surface molecules (PTPRC, ITGB2, HLA-DRA), the caspase cascade (CASP1), complement pathway (C1QA, C1QB), CTL-mediated immune responses to target cells (ITGB2, CD3D, GZMB), dendritic

cell regulation of Th1 and Th2 development (CD2, IL7R), granzyme-mediated apoptosis (GZMA, GZMB), IL12-mediated signaling events (CD3D, HLA-DRA, GZMA, LCK), the IL2 signaling pathway (LCK), interleukin-3, 5 and GM-CSF signaling (HCK, BLNK, CSF2RB), T-cell surface molecules (PTPRC, CD3D, CD2, ITGB2), and the T-cell receptor signaling pathway (PTPRC, CD3D, HLA-DRA, LCK).

Burstein *et al.*²⁵ identified four different TNBC subtypes (LAR, MES, BLIS, BLIA) with the identification of similar pathways and a prognostic value for the BLIA subgroup similar to that for the signature identified in our study. This subgroup displays an upregulation of B-cell, T-cell, and natural killer cell immune-regulating pathways and an activation of STAT transcription factor-mediated pathways. The authors showed that the prognosis was worse for basal-like immune-suppressed tumors than for basal-like immune-activated tumors, for both disease-free survival ($p=0.04$) and disease-specific survival ($p=0.039$).

Several recent studies have demonstrated the importance of tumor-infiltrating lymphocytes (TILs) in controlling the clinical progression of various epithelial cancers³⁵. In breast cancer, recent advances in gene expression profiling have revealed an association between immune signatures and favorable outcomes^{29,36}. A gene signature enriched in cytotoxic CD8⁺ T-cell genes and genes associated with natural killer cell activity has been reported³⁷. However, the ability of CD8⁺ T cells to control human breast cancer is probably counteracted by the presence of immunosuppressive cells, CD4⁺ T-regulatory cells or macrophages: immunohistochemistry (IHC) analysis of tissue microarray data for 179 treatment-naive breast tumors revealed that high levels of macrophages and CD4⁺ T cells were correlated with poor overall survival, whereas a combination of high levels of CD8⁺ T cells and low levels of macrophages and CD4⁺ T cells was correlated with higher

overall survival³⁸. Intratumoral B cells have also been associated with a favorable prognosis in breast cancer³⁹. In ER-negative breast cancers, a STAT1 signaling metagene¹⁶, and a B-cell metagene¹⁹ were found to be associated with better outcomes. Another group identified an immune response-based prognostic gene module (C1QA, XCL2SPP1, TNFRSF17, LY9, IGLC2, HLA-F) associated with a better prognosis than for other ER-negative breast cancers, regardless of lymph node status and lymphocytic infiltration⁴⁰. According to Bertucci *et al.*⁴¹, the immunomodulatory subtype (overlapping with medullary breast cancers, a rare form of TNBC with a prominent lymphocytic reaction) is associated with a favorable prognosis. The two Immunity modules identified in this study had many biological connections with other eight immune prognosis signatures published for TNBC^{18,19,20,21,22,23,24,25}.

Neoadjuvant chemotherapy is increasingly being used for TNBC, because these tumors have a poor prognosis, are assumed to be chemosensitive and no alternative specific systemic treatment is available. Patients with a complete pathologic response (pCR) after neoadjuvant chemotherapy have a better outcome than those with residual disease, and pCR is a good surrogate for long-term survival and cure in this specific subgroup^{9,42}.

The Immunity2 metagene was not found to be predictive of response to neoadjuvant chemotherapy in TNBC (272 fine needle aspirations of TNBC samples for which information about pCR or its absence was available from the eight datasets previously published by Ignatiadis *et al.*¹⁶) (data not shown). This lack of relationship may have resulted from the use of fine needle aspiration biopsy samples. The Immunity2 genes, which are largely expressed in the stromal environment, were less

strongly expressed in fine needle aspiration samples than in tumor samples (Supplemental Figure S23).

However, intratumoral immune responses are known to be correlated with clinical outcomes in TNBC. This may reflect the role of immune cells in the activity of cytotoxic chemotherapeutic agents. Some chemotherapeutic drugs, such as anthracyclines, act not only through direct cytotoxic effects, but also by activating CD8⁺ T-cell responses. Conflicting results have been published on the ability of other immune-based classifiers to predict outcome in TNBC. High intratumoral levels of CD8⁺ T cells⁴³ or TILs^{36,44} are associated with better clinical responses to anthracycline-based chemotherapy. West *et al.*⁴⁵ reported that high levels of lymphocyte gene expression were associated with a high rate (74%) of complete pathological responses to neoadjuvant anthracycline-based chemotherapy. In 2011, Sabatier *et al.*²⁰ showed, by gene-expression profiling, that 'Immune High' patients (59%) were more likely to present pCR than 'Immune Low' patients (43%), but this difference was not significant ($p=0.29$). In 2014⁴⁶, they showed that 'PDL1 mRNA expression high' (57%) patients presented higher rates of pCR than 'PDL1 mRNA expression low' (43%) patients ($p<0.001$). Wimberley *et al.*⁴⁷ showed that PDL1 protein levels in the epithelium and stroma were correlated with pCR only in hormone receptor-positive and HER2-amplified breast cancers. Denkert *et al.*⁴⁴ demonstrated the importance of TIL and immune gene expression signatures for predicting pCR in breast carcinoma. However no significant difference in pCR rate was detected between lymphocyte-predominant breast cancer (LPBC) and no-LPBC in the anthracycline-taxane subgroup.

However, the results of these studies suggest that clinical outcomes in ER-negative breast cancers, including TNBC in particular, are strongly influenced by tumor

immune responses and are, thus, highly responsive to immunotherapies. The possible use of immunotherapy approaches to treat TNBC (tumor vaccine approaches, immune-checkpoint inhibitors, antagonists of immunosuppressive molecules and adoptive cell therapies) should be investigated in detail ⁴⁸.

The other metagenes studied had no significant prognostic or predictive value. However, they identified sound biological networks providing opportunities for therapeutic intervention. The Immunity1 metagene included genes involved in the interferon α/β signaling pathway or cytokine signaling (STAT1, IRF7, IRF27, OAS1, OAS2, PMSB8, XAF1, IFIT1, IFITM1, ISG15, IGS20, IF6, MX1), the Toll-like receptor signaling pathway (STAT1, CXCL9, CXCL10, CXCL11, CCL5, IRF7), cell-cycle checkpoints and DNA synthesis (PSMB8, PSMB9). Patients displaying strong expression of this metagene often also had high levels of Immunity2 and Proliferation/DNA damage metagene expression, suggesting the possible existence of common pathways. The IDO1 (indoleamine 2,3-dioxygenase 1) gene is a particularly interesting potential target. It encodes a tryptophan-degrading enzyme known to suppress antitumor CD8⁺ T cells and it contributes to the inhibition of anticancer immune responses ⁴⁸. This immunosuppressive enzyme is actually investigated as a promising candidate target in cancer immunotherapy.

A subset of TNBC tumors strongly expresses AR-regulated genes ⁴⁹. AR expression has been reported to be lower in triple-negative breast tumor cells than in other types of breast cancer. The overall frequency of AR expression in carcinoma cells varies considerably between studies (0-53%) ^{50,51}. We identified strong expression of AR pathway genes in 25% of our population. The biological role of androgens in TNBC remains a matter of debate. Immunohistochemical studies investigating the presence of AR in tumor cells have reported conflicting results for

clinical outcome; some studies have suggested that AR expression is advantageous for survival⁵²⁻⁵⁴, whereas others found no significant effect⁵⁵. Lehmann *et al.* found that the LAR subtype of TNBC displayed the lowest frequency of pCR (10%). The presence of AR in a subset of TNBC patients suggests that androgenic pathways in tumor cells could be targeted in at least some TNBC patients. The widespread availability of agents targeting the AR also makes this approach potentially appealing, as it would be straightforward to incorporate such treatment into clinical practice.

The Matrix/Invasion1 metagene included genes associated with β 1 integrin cell surface interactions, ECM-receptor interaction or integrin family cell surface interactions (NID1, TGFBI, COL5A1, COL5A2, COL6A3, COL3A1, COL1A1, COL1A2, COL11A1, FN1, FBN1, THBS1, THBS2), the TGF β signaling pathway (DCN, COMP, THBS1, THBS2), the inhibition of matrix metalloproteases (MMP2, TIMP3), and the AP-1 transcription factor network (DCN, COL1A2, MMP2). Metalloproteinases (MMPs) and their tissue inhibitors are involved in several key pathways of tumor growth, invasion and metastasis^{56,57}. The expression and activity of MMPs has been linked to advanced stages of breast cancer, greater tumor invasion and the construction of metastatic formations^{58,59,60}. Some studies have highlighted the importance of matrix MMP expression by stromal cells a prognostic factor in the TNBC subtype⁶¹. These molecules are thus attractive targets for drug development⁶².

The Matrix2 metagene included genes associated with the AP-1 transcription factor network (FOS, EGR1, FABP4, DUSP1), the EGR receptor signaling pathway (FOS, DUSP1, EGR1), the Wnt or ALK signaling pathway (CAV1), the MAPK signaling

pathway (FOS, DUSP1) or Trk receptor signaling mediated by the MAPK pathway (FOS, EGR1), the mTOR signaling pathway (IGF1), the PPAR signaling pathway (ADIPOQ, CD36, FABP4), and androgen-mediated signaling (FOS, EGR1). These pathways may contribute to cell motility and tumor cell invasion⁶³ and play a prominent role in epithelial-mesenchymal transition (EMT) and in stem cells. These metagenes are strongly expressed in mesenchymal cells and metaplastic breast cancers 4. Metaplastic breast cancers have lineage plasticity, including spindle cell foci, and display osseous or cartilaginous differentiation⁶⁴. Some drugs targeting the pathways relating to the metagenes identified here may be of particular interest for the treatment of TNBC (PI3K/mTOR inhibitor, Wnt/ β catenin inhibitor).

Conclusion

In conclusion, our 167-gene TNBC molecular signature, consisting of six metagenes, appears to be particularly robust for the identification of TNBC subtypes. Furthermore, expression of the Immunity2 metagene was strongly correlated with prognosis, and many biological targets have been identified within the corresponding biological network. These findings open up interesting new possibilities for the development of new therapeutic interventions.

Patients and Methods

Data normalization and quality control

We collected 21 publicly available datasets (described in the supplementary data) containing raw gene expression data from microarray analyses (Affymetrix® GeneChip Human Genome HG-U133A and HG-U133Plus2) of 3247 primary human breast cancer samples. The data were normalized by the robust multichip average

(RMA) procedure from the EMA R package⁶⁵. The datasets were split into training (HGU-133A Affymetrix© arrays, 12 datasets, $n=1,995$) and validation (HGU-133Plus2 Affymetrix© arrays (9 datasets, $n=1,014$) sets. We also collected two large datasets, to validate our classification: The Ignatiadis dataset ($n=996$) and the METABRIC dataset ($n=1992$). Data processing for these two datasets has been described elsewhere^{16,17}.

Determination and preprocessing of triple-negative breast cancer samples

We identified the TNBC samples in each dataset, using a bimodal mixture of two Gaussian distributions for ER and *HER2* gene expression, and the median value for PR expression.

The training, validation and Ignatiadis datasets

Batch effects were eliminated by the median centering of each probe-set across arrays and by a, independent quantile normalization of all arrays for each dataset. We controlled for outliers with the Array Quality Metrics R package.

The METABRIC set

We fitted a linear model (limma R package) to remove the batch effect and probes were filtered according to three criteria: probe quality⁶⁶, GC content and presence in more than 5% of the samples. We centered expression values, using the R function *scale()*.

Gene selection process

Consensus clustering was applied to the training set, to determine the optimal number of robust gene clusters for the most variant genes (standard deviation>0.8). We investigated the enrichment of each gene cluster in particular types of genes. We

then identified known biological networks, for each gene cluster separately, using String© database software version 9.1 (<http://string-db.org/>)⁶⁷.

We then applied a two-step selection process: 1) we selected strong biological networks by retaining only genes for which connection scores of at least 0.7 were obtained with String© database software, 2) within each biological network, we selected groups of genes with for which expression levels were correlated, with a correlation coefficient of at least 0.5.

For each dataset (the training, validation, Ignatiadis and METABRIC sets), we applied a hierarchical clustering procedure to the TNBC gene expression (GE) profiles, using the selected genes to visualize the optimal number of stable TNBC subtypes.

Prognostic analysis

Prognostic analysis was performed on the METABRIC set published by Curtis *et al.*¹⁷.

Expression data were summarized by a metagene for each gene cluster (details in the supplementary material). The clinical and pathological variables available for each dataset are described in the supplementary data. Qualitative variables were compared in χ^2 tests or Fisher's exact tests, as appropriate. Quantitative variables were analyzed in Student's *t*-tests. Survival analyses were performed separately for patients with and without chemotherapy. Survival analyses were performed, with the Kaplan-Meier estimate of the survival function. The endpoint of these analyses was breast cancer-specific survival (BCSS). Survival curves were compared in log rank tests. Hazard ratios were estimated with Cox's proportional hazard model.

Expression of the gene signature in human triple-negative breast cancer cell lines

We downloaded the gene expression profiles of the human cancer cell lines from the Cancer Cell Line Encyclopedia (CCLE)⁶⁸ of Novartis/the Broad Institute and the Cancer Genome Project (CGP)⁶⁹ of the Sanger Institute. We normalized all the cell lines from different tissues together.

All statistical analyses were performed with R software (www.cran.r-project.org). *P*-values < 0.05 were considered statistically significant.

Abbreviations

TNBC: Triple-negative breast cancer

ER: Estrogen receptor

PR: Progesterone receptor

HER2: Human epidermal growth factor receptor 2

BC: Breast cancer

RMA: Robust multichip average

GE: Gene expression

BCSS: Breast cancer-specific survival

CCLE: Cancer Cell Line Encyclopedia

CGP: Cancer Genome Project

CDF: Consensus distribution function

AR: Androgen receptor

M: Mesenchymal

MSL: Mesenchymal stem-Like

IM: Immunomodulatory

LAR: Luminal androgen receptor

pCR: Pathological complete remission

no CT: No chemotherapy

NPI: Nottingham Prognostic Index

BC_CL: Breast cancer cell lines

TNBC_CL: Triple-negative breast cancer cell lines

TILs: Tumor-infiltrating lymphocytes

IHC: Immunohistochemistry

EMT: Epithelial-mesenchymal transition

Acknowledgments

The authors thank Sergio Roman-Roman for the reviewing of the study and the manuscript.

This work was supported by Institut Curie, INCa (the french National Cancer Institute) Grant INCa-DGOS-4654, ANR-10-IDEX-0001-02 PSL, ANR-11-LABX-0043, CIC IGR Curie 1428 and Fondation ARC (association for Research Against Cancer).

References

1. Hu Z, Fan C, Oh DS, et al. The molecular portraits of breast tumors are conserved across microarray platforms. *BMC genomics*. 2006;7:96. doi:10.1186/1471-2164-7-96.
2. Sørlie T, Perou CM, Tibshirani R, et al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc Natl Acad Sci U S A*. 2001;98(19):10869-10874. doi:10.1073/pnas.191367098.
3. Parker JS, Mullins M, Cheang MCU, et al. Supervised risk predictor of breast cancer based on intrinsic subtypes. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*. 2009;27(8):1160-1167. doi:10.1200/JCO.2008.18.1370.
4. Hennessy BT, Gonzalez-Angulo A-M, Stemke-Hale K, et al. Characterization of a naturally occurring breast cancer subset enriched in epithelial-to-mesenchymal transition and stem cell characteristics. *Cancer Res*. 2009;69(10):4116-4124. doi:10.1158/0008-5472.CAN-08-3441.
5. Foulkes WD, Smith IE, Reis-Filho JS. Triple-negative breast cancer. *N Engl J Med*. 2010;363(20):1938-1948. doi:10.1056/NEJMra1001389.
6. Turner NC, Reis-Filho JS. Tackling the diversity of triple-negative breast cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research*. 2013;19(23):6380-6388. doi:10.1158/1078-0432.CCR-13-0915.
7. Von Minckwitz G, Blohmer JU, Costa SD, et al. Response-guided neoadjuvant chemotherapy for breast cancer. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*. 2013;31(29):3623-3630. doi:10.1200/JCO.2012.45.0940.
8. Liedtke C, Mazouni C, Hess KR, et al. Response to neoadjuvant therapy and long-term survival in patients with triple-negative breast cancer. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*. 2008;26(8):1275-1281. doi:10.1200/JCO.2007.14.4147.
9. Cortazar P, Zhang L, Untch M, et al. Pathological complete response and long-term clinical benefit in breast cancer: the CTNeoBC pooled analysis. *Lancet*. 2014;384(9938):164-172. doi:10.1016/S0140-6736(13)62422-8.
10. Lehmann BD, Bauer JA, Chen X, et al. Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies. *J Clin Invest*. 2011;121(7):2750-2767. doi:10.1172/JCI45014.
11. Chen X, Li J, Gray WH, et al. TNBCtype: A Subtyping Tool for Triple-Negative Breast Cancer. *Cancer informatics*. 2012;11:147-156. doi:10.4137/CIN.S9983.

12. Sontrop HMJ, Moerland PD, van den Ham R, Reinders MJT, Verhaegh WFJ. A comprehensive sensitivity analysis of microarray breast cancer classification under feature variability. *BMC bioinformatics*. 2009;10:389. doi:10.1186/1471-2105-10-389.
13. Abraham G, Kowalczyk A, Loi S, Haviv I, Zobel J. Prediction of breast cancer prognosis using gene set statistics provides signature stability and biological context. *BMC bioinformatics*. 2010;11:277. doi:10.1186/1471-2105-11-277.
14. Fröhlich H. Network based consensus gene signatures for biomarker discovery in breast cancer. *PLoS One*. 2011;6(10):e25364. doi:10.1371/journal.pone.0025364.
15. Cun Y, Fröhlich HF. Prognostic gene signatures for patient stratification in breast cancer: accuracy, stability and interpretability of gene selection approaches using prior knowledge on protein-protein interactions. *BMC bioinformatics*. 2012;13:69. doi:10.1186/1471-2105-13-69.
16. Ignatiadis M, Singhal SK, Desmedt C, et al. Gene modules and response to neoadjuvant chemotherapy in breast cancer subtypes: a pooled analysis. *J Clin Oncol*. 2012;30(16):1996-2004. doi:10.1200/JCO.2011.39.5624.
17. Curtis C, Shah SP, Chin S-F, et al. The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups. *Nature*. 2012;486(7403):346-352. doi:10.1038/nature10983.
18. Bianchini G, Qi Y, Alvarez RH, et al. Molecular anatomy of breast cancer stroma and its prognostic value in estrogen receptor-positive and -negative cancers. *Journal of Clinical Oncology*. 2010;28(28):4316-4323. doi:10.1200/JCO.2009.27.2419.
19. Rody A, Karn T, Liedtke C, et al. A clinically relevant gene signature in triple negative and basal-like breast cancer. *Breast cancer research: BCR*. 2011;13(5):R97. doi:10.1186/bcr3035.
20. Sabatier R, Finetti P, Cervera N, et al. A gene expression signature identifies two prognostic subgroups of basal breast cancer. *Breast Cancer Research and Treatment*. 2011;126:407-420. doi:10.1007/s10549-010-0897-9.
21. Teschendorff AE, Miremadi A, Pinder SE, Ellis IO, Caldas C. An immune response gene expression module identifies a good prognosis subtype in estrogen receptor negative breast cancer. *Genome Biol*. 2007;8(8):R157. doi:10.1186/gb-2007-8-8-r157.
22. Desmedt C, Haibe-Kains B, Wirapati P, et al. Biological processes associated with breast cancer clinical outcome depend on the molecular subtypes. *Clin Cancer Res*. 2008;14(16):5158-5165. doi:10.1158/1078-0432.CCR-07-4756.

23. Gu-Trantien C, Loi S, Garaud S, et al. CD4+ follicular helper T cell infiltration predicts breast cancer survival. *The Journal of clinical investigation*. 2013;123(7):1-20. doi:10.1172/JCI67428. Traditionally.
24. Karn T, Pusztai L, Holtrich U, et al. Homogeneous datasets of triple negative breast cancers enable the identification of novel prognostic and predictive signatures. *PloS one*. 2011;6(12):e28403. doi:10.1371/journal.pone.0028403.
25. Burstein MD, Tsimelzon A, Poage GM, et al. Comprehensive Genomic Analysis Identifies Novel Subtypes and Targets of Triple-negative Breast Cancer. *Clinical cancer research: an official journal of the American Association for Cancer Research*. 2014. doi:10.1158/1078-0432.CCR-14-0432.
26. Gatz ML, Lucas JE, Barry WT, et al. A pathway-based classification of human breast cancer. *Proceedings of the National Academy of Sciences of the United States of America*. 2010;107:6994-6999. doi:10.1073/pnas.0912708107.
27. Palmer C, Diehn M, Alizadeh A a, Brown PO. Cell-type specific gene expression profiles of leukocytes in human peripheral blood. *BMC genomics*. 2006;7:115. doi:10.1186/1471-2164-7-115.
28. Isella C, Terrasi A, Bellomo SE, et al. Stromal contribution to the colorectal cancer transcriptome. *Nature Genetics*. 2015;(August 2014):1-11. doi:10.1038/ng.3224.
29. Schalper K a., Velcheti V, Carvajal D, et al. In situ tumor PD-L1 mRNA expression is associated with increased tils and better outcome in breast carcinomas. *Clinical Cancer Research*. 2014;20:2773-2782. doi:10.1158/1078-0432.CCR-13-2702.
30. Sabatier R, Finetti P, Mamessier E, et al. Prognostic and predictive value of PDL1 expression in breast cancer. 6(7).
31. Weigelt B, Mackay A, A'hern R, et al. Breast cancer molecular profiling with single sample predictors: a retrospective analysis. *The Lancet Oncology*. 2010;11(4):339-349. doi:10.1016/S1470-2045(10)70008-5.
32. De Wever O, Mareel M. Role of tissue stroma in cancer cell invasion. *J Pathol*. 2003;200(4):429-447. doi:10.1002/path.1398.
33. Lum DH, Matsen C, Welm AL, Welm BE. Overview of human primary tumorgraft models: comparisons with traditional oncology preclinical models and the clinical relevance and utility of primary tumorgrafts in basic and translational oncology research. *Curr Protoc Pharmacol*. 2012;Chapter 14(801):Unit 14.22. doi:10.1002/0471141755.ph1422s59.
34. Sanavia T, Aioli F, Da San Martino G, Bisognin A, Di Camillo B. Improving biomarker list stability by integration of biological knowledge in the learning process. *BMC Bioinformatics*. 2012;13 Suppl 4(Suppl 4):S22. doi:10.1186/1471-2105-13-S4-S22.

35. Fridman WH, Pagès F, Sautès-Fridman C, Galon J. The immune contexture in human tumours: impact on clinical outcome. *Nat Rev Cancer*. 2012;12(4):298-306. doi:10.1038/hrc3245.
36. Denkert C, Loibl S, Noske A, et al. Tumor-associated lymphocytes as an independent predictor of response to neoadjuvant chemotherapy in breast cancer. *J Clin Oncol*. 2010;28(1):105-113. doi:10.1200/JCO.2009.23.7370.
37. Finak G, Bertos N, Pepin F, et al. Stromal gene expression predicts clinical outcome in breast cancer. *Nat Med*. 2008;14(5):518-527. doi:10.1038/nm1764.
38. DeNardo DG, Brennan DJ, Rexhepaj E, et al. Leukocyte complexity predicts breast cancer survival and functionally regulates response to chemotherapy. *Cancer Discov*. 2011;1(1):54-67. doi:10.1158/2159-8274.CD-10-0028.
39. Schmidt M, Böhm D, von Törne C, et al. The humoral immune system has a key prognostic impact in node-negative breast cancer. *Cancer Res*. 2008;68(13):5405-5413. doi:10.1158/0008-5472.CAN-07-5206.
40. Teschendorff AE, Miremadi A, Pinder SE, Ellis IO, Caldas C. An immune response gene expression module identifies a good prognosis subtype in estrogen receptor negative breast cancer. *Genome biology*. 2007;8(8):R157. doi:10.1186/gb-2007-8-8-r157.
41. Bertucci F, Finetti P, Cervera N, et al. Gene expression profiling shows medullary breast cancer is a subgroup of basal breast cancers. *Cancer Res*. 2006;66(9):4636-4644. doi:10.1158/0008-5472.CAN-06-0031.
42. Von Minckwitz G, Untch M, Blohmer J-U, et al. Definition and impact of pathologic complete response on prognosis after neoadjuvant chemotherapy in various intrinsic breast cancer subtypes. *Journal of clinical oncology: official journal of the American Society of Clinical Oncology*. 2012;30(15):1796-1804. doi:10.1200/JCO.2011.38.8595.
43. Mattarollo SR, Loi S, Duret H, Ma Y, Zitvogel L, Smyth MJ. Pivotal role of innate and adaptive immunity in anthracycline chemotherapy of established tumors. *Cancer Res*. 2011;71(14):4809-4820. doi:10.1158/0008-5472.CAN-11-0753.
44. Denkert C, von Minckwitz G, Brase JC, et al. Tumor-Infiltrating Lymphocytes and Response to Neoadjuvant Chemotherapy With or Without Carboplatin in Human Epidermal Growth Factor Receptor 2-Positive and Triple-Negative Primary Breast Cancers. *Journal of Clinical Oncology*. 2014. doi:10.1200/JCO.2014.58.1967.
45. West NR, Milne K, Truong PT, Macpherson N, Nelson BH, Watson PH. Tumor-infiltrating lymphocytes predict response to anthracycline-based chemotherapy in estrogen receptor-negative breast cancer. *Breast cancer research: BCR*. 2011;13(6):R126. doi:10.1186/bcr3072.

46. Sabatier R, Finetti P, Guille A, et al. Claudin-low breast cancers: clinical, pathological, molecular and prognostic characterization. *Molecular Cancer*. 2014;13:228. doi:10.1186/1476-4598-13-228.
47. Wimberly H, Brown JR, Schalper K, et al. PD-L1 Expression Correlates with Tumor-Infiltrating Lymphocytes and Response to Neoadjuvant Chemotherapy in Breast Cancer. *Cancer Immunology Research*. 2014;3(April):326-333. doi:10.1158/2326-6066.CIR-14-0133.
48. Stagg J, Allard B. Immunotherapeutic approaches in triple-negative breast cancer: latest research and clinical prospects. *Therapeutic advances in medical oncology*. 2013;5(3):169-181. doi:10.1177/1758834012475152.
49. Doane a S, Danso M, Lal P, et al. An estrogen receptor-negative breast cancer subset characterized by a hormonally regulated transcriptional program and response to androgen. *Oncogene*. 2006;25(28):3994-4008. doi:10.1038/sj.onc.1209415.
50. Ogawa Y, Hai E, Matsumoto K, et al. Androgen receptor expression in breast cancer: relationship with clinicopathological factors and biomarkers. *International journal of clinical oncology*. 2008;13(5):431-435. doi:10.1007/s10147-008-0770-6.
51. He J, Peng R, Yuan Z, et al. Prognostic value of androgen receptor expression in operable triple-negative breast cancer: a retrospective analysis based on a tissue microarray. *Med Oncol*. 2012;29(2):406-410. doi:10.1007/s12032-011-9832-0.
52. Luo X, Shi Y-X, Li Z-M, Jiang W-Q. Expression and clinical significance of androgen receptor in triple negative breast cancer. *Chin J Cancer*. 2010;29(6):585-590. <http://www.ncbi.nlm.nih.gov/pubmed/20507730>.
53. Rakha E a, El-Sayed ME, Green AR, Lee AHS, Robertson JF, Ellis IO. Prognostic markers in triple-negative breast cancer. *Cancer*. 2007;109(1):25-32. doi:10.1002/cncr.22381.
54. McNamara KM, Yoda T, Miki Y, et al. Androgenic pathway in triple negative invasive ductal tumors: its correlation with tumor cell proliferation. *Cancer science*. 2013;104(5):639-646. doi:10.1111/cas.12121.
55. Peters A a, Buchanan G, Ricciardelli C, et al. Androgen receptor inhibits estrogen receptor-alpha activity and is prognostic in breast cancer. *Cancer Res*. 2009;69(15):6131-6140. doi:10.1158/0008-5472.CAN-09-0452.
56. Jiang Y, Goldberg ID, Shi YE. Complex roles of tissue inhibitors of metalloproteinases in cancer. 2002;(October 2001):2245-2252. doi:10.1038/sj/onc/1205291.
57. Deryugina EI, Quigley JP. Matrix metalloproteinases and tumor metastasis. *Cancer metastasis reviews*. 2006;25(1):9-34. doi:10.1007/s10555-006-7886-9.

58. Vizoso FJ, González LO, Corte MD, et al. Study of matrix metalloproteinases and their inhibitors in breast cancer. *British journal of cancer*. 2007;96(6):903-911. doi:10.1038/sj.bjc.6603666.
59. McGowan PM, Duffy MJ. Matrix metalloproteinase expression and outcome in patients with breast cancer: analysis of a published database. *Annals of oncology: official journal of the European Society for Medical Oncology / ESMO*. 2008;19(9):1566-1572. doi:10.1093/annonc/mdn180.
60. Figueira RCS, Gomes LR, Neto JS, Silva FC, Silva IDCG, Sogayar MC. Correlation between MMPs and their inhibitors in breast cancer tumor tissue specimens and in cell lines with different metastatic potential. *BMC cancer*. 2009;9:20. doi:10.1186/1471-2407-9-20.
61. González LO, Corte MD, Junquera S, et al. Expression and prognostic significance of metalloproteases and their inhibitors in luminal A and basal-like phenotypes of breast carcinoma. *Human pathology*. 2009;40(9):1224-1233. doi:10.1016/j.humpath.2008.12.022.
62. Fingleton B. Matrix metalloproteinases as valid clinical targets. *Current pharmaceutical design*. 2007;13:333-346. doi:10.2174/138161207779313551.
63. Shin S-Y, Rath O, Zebisch A, Choo S-M, Kolch W, Cho K-H. Functional roles of multiple feedback loops in extracellular signal-regulated kinase and Wnt signaling pathways that regulate epithelial-mesenchymal transition. *Cancer Res*. 2010;70(17):6715-6724. doi:10.1158/0008-5472.CAN-10-1377.
64. Gibson GR, Qian D, Ku JK, Lai LL. Metaplastic breast cancer: clinical features and outcomes. *Am Surg*. 2005;71:725-730.
65. Servant N, Gravier E, Gestraud P, et al. EMA - A R package for Easy Microarray data analysis. *BMC Res Notes*. 2010;3(1):277. doi:10.1186/1756-0500-3-277.
66. Barbosa-Morais NL, Dunning MJ, Samarajiwa S a, et al. A re-annotation pipeline for Illumina BeadArrays: improving the interpretation of gene expression data. *Nucleic Acids Res*. 2010;38(3):e17. doi:10.1093/nar/gkp942.
67. Jensen LJ, Kuhn M, Stark M, et al. STRING 8--a global view on proteins and their functional interactions in 630 organisms. *Nucleic acids research*. 2009;37(Database issue):D412-D416. doi:10.1093/nar/gkn760.
68. Barretina J, Caponigro G, Stransky N, et al. NIH Public Access of anticancer drug sensitivity. 2012;483(7391):603-607. doi:10.1038/nature11003.The.
69. Garnett MJ, Edelman EJ, Heidorn SJ, et al. Europe PMC Funders Group Systematic identification of genomic markers of drug sensitivity in cancer cells. *Nature*. 2012;483(7391):570-575. doi:10.1038/nature11005.Systematic.

Table 1A. Survival analysis (disease-specific survival). Chemotherapy-treated population. Univariate and multivariate analysis.

139 triple-negative breast cancer patients		Univariate analysis		Multivariate analysis			
		DS-survival HR [95% CI]	p value	DS-survival HR [95% CI]	p value		
Menopausal status	Pre	1					
	Post	1.56 [0.95-2.55]	0.08				
Tumor size (mm)	<20 mm	1					
	>20 mm	1.03 [0.58-1.82]	0.92				
Tumor grade	II	1					
	III	1.23 [0.45-3.39]	0.69				
Lymph node status	0	1					
	1	0.84 [0.42-1.65]	0.61				
NPI score	<5.4	1				1	
	>5.4	2.15 [1.28-3.60]	0.003			2.30 [1.36-3.89]	0.002
Cellularity	Low	1					
	Moderate	0.57 [0.22-1.46]	0.24				
	High	0.59 [0.25-1.39]	0.23				
P53 status	Wild-type	1					
	Mutant	2.42 [1.15-5.09]	0.02				
Immunity1 metagene expression	High	1					
	Low	0.97 [0.60-1.58]	0.91				
Immunity2 metagene expression	High	1				1	/
	Low	2.59 [1.54-4.34]	0.0002			2.68 [1.59-4.52]	0.0002
Proliferation/DNA damage metagene expression	High	1					
	Low	1.13 [0.69-1.84]	0.63				
AR-like metagene expression	High	1					
	Moderate	1.07 [0.59-1.94]	0.82				
	Low	0.98 [0.50-1.94]	0.96				
Matrix/Invasion1 metagene expression	High	1					
	Low	1.23 [0.76-2.01]	0.40				
Matrix2 metagene expression	High	1					
	Low	0.99 [0.61-1.61]	0.96				

Abbreviations: NPI, Nottingham Prognostic Index; AR, androgen receptor; HR, hazard ratio; CI, confidence interval.

Table 1B. Survival analysis (disease-specific survival). Chemotherapy-treated population. Two univariate models. Combination of NPI score and Immunity2 metagene expression.

139 triple-negative breast cancer patients		DS-survival HR [95% CI]	p value
NPI score/Immunity2 metagene expression	NPI1-2/HighI2	1	
	NPI1-2/LowI2	2.90 [1.51-5.56]	0.001
	NPI3	3.98 [2.00-7.92]	8.72 ^{10⁻⁵}

139 triple-negative breast cancer patients		DS-survival HR [95% CI]	p value
NPI score/Immunity2 metagene expression	NPI1-2/HighI2	1	
	NPI1-2/LowI2	2.91 [1.51-5.59]	0.001
	NPI3/HighI2	2.31 [0.96-5.57]	0.06
	NPI3/LowI2	6.30 [2.89-13.78]	3.87 ^{10⁻⁶}

Abbreviations: NPI, Nottingham Prognostic Index; I2, Immunity2; HR, hazard ratio; CI, confidence interval.

$$\text{NPI} = [0.2 \times \text{S}] + \text{N} + \text{G}$$

S: tumor size (cm)

N: number of lymph nodes involved (0=1, 1-3=2, >3=3)

G: tumor grade according to Elston and Ellis (Grade I=1, Grade II=2, Grade III=3)

Table 2A. Survival analysis (disease-specific survival). Chemotherapy-naive population. Univariate and multivariate analysis.

115 triple-negative breast cancer patients		Univariate analysis		Multivariate analysis			
		DS-survival HR [95% CI]	p value	DS-survival HR [95% CI]	p value		
Menopausal status	Pre	1					
	Post	1.31 [0.56-3.06]	0.53				
Tumor size (mm)	<20 mm	1					
	>20 mm	2.36 [1.01-5.48]	0.04				
Tumor grade	I-II	1					
	III	1.33 [0.51-3.49]	0.56				
Lymph node status	0	1					
	1	3.66 [1.65-8.11]	0.001				
NPI score	<3.4	1				1	
	3.4-5.4	1.36 [0.47-3.96]	0.57			1.55 [0.53-4.51]	0.43
	>5.4	10.69 [2.74-41.76]	0.001			12.03 [3.05-47.50]	0.0004
Cellularity	Low	1					
	Moderate	1.91 [0.54-6.71]	0.31				
	High	1.42 [0.41-4.90]	0.58				
P53 status	Wild-type	1					
	Mutant	0.90 [0.17-4.63]	0.90				
Immunity1 metagene expression	High	1					
	Low	1.56 [0.76-3.19]	0.22				
Immunity2 metagene expression	High	1		1			
	Low	2.33 [1.09-4.95]	0.03	2.42 [1.13-5.16]	0.02		
Proliferation/DNA damage metagene expression	High	1					
	Low	1.14 [0.56-2.32]	0.72				
AR Like metagene expression	High	1					
	Moderate	0.96 [0.42-2.20]	0.92				
	Low	0.74 [0.28-2.00]	0.56				
Matrix/Invasion1 metagene expression	High	1					
	Low	0.48 [0.23-1.01]	0.06				
Matrix2 metagene expression	High	1					
	Low	1.31 [0.64-2.66]	0.46				

Abbreviations: NPI, Nottingham Prognostic Index; AR, androgen receptor; HR, hazard ratio; CI, confidence interval.

Table 2B. Survival analysis (disease-specific survival). Chemotherapy-naive population. Univariate analysis. Combination of NPI score and Immunity2 metagene expression.

115 triple-negative breast cancer patients		DS-survival hazard Ratio [95% CI]	p value
NPI score/Immunity2 metagene expression	NPI1-2/HighI2	1	
	NPI1-2/LowI2	2.13 [0.95-4.78]	0.07
	NPI3	12.89 [4.07-40.82]	1.37 x 10⁻⁵

Abbreviations: NPI, Nottingham Prognostic Index; I2, Immunity2; HR, hazard ratio; CI, confidence interval.

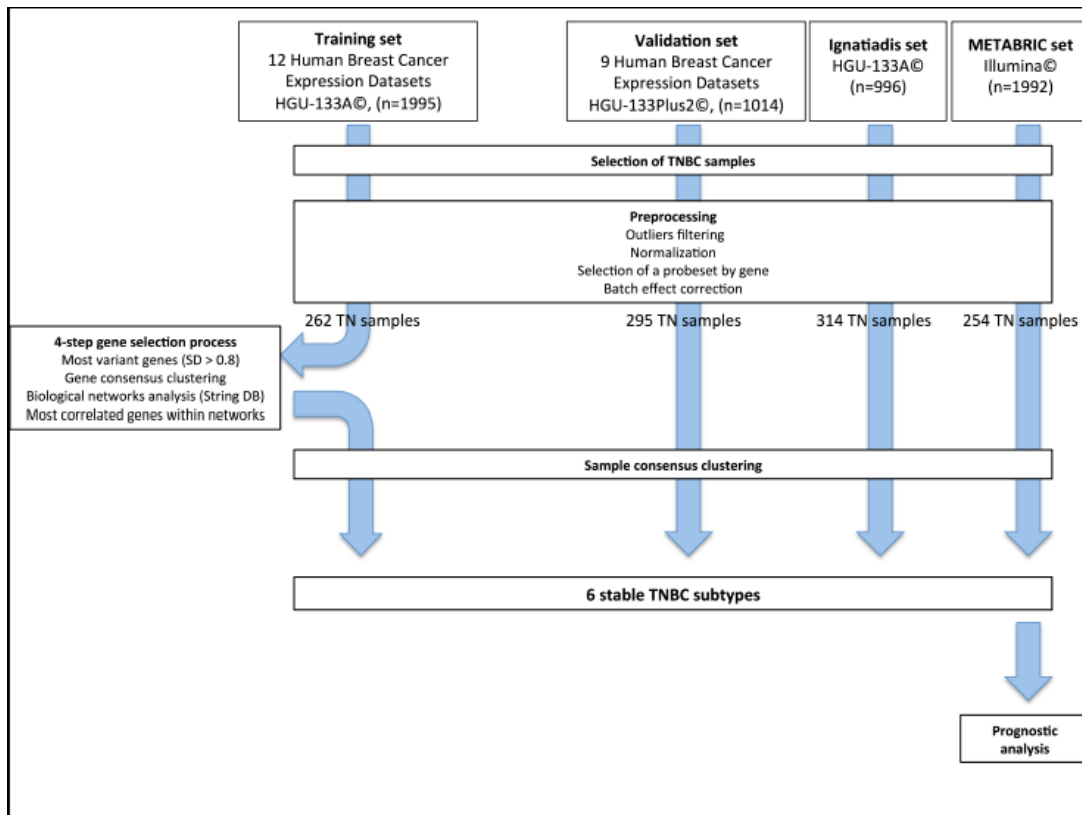


Figure 1

Methodology flow chart

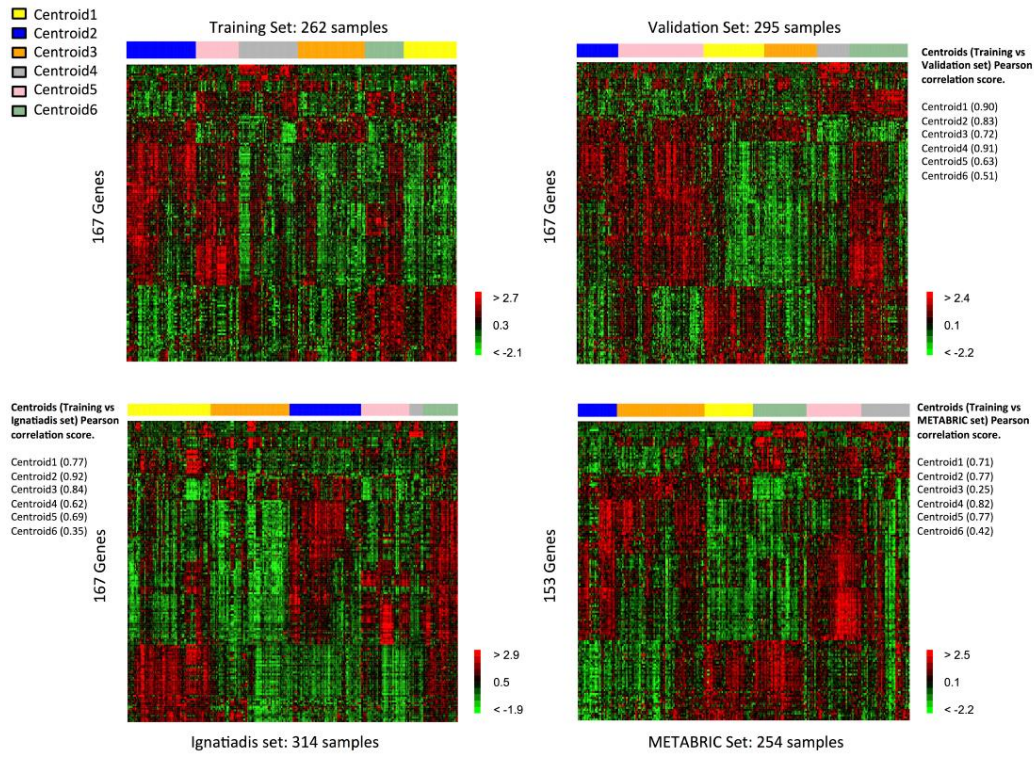


Figure 2

Heatmaps of the selected genes in the TNBC training set (upper left) and the TNBC validation sets (upper right: validation, lower left: Ignatiadis, lower right: METABRIC).

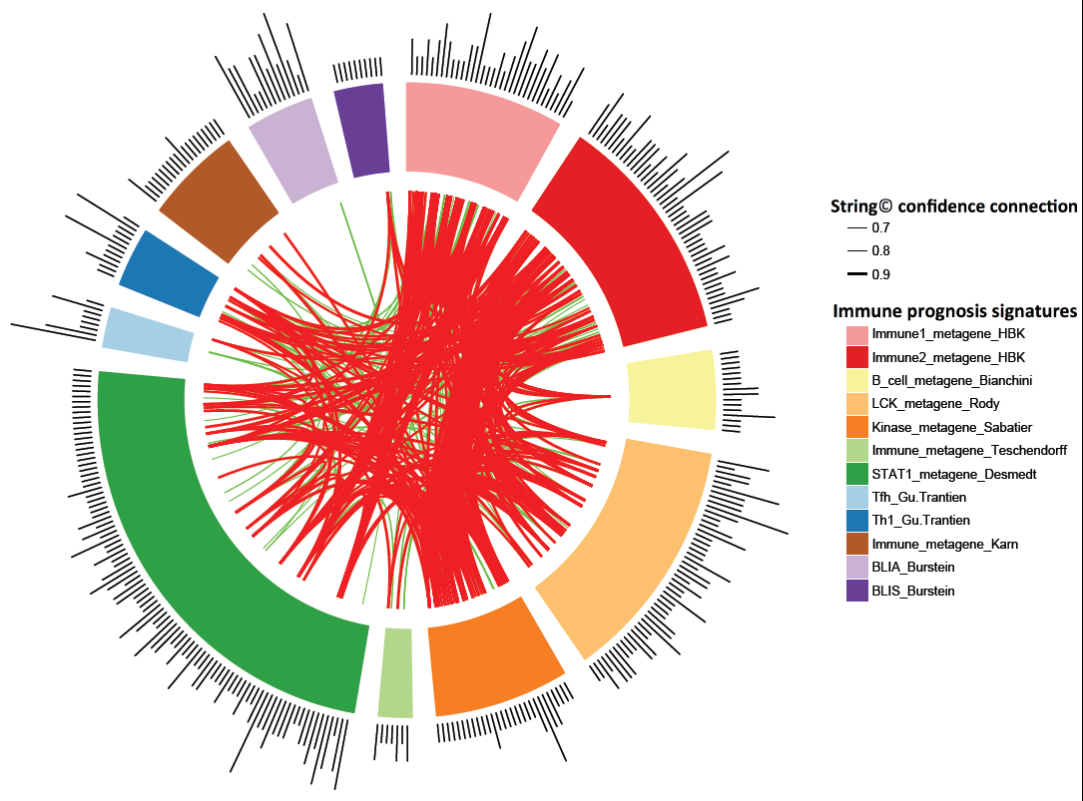
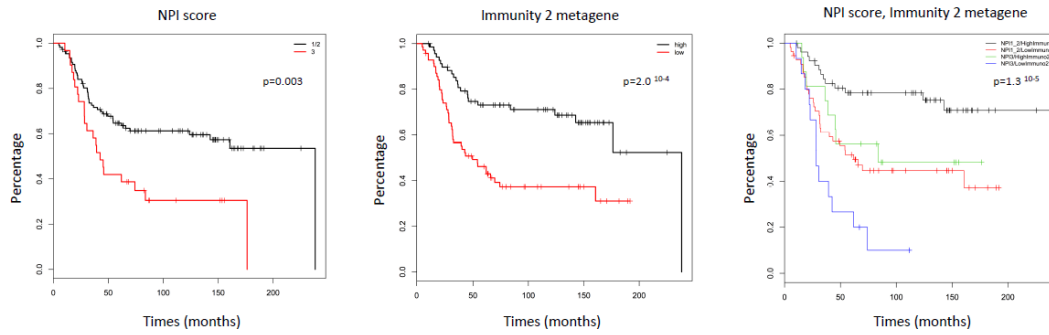
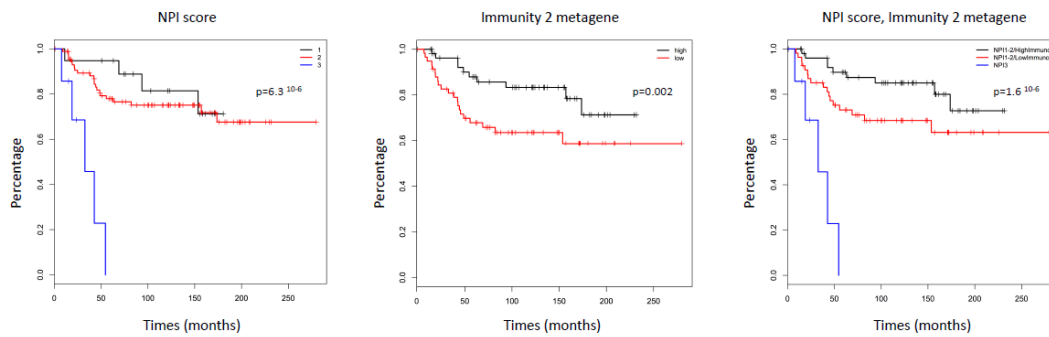


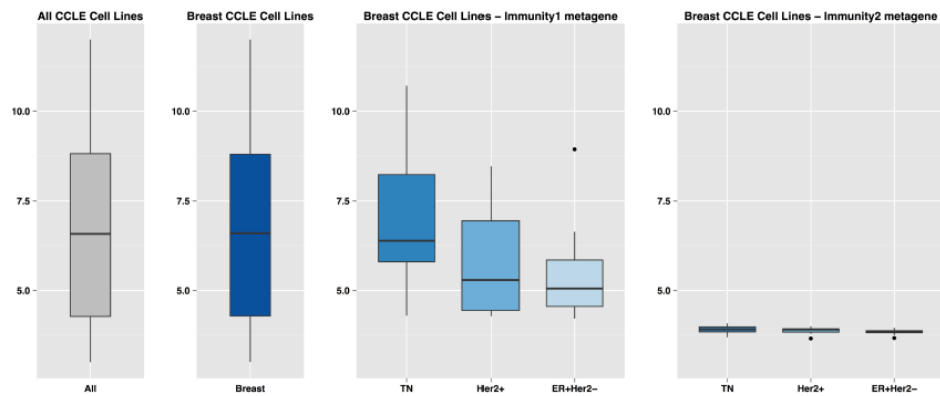
Figure 3

String Software connections between our Immunity1 and Immunity 2 genes and the genes of eight previously published prognostic immune signatures. Stronger associations between genes are represented by thicker lines. Associations between genes with a coefficient < 0.9 are shown in green. Associations between genes with a coefficient ≥ 0.9 are shown in red. Associations between genes with a coefficient between 0.4 to 0.7 are not shown.

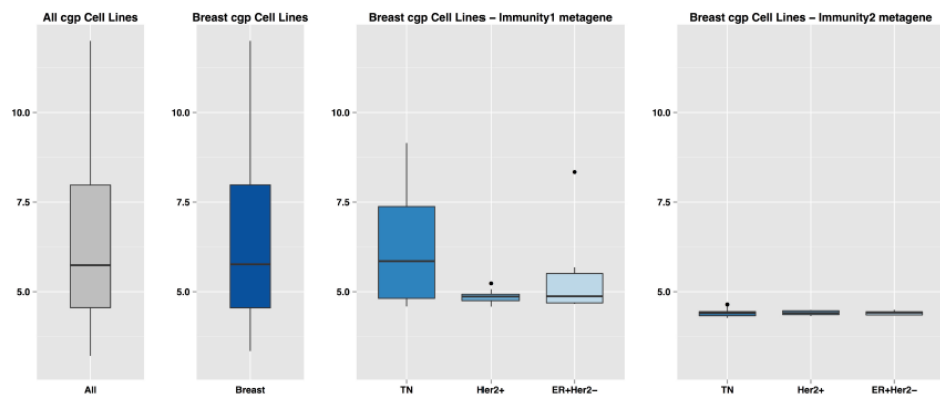
A TNBC chemotherapy-treated population: Disease-specific survival (n=139)**B** TNBC chemotherapy-naïve population: Disease-specific survival (n=115)**Figure 4**

- (A) Kaplan-Meier plots. Disease-specific survival of the chemotherapy-treated population (n=139). NPI score. Immunity2 metagene. NPI score/Immunity2 metagene.
- (B) Kaplan-Meier plots. Disease-specific survival of the noCT population (n=115). NPI score. Immunity2 metagene. NPI score/Immunity2 metagene.

Cancer Cell Line encyclopedia (CCLE)- Cell Lines



Cancer Genome Project (CGP)- Cell Lines

**Figure 5**

- (A) Boxplots of gene expression for the Immunity 1 and Immunity 2 metagenes, in each breast cancer cell line subtype from the CCLE.
- (B) Boxplots of gene expression for the Immunity 1 and Immunity 2 metagenes in each breast cancer cell line subtype from the CGP.

We collected eight publicly available datasets from the following studies: EORTC10994, I-SPY-1, LBJ/INEN/GEICAM, MDACC trial, TOP, MAQCII/MDACC, MAQCIII, USO-02103, all of which used Affymetrix GeneChip Human Genome HG-U133A arrays. This series was described by Ignatiadis *et al.*² for an analysis of the responses of various TNBC molecular subtypes to neoadjuvant chemotherapy (anthracycline, with or without taxane; $n=996$). Raw GE values for each dataset were normalized independently, for identification of the TNBC samples in each dataset.

The METABRIC set

We collected the METABRIC -Molecular Taxonomy of Breast Cancer International Consortium- dataset published by Curtis *et al.*³, which was established for analysis of the prognosis of the various molecular subtypes of TNBC. We normalized the 1992 samples together, using scripts and Rdata provided by the authors.

Determination of triple-negative status

The Affymetrix probes 205225_at, 208305_at and 216836_s_at were chosen, to provide information about ER, PR and *HER2* expression, respectively⁴. For the Metabric dataset, after a quality control test, the Illumina probes ILMN_1678535, ILMN_1811014, and ILMN_2352131 were chosen to provide information about ER, PR and *HER2* expression, respectively. A density plot of ER, PR and *HER2* gene expression in each dataset showed a bimodal distribution for ER and *HER2*, but not for PR (Supplemental Figure S24). We identified the TNBC samples in each dataset as follows: the distribution of ER and *HER2* expression was analyzed empirically, with a two-component Gaussian mixture model, and parameters for bimodal filtering were

estimated with the R Mclust package. The median value was used as the cutoff for PR expression in each dataset.

Preprocessing of triple-negative samples

Affymetrix©_platform

For each dataset (training, validation and Ignatiadis), we used the R arrayQualityMetrics package on an Affybatch object to filter out outliers. We excluded samples detected as outliers by at least two of the following methods: distances between arrays, boxplots, relative log expression (RLE), normalized unscaled standard error (NUSE), MA plots, spatial distribution of M. Raw GE values for the TNBC samples in each dataset (training, validation and Ignatiadis) were normalized independently. The optimal microarray probe set to represent a gene was selected with the R JetSet package. This package developed scoring methods for the assessment of each probe set for specificity, coverage, and degradation resistance. For each dataset, batch effects were removed by the median centering of each probe set across arrays and the quantile normalization of all arrays separately for each set.

Illumina©_platform

We used the Illumina© probe quality score introduced by Barbosa-Morais in 2010 to ensure that all the probes used were of high quality (deleting probes scored as “bad” or “no match”). Probes were filtered on the basis of three criteria: probe quality⁵, GC content between 38% and 64% and presence in more than 5% of the samples ($n = 20,009$ probes). We eliminated any batch effects associated with sample collection sites, by fitting a linear model (R limma package). Three samples were

excluded because of missing data. We then chose the most variant probes as the optimal probe set. We centered expression values, using the R function scale.

1) Establishment of the TNBC classifier

Gene selection process

Consensus clustering was applied to the training set, to determine the optimal number of robust gene clusters from the most variant genes (standard deviation > 0.8) (ConsensusClusterPlus R package). Cluster robustness was assessed by hierarchical clustering (1,000 iterations) with a ward inner, final linkage and Pearson distance. The optimal number of clusters was determined from the cumulative distribution function (CDF), which plots the corresponding empirical cumulative distribution, defined over the range [0,1], and by calculating the proportional increase in the area under the CDF curve. The number of clusters was set as that at which an increase in cluster number (k) did not lead to a corresponding marked increase in CDF area. We calculated Pearson's correlation coefficient for the relationships between genes within the same cluster, to assess the heterogeneity of each gene cluster.

Each gene cluster was tested for gene enrichment (biological process (BP), molecular function (MF), cellular component (CC)) by a conditional test for overrepresentation, in the R runHyperGO package.

We then used the String© database (<http://string-db.org/help/index.jsp?topic=/org.string-db.docs/ch04.html>)⁶ to identify biological gene networks. String© is a database of known and predicted protein interactions. The interactions include physical and functional associations derived from four sources: genomic context, high-throughput experiments, conserved coexpression, previous knowledge. For each gene cluster, we excluded genes that were not connected to any

of the other genes present in the cluster. We then applied a two-step selection process: 1) we selected strong biological networks, by retaining genes with connection scores of at least 0.7 to each other, according to the String database 2) within each biological network, we then selected groups of genes with correlated patterns of expression, with correlation coefficients of at least 0.5. For this step, we used Cytoscape (<http://cytoscapeweb.cytoscape.org>), an open-source software platform for visualizing complex networks and integrating them with any type of attribute data. Attribute data, like correlation, variance and interquartile range, were calculated from the expression data matrix.

After selection, we checked that the various genes selected from the same cluster clustered together again (R package ConsensusClusterPlus). We also assessed gene enrichment in each of the gene clusters.

Metagene identification

For each dataset (training, validation, Ignatiadis and METABRIC), each gene cluster was used to define a metagene (for the Affymetrix© platform: from the 167 selected genes; for the METABRIC set, we used the 153 genes common to the preprocessed METABRIC expression matrix and the selected genes matrix from the Affymetrix© platform). Metagene expression was assessed by calculating the median value for the normalized expression values of all probesets in the respective gene clusters for each sample. For each dataset, we calculated the correlation between expression levels for the various metagenes, using the R psych package. We generated a scatter plot of the metagene matrices, with bivariate scatter plots, histograms, and Pearson's correlation coefficient. The metagene value for each

sample was then discretized as “high” or “low” expression or as “low”, “moderate” and “high” expression, depending on the distribution of expression values for the metagene concerned.

Classification of TNBC samples

In each dataset, hierarchical clustering was applied to the TNBC GE profiles, using the selected genes to visualize the optimal number of stable TNBC subtypes (ConsensusClusterPlus R package). We identified six stable TNBC subtypes, using the same decision rules as described in Materials and Methods (gene selection process section). We checked the concordance between each of the validation sets and the training set (Pearson’s correlation coefficient for the relationship between centroids). Centroid expression values were determined by calculating the mean normalized expression values of all samples in the sample cluster, for each probe set.

For each TNBC sample, we compared our classification with those of Lehmann *et al.* and Curtis *et al.* (χ^2 test *p* value).

2) Analysis of prognosis

We assessed the prognostic classification, by collecting the independent dataset described above (METABRIC set), which contained data for gene expression and clinical variables. We selected TNBC samples and the data were preprocessed as described above.

The following clinical and pathological variables were available: age, menopausal status, histological type, tumor size, tumor grade according to the Elston and Ellis grading system, number of lymph nodes involved, number of lymph nodes

removed, Nottingham Prognostic Index score, tumor cellularity, *p53* mutation status, treatment type, last follow-up status and the time at which last-follow-up occurred.

We performed descriptive statistics and survival analyses separately for the samples of patients with and without chemotherapy, as follows.

1) We assessed the expression of the metagenes in each population. Given their unimodal distribution, the expression of each metagene was classified as “low” or “high”, based on the median value for five metagenes (Immunity1, Immunity2, Proliferation/DNA damage, Matrix/Invasion1 and Matrix2). For the AR-like metagene, we used three classes (low expression: up to and including the first quartile, moderate expression: between the first and third quartiles, high expression: third quartile or higher).

2) We performed a descriptive analysis of these two populations, as described above.

3) Survival analyses were performed by calculating Kaplan-Meier estimates of the survival function. The endpoint of these analyses was breast cancer-specific survival (BCSS) (death from breast cancer). Time-censoring analyses were performed with a right censoring of events from 1 to 20 years. Log rank tests were used to compare survival curves. Hazard ratios were estimated with Cox's proportional hazards model. Only variables significant in the univariate analysis were included in the multivariate model. However, some variables (size, grade and lymph node status) were systematically excluded due to redundancy with NPI score and *p53* status the large proportion of missing data.

3) Expression of our gene signature in human triple-negative breast cancer cell lines

We assessed the expression of our signature in “*in vitro*” models, as a means of validating our classification and its prognostic value.

We used the gene expression profiles of the human cancer cell lines from the Cancer Cell Line Encyclopedia (CCLE)⁷ of Novartis/the Broad Institute and from the Cancer Genome Project (CGP)⁸ of the Sanger Institute. All cell lines from different tissues were normalized together. The global gene expression signal is shown for all cancer cell lines and breast cancer cell lines from the CCLE (1036 and 58, respectively) and CGP (727 and 39, respectively). Box plots were generated for the expression of the Immunity 1 and Immunity 2 metagenes in the CCLE and CGP, according to breast cancer subtype (CCLE: 31 TN, 15 Her2⁺ and 13 ER⁺Her2⁻ cell lines; CGP: 23 TN, 8 Her2⁺ and 8 ER⁺Her2⁻ cell lines).

1. Servant N, Gravier E, Gestraud P, et al. EMA - A R package for Easy Microarray data analysis. *BMC Res Notes*. 2010;3(1):277. doi:10.1186/1756-0500-3-277.
2. Ignatiadis M, Singhal SK, Desmedt C, et al. Gene modules and response to neoadjuvant chemotherapy in breast cancer subtypes: a pooled analysis. *J Clin Oncol*. 2012;30(16):1996-2004. doi:10.1200/JCO.2011.39.5624.
3. Curtis C, Shah SP, Chin S-F, et al. The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups. *Nature*. 2012;486(7403):346-352. doi:10.1038/nature10983.
4. Karn T, Metzler D, Ruckhaberle E, et al. Data-driven derivation of cutoffs from a pool of 3,030 Affymetrix arrays to stratify distinct clinical types of breast cancer. *Breast Cancer Research and Treatment*. 2010;120(3):567-579. doi:10.1007/s10549-009-0416-z.
5. Barbosa-Morais NL, Dunning MJ, Samarajiwa S a, et al. A re-annotation pipeline for Illumina BeadArrays: improving the interpretation of gene expression data. *Nucleic Acids Res*. 2010;38(3):e17. doi:10.1093/nar/gkp942.

6. Jensen LJ, Kuhn M, Stark M, et al. STRING 8--a global view on proteins and their functional interactions in 630 organisms. *Nucleic Acids Research*. 2009;37(Database issue):D412-D416. doi:10.1093/nar/gkn760.
7. Barretina J, Caponigro G, Stransky N, et al. NIH Public Access of anticancer drug sensitivity. 2012;483(7391):603-607. doi:10.1038/nature11003.
8. Garnett MJ, Edelman EJ, Heidorn SJ, et al. Europe PMC Funders Group Systematic identification of genomic markers of drug sensitivity in cancer cells. *Nature*. 2012;483(7391):570-575. doi:10.1038/nature11005.

Our signature					
Immunity1	Immunity2	Proliferation, AR_Like		Matrix/Invas	Matrix2
IFITM1	CYTIP	CKS2	PIP	COL6A3	CD36
PSMB8	CXCL13	NDC80	DDC	LOX	EGR1
XAF1	LY96	TYMS	FOXA1	ASPN	CAV1
ISG15	SELL	MAD2L1	AGR2	THBS1	COL14A1
IFIH1	ITGB2	RAD51AP1	XBP1	DCN	SFRP4
PSMB9	CCL8	FOXO1	AR	FN1	IGF1
IDO1	BLNK	CDC20	SCGB2A1	MMP11	CAV2
DDX58	GZMK	TRIP13	SCGB1D2	MYLK	FOS
ISG20	GIMAP6	EZH2	SCGB2A2	ACTA2	FABP4
OAS2	TYROBP	TOP2A	HIST1H1C	FBN1	DUSP1
WARS	CD3D	DLGAP5	HIST1H2BK	COL1A1	ADIPOQ
BST2	GZMA	BCL2	HIST1H4H	IGFBP3	CFD
MX1	IL7R	MYC	HIST1H2AE	SPOCK1	CXCL12
CXCL9	CCL19	SFRP1	HIST1H2BD	ACTG2	CILP
APOBEC3G	IGJ	TP53	HIST1H2BG	COL1A2	
IRF7	CCL2			MMP3	
GBP1	FGL2			COL10A1	
HERC5	PTPRC			FAP	
IFI44	HLA-DRA			TGFBI	
RSAD2	RAC2			CTGF	
CCL5	CD2			COMP	
STAT1	NKG7			FSTL1	
HERC6	LCK			COL11A1	
IFI44L	EVI2A			NID1	
CXCL10	CASP1			TPM2	
DDX60	HLA-DMA			NNMT	
CXCL11	CSF2RB			MFAP5	
OAS1	TNFRSF17			MYL9	
TRIM22	EVI2B			LUM	
IFI27	POU2AF1			COL5A2	
RTP4	HLA-DPA1			CDH11	
IFI6	MS4A1			THBS2	
IFIT1	CD74			TAGLN	
	C1QA			MMP2	
	MNDA			BGN	
	CCR7			SPARC	
	C1QB			COL5A1	
	CD163			COL3A1	
	GPR18			TIMP3	
	HLA-DPB1			CTSK	
	GNLY			POSTN	
	NCF2			COL15A1	
	HCLS1			IGFBP5	
	CD69				
	HCK				
	GZMB				
	CD14				

Figure S1

Identification of gene clusters.

- (A) The consensus distribution function (CDF), showing the cumulative distribution from consensus matrices at a given cluster number (k).
- (B) The optimal cluster number is 4, corresponding to the point in which the relative change in area (Δ) under the CDF plot does not change with increasing k .
- (C) Consensus clustering for four gene clusters (1,000 iterations, ward inner and final linkage, Pearson distance).
- (D) Tracking plot showing the consensus clusters of genes (in columns) for each k (in rows).

Figure S2

Gene ontology analysis for the four main gene clusters identified.

Figure S3

- (A) Heatmap showing the 830 most variant genes in the 262 TNBC samples (training set).
- (B) Distribution of 2 by 2 gene expression correlation (Pearson score) for each of the four main gene clusters.

Figure S4

String database software confidence view of the Immunity genes cluster. Stronger associations between genes are represented by thicker lines (medium confidence score=0.4).

Figure S5

Cytoscape View for the Immunity genes cluster. GE correlations between genes are indicated by colored lines, from green to red. GE correlation > 0.5 (third quartile of the distribution of correlations), shown in red. GE variability is indicated by points, the size and color (from green to red) of which are proportional to the variability.

Figure S6

- (A) Heatmap showing the relative expression of 167 selected genes in 262 TNBC samples from the training set.
- (B) Table of Pearson correlation coefficient values for the correlations between metagenes.

Figure S7

Heatmaps generated after consensus gene clustering before and after the gene selection process and the consensus distribution function (CDF) curve associated.

Figure S8

Cytoscape view of a “Prototype Immunity2 TNBC Sample”. Gene expression levels are represented by a circle, the size and color (from green to red) of which are proportional to the expression level.

Figure S9

Comparison between our classification (HBK classification) and those of Lehmann and Curtis for each TNBC sample from the METABRIC set.

Figure S10

- (A) Comparison between our TNBC classification and Lehmann’s classification, for the METABRIC set. Barplots representing the percentage of samples assigned to each of Lehmann’s subtypes, for each of our subtypes.
- (B) Comparison between Lehmann’s TNBC classification and our classification, for the METABRIC set. Barplots representing the percentage of samples assigned to each of our subtypes, for each of Lehmann’s subtypes.

Figure S11

Heatmap of the gene expression profiles of the eight immune prognostic signatures previously published, applied to the METABRIC dataset. The samples were ordered according to our classification of Low/High ‘Immunity2’ metagene expression.

Figure S12

Kaplan-Meier plots. Disease-specific survival of the METABRIC population ($n=254$), according to each of the eight previously published immune prognostic signatures.

Figure S13

Distribution histograms for the eight previously published immune prognostic metagenes and for the Immunity 2 metagene. Pearson correlation coefficient values and pairwise scatter plots.

Figure S14

Distribution histograms for the Immune metagenes and the immune pathway metagenes published by Gatza *et al.* and Palmer *et al.*. Pearson correlation coefficient values and pairwise scatter plots.

Figure S15

Boxplots of gene expression for the Immune metagenes and immune pathway metagenes (published by Gatza *et al.* and Palmer *et al.*) in all cancer cell lines from the CCLE and the CGP.

Figure S16

Boxplots of gene expression for the Immune metagenes and immune pathway metagenes (published by Gatza *et al.* and Palmer *et al.*) in each subtype of breast cancer cell lines from the CCLE and the CGP.

Figure S17

Heatmap generated after consensus clustering of the Immunity 2 genes in the white blood cell populations from Palmer *et al.*

Figure S18

Boxplots of the stromal contribution to global gene expression evaluated with PDX RNAseq data (Isella *et al.*), for each of the gene clusters for our signature.

Figure S19

Representation of the various genes available (with or without US Food and Drug Administration approval) for the targeting of each of the metagenes in our signature. First line: point size is proportional to the number of genes in the metagene divided by the total number of genes in the signature; Second line: point size is proportional to the number of drugs in the metagene divided by the total number of drugs; Third line: point size is proportional to the number of genes in the metagene divided by the number of drugs targeting the metagene.

Figure 20

Distribution histograms for the Immunity 2 metagene, TILs metagene and the PD1, PDL1, CTLA4 genes and their metagenes, Pearson correlation coefficient values and pairwise scatter plots.

Figure S21

Boxplots of gene expression for Immune metagenes, the TILs metagene and the PD1, PDL1, CTLA4 genes and their metagenes, in all cancer cell lines from the CCLE and the CGP.

Figure S22

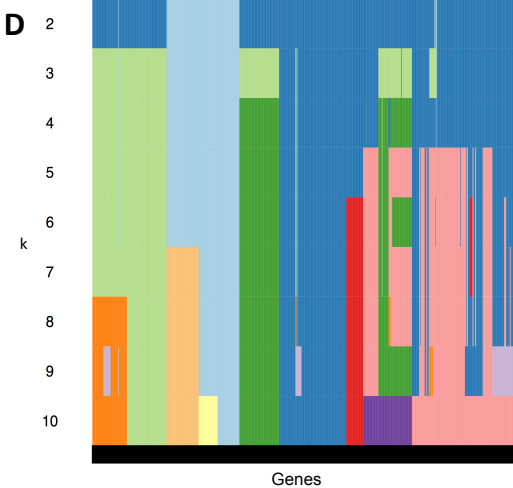
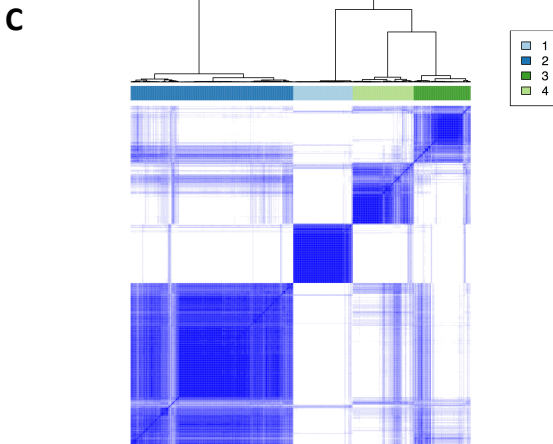
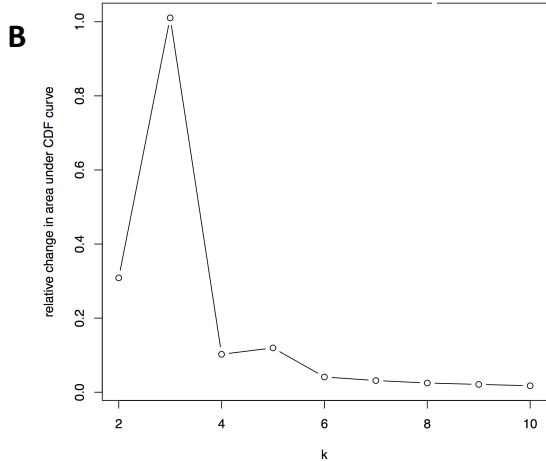
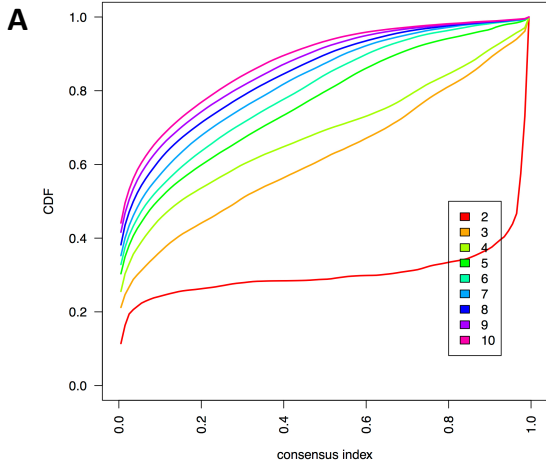
Kaplan-Meier plots. Disease-specific survival of the METABRIC population ($n=254$) according to expression of the PD1, PDL1, CTLA4, TILs metagenes.

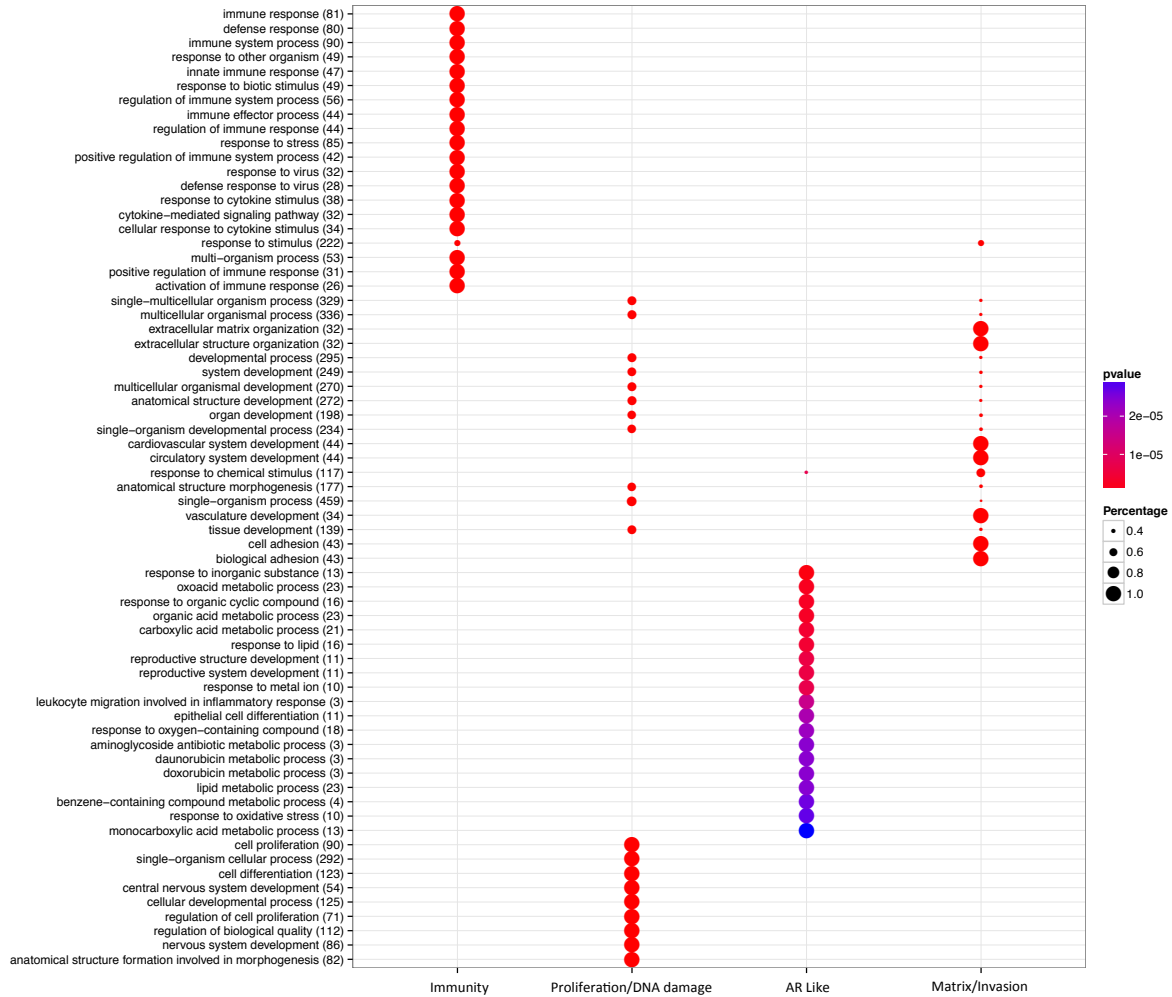
Figure S23

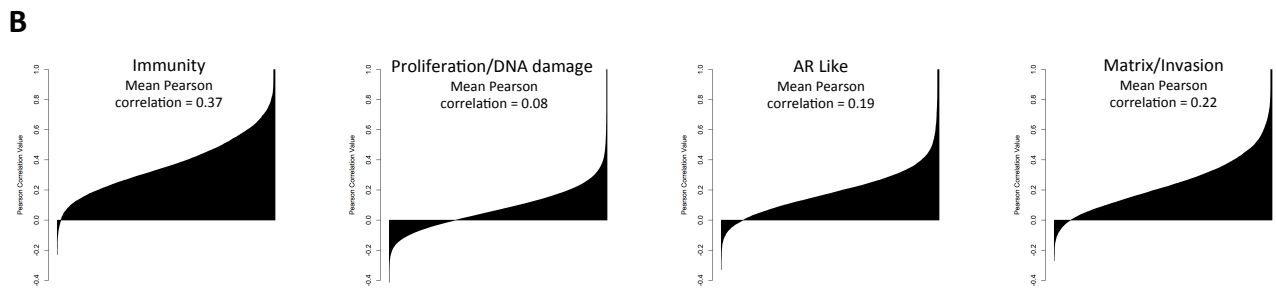
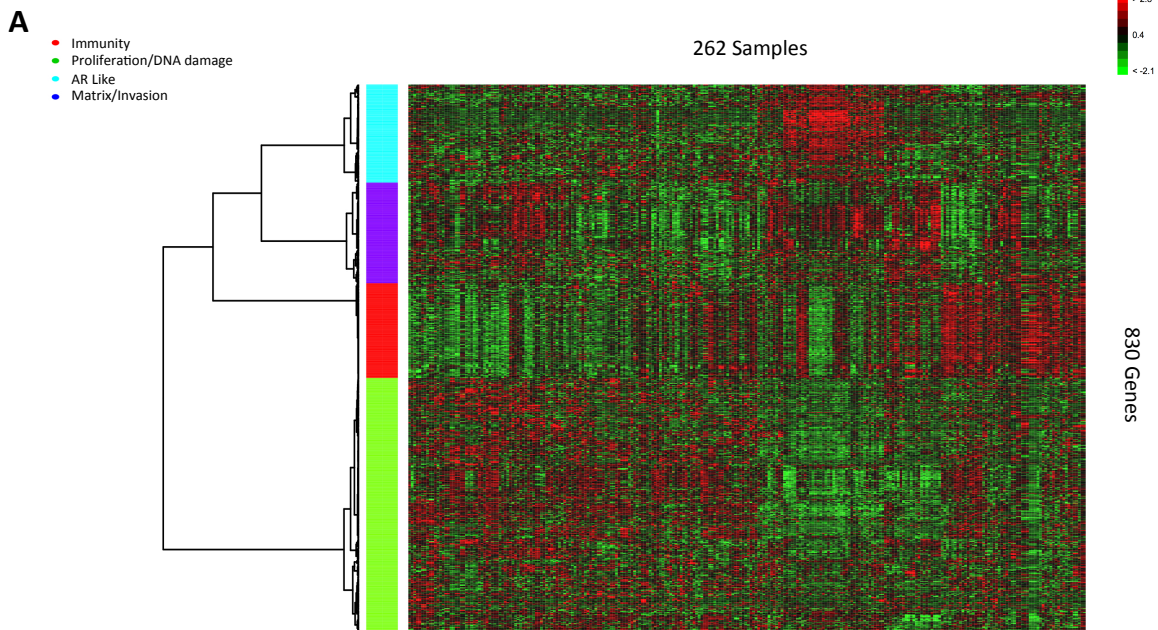
Boxplots of gene expression for each gene cluster of our signature in tumor samples, fine needle aspiration samples and core biopsy samples, as assessed on the Affymetrix platform.

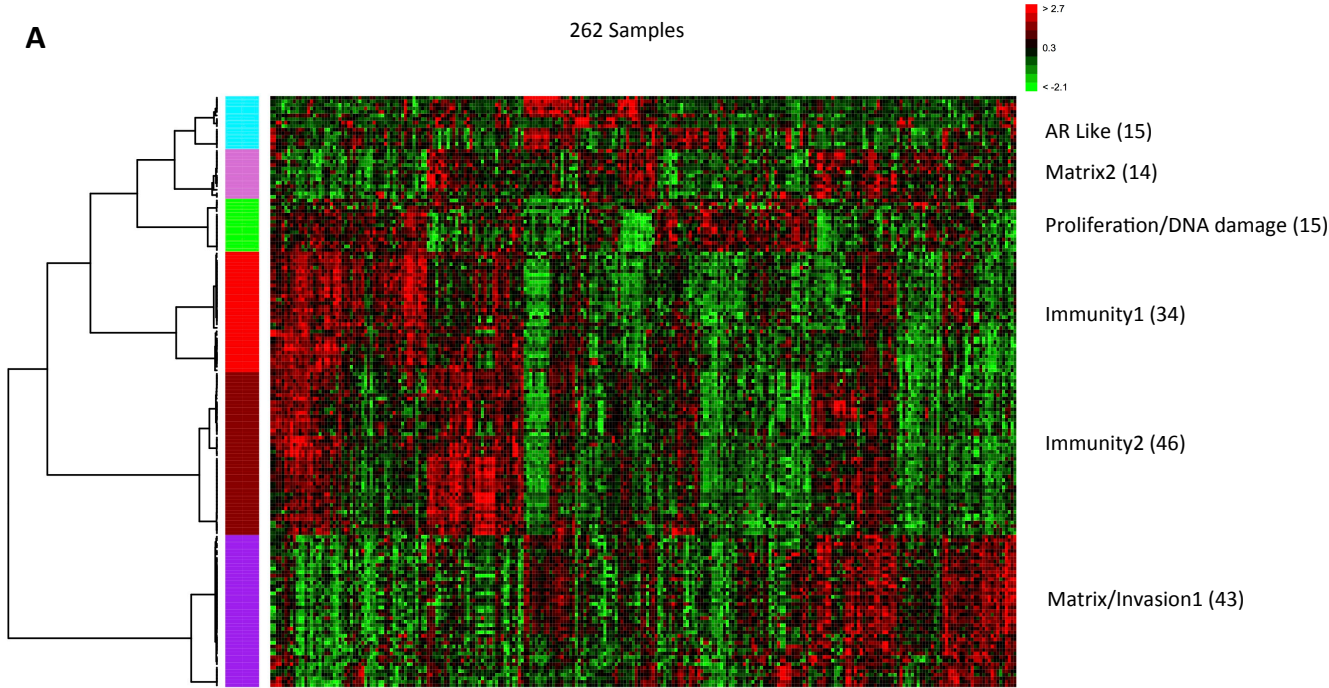
Figure S24

Histograms of ER, HER2 and PR gene expression distribution in the training, validation and Metabric sets, and density plots of groups obtained by bimodal filtering (R Mclust package).





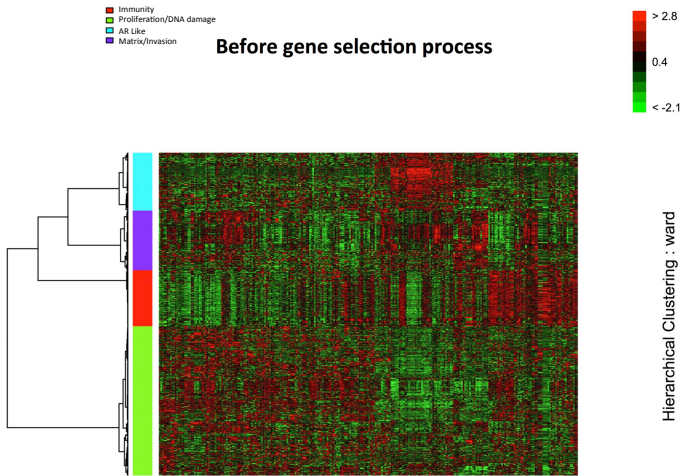




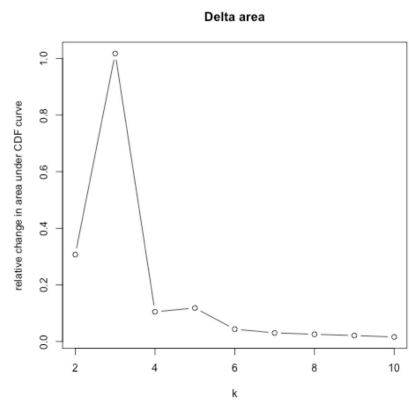
B

	Immunity1				
0.58	Immunity2				
0.29	-0.13	Proliferation/ DNA damage			
-0.14	-0.12	-0.28	AR Like		
-0.19	-0.06	-0.43	0.11	Matrix/ Invasion1	
-0.20	0.18	-0.60	0.08	0.48	Matrix2

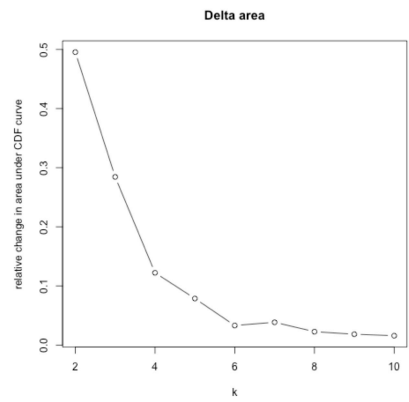
2-2 Metagene Pearson correlation score

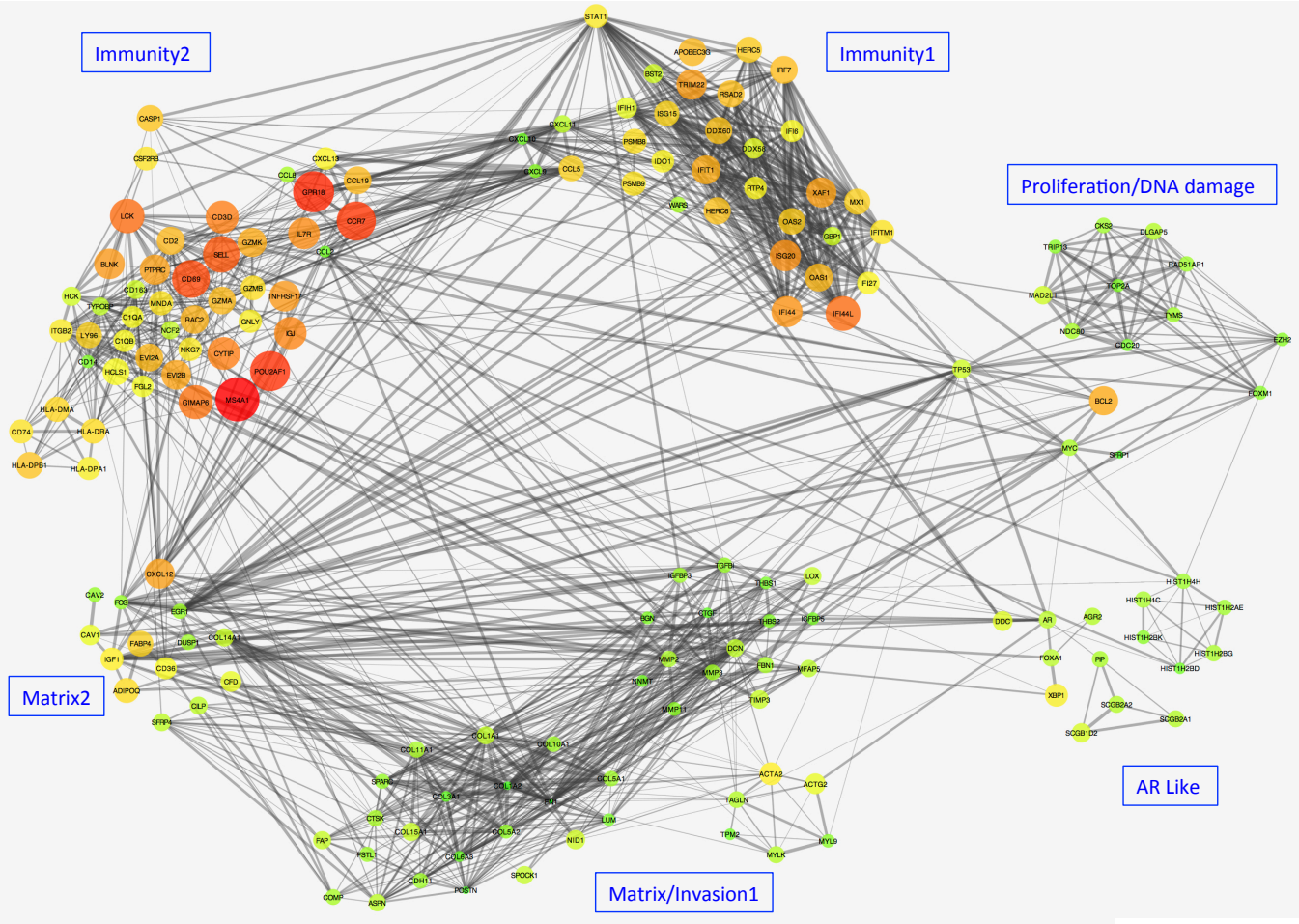


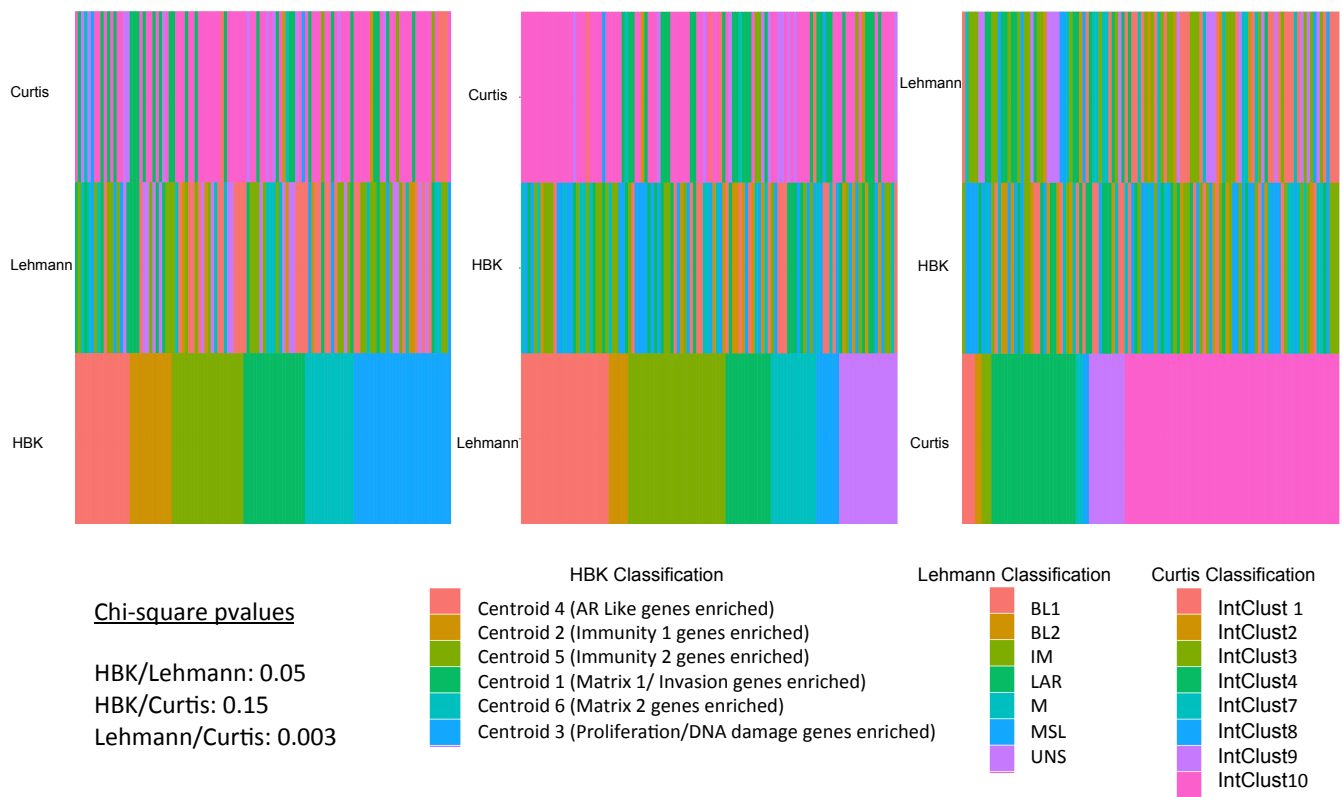
CDF curve of the consensus clustering before gene selection process



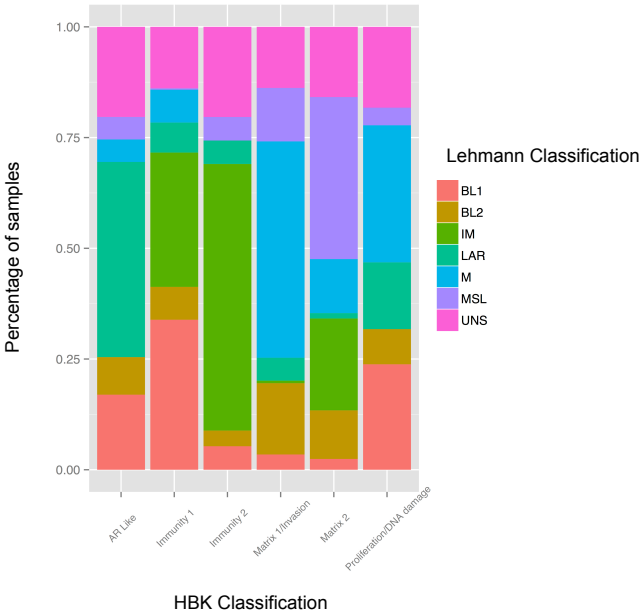
CDF curve of the consensus clustering after gene selection process



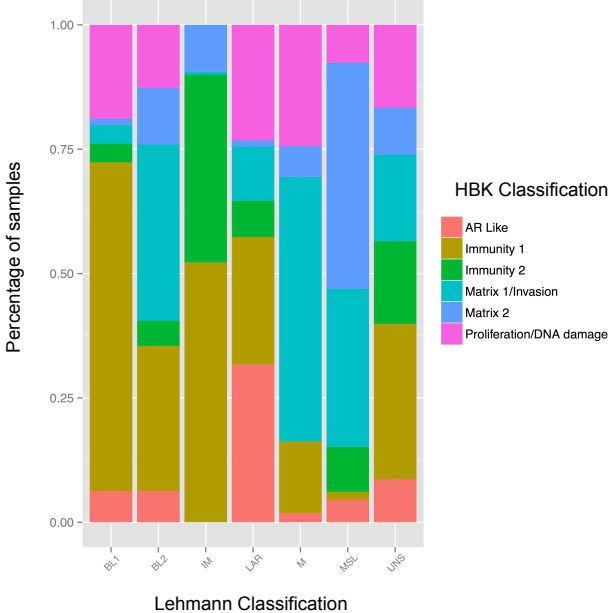


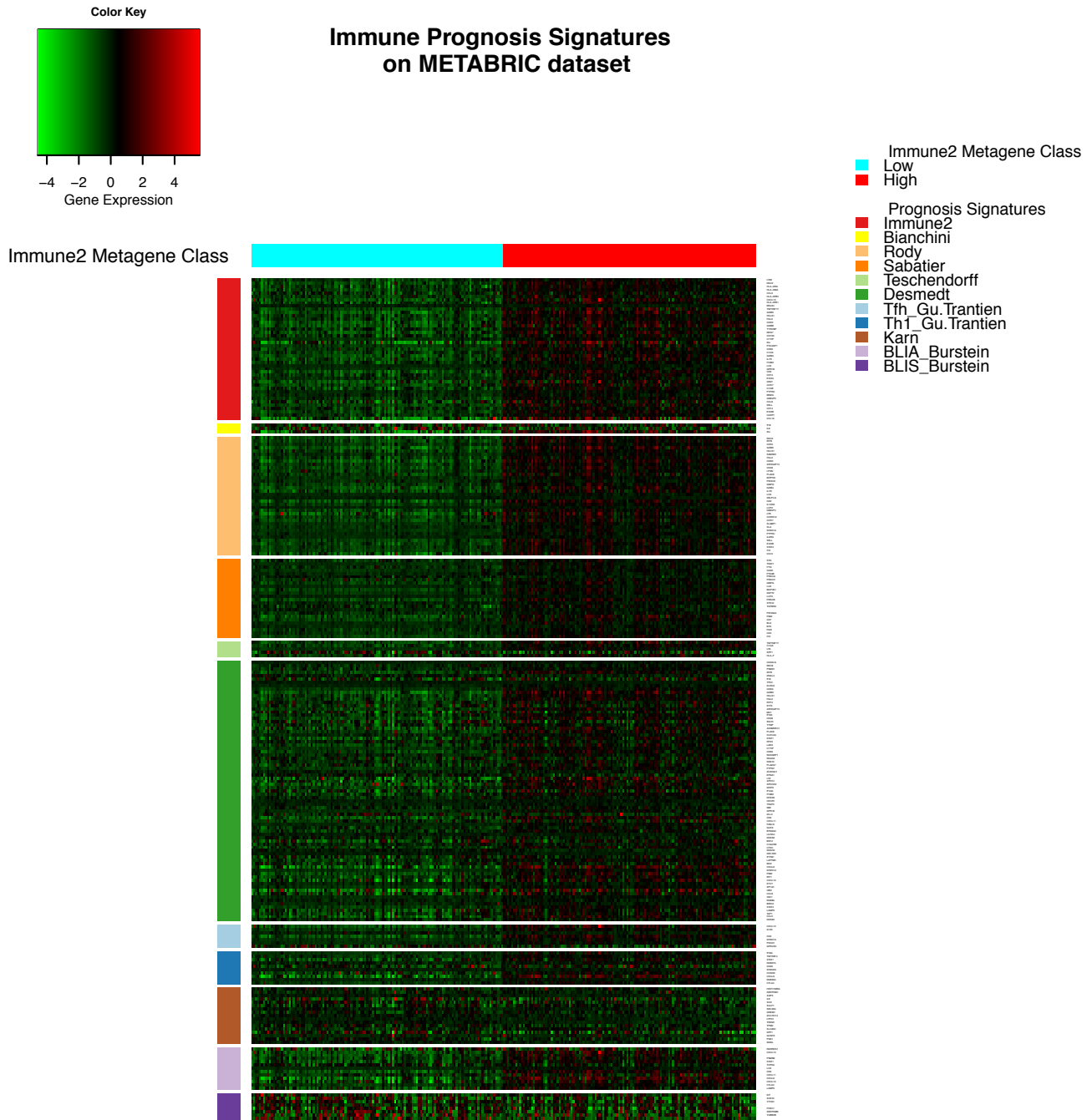


A

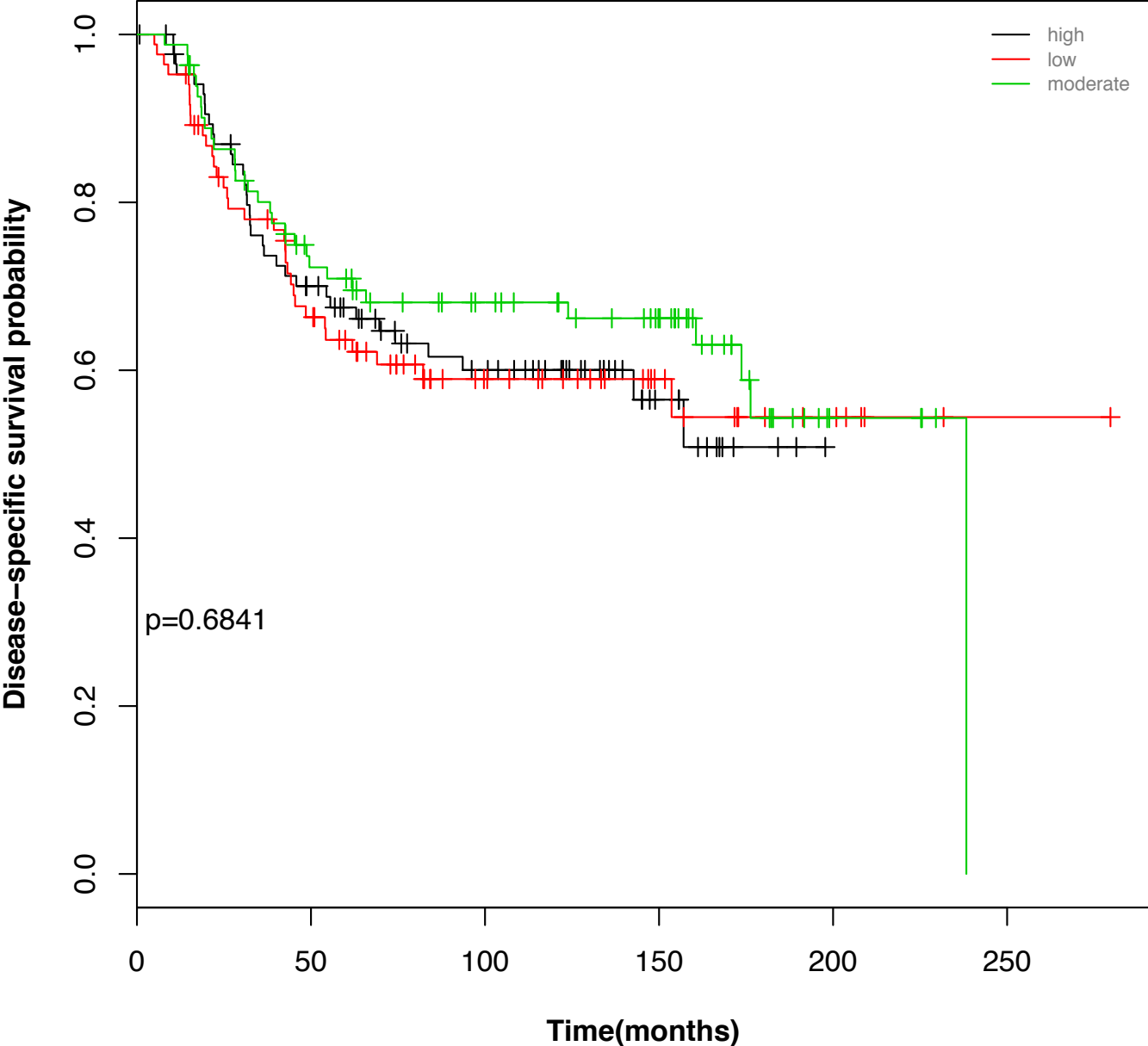


B

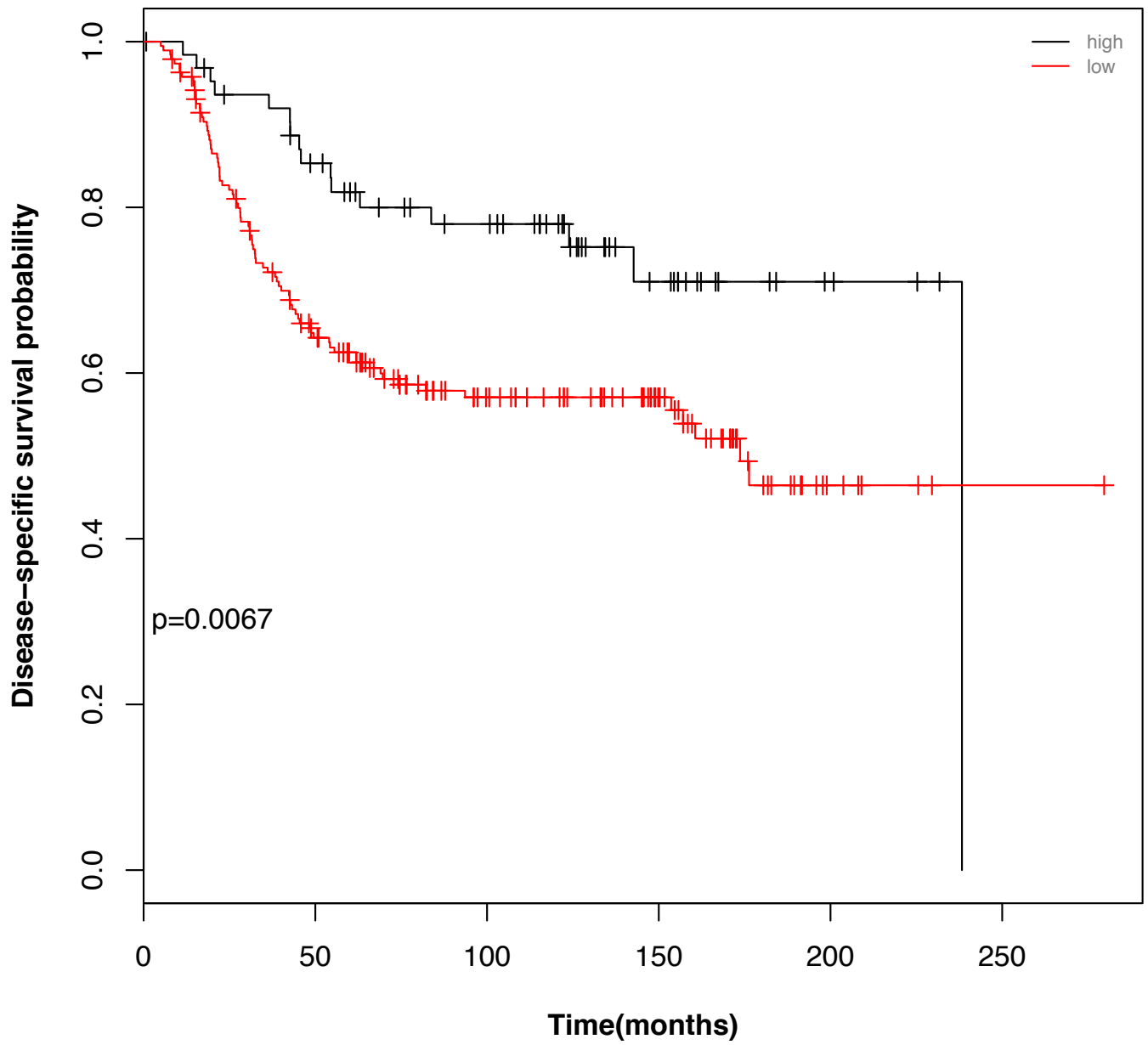




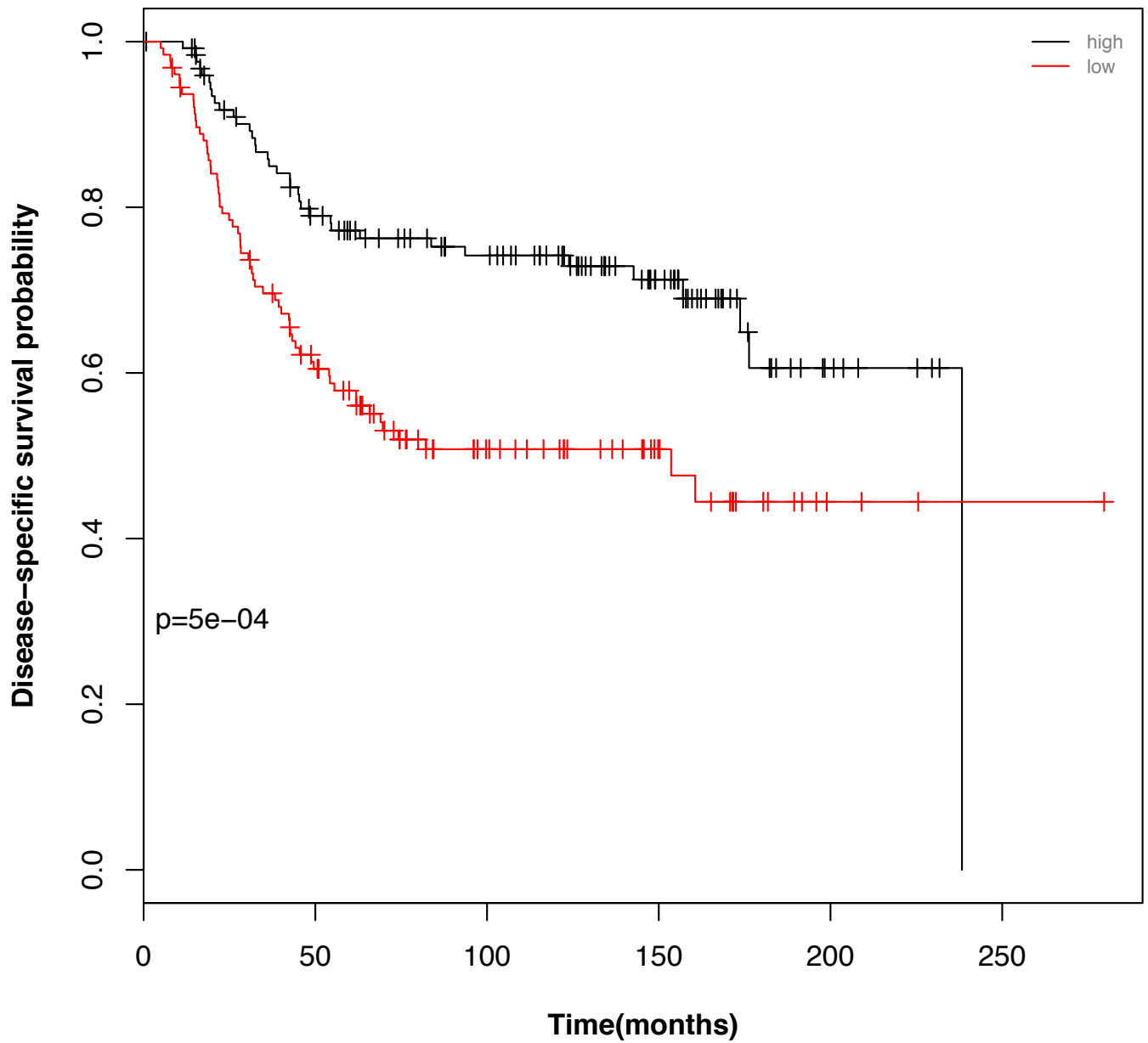
Kaplan Meier plot of disease-specific survival for the Bianchini metagene



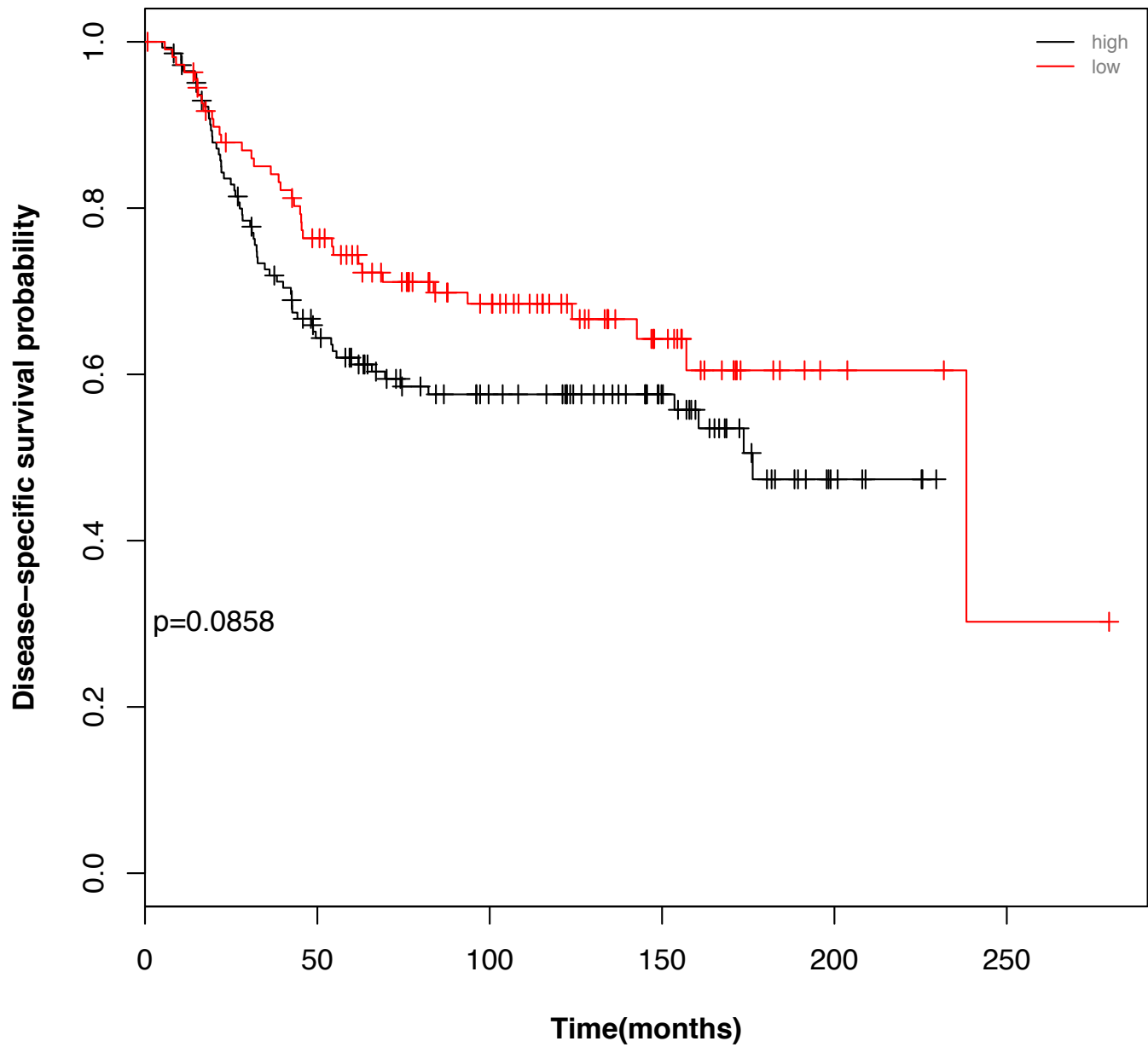
Kaplan Meier plot of disease-specific survival for the Rody metagene



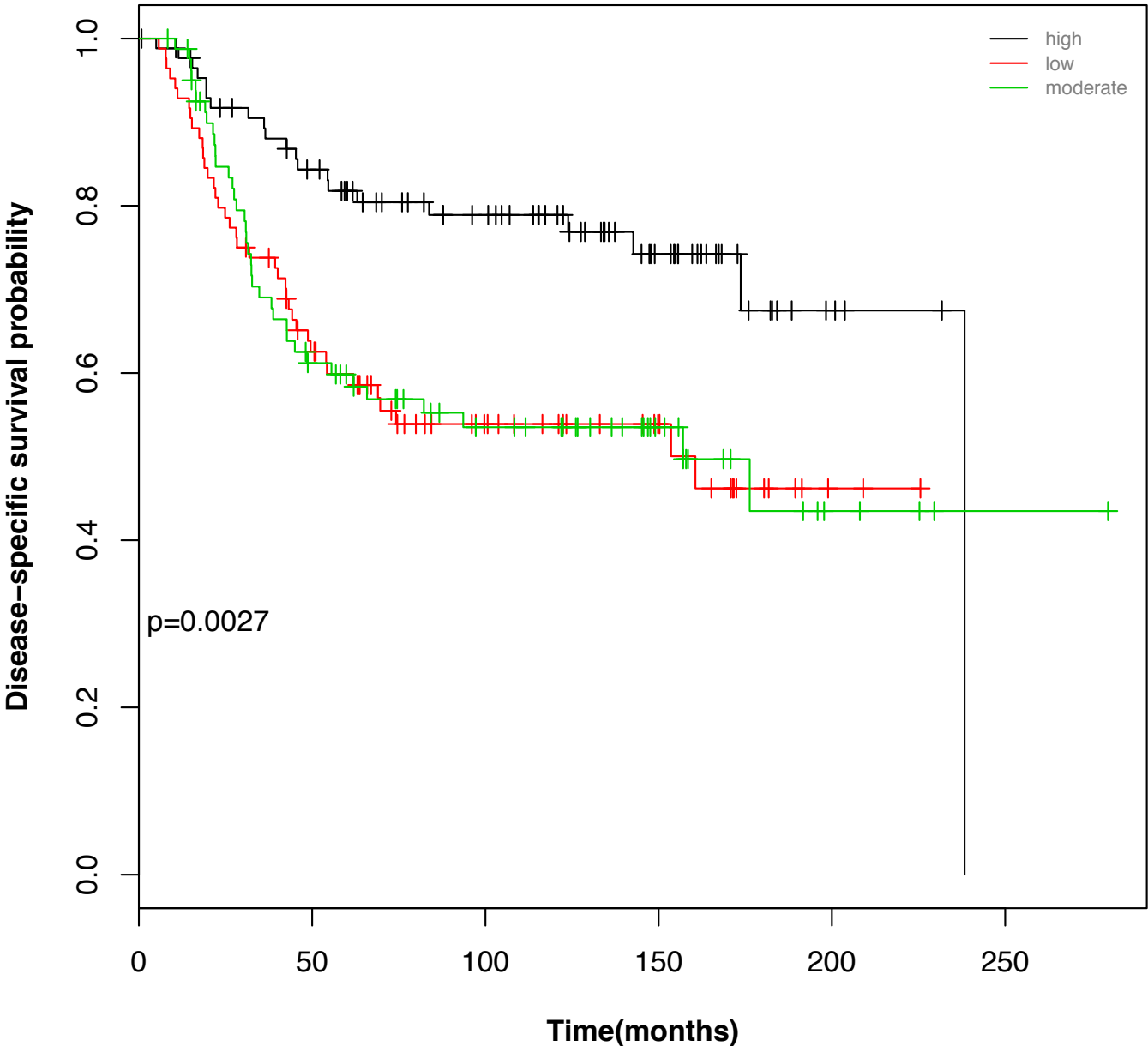
Kaplan Meier plot of disease-specific survival for the Sabatier metagene



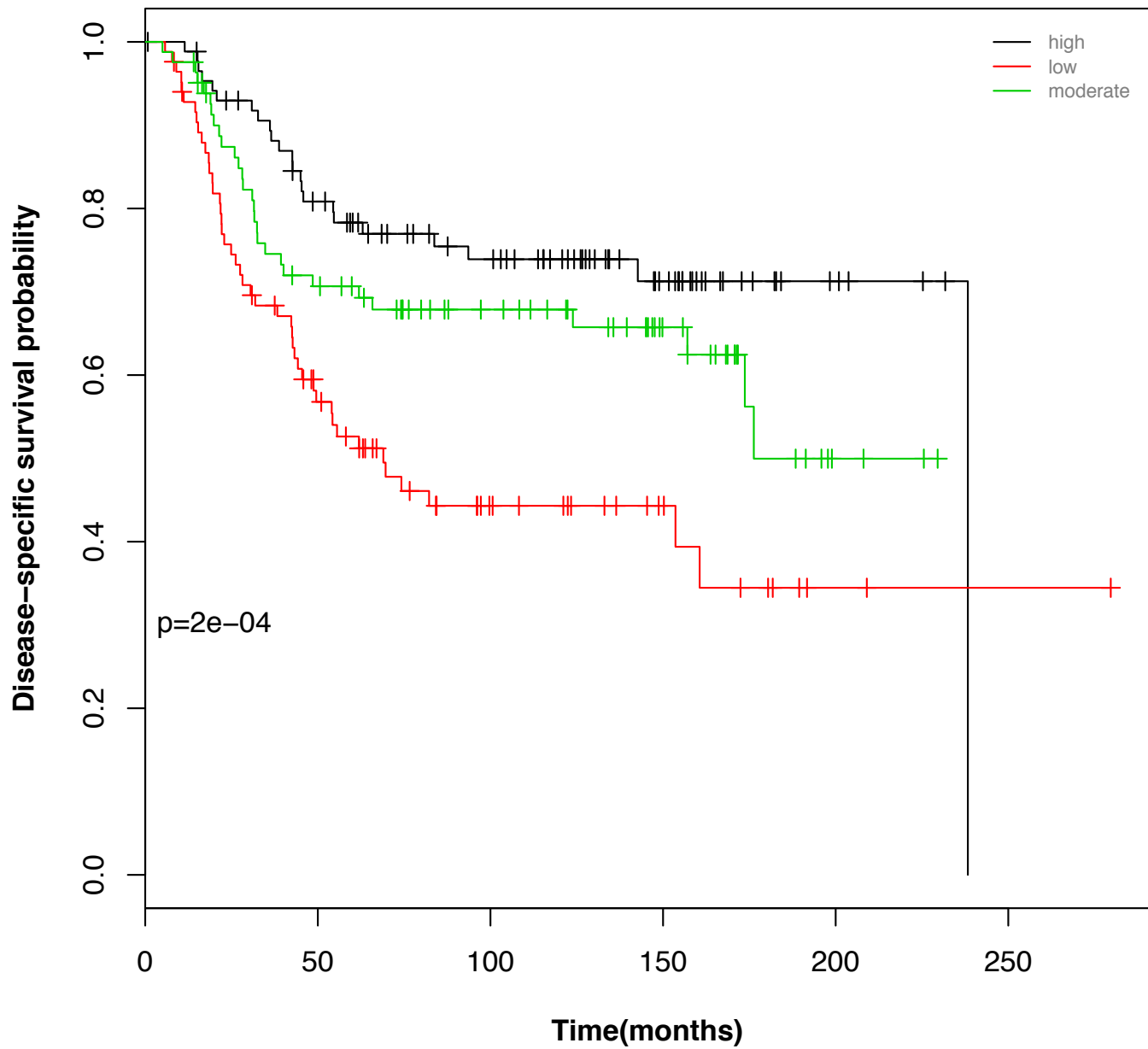
Kaplan Meier plot of disease-specific survival for the Teschendorff metagene



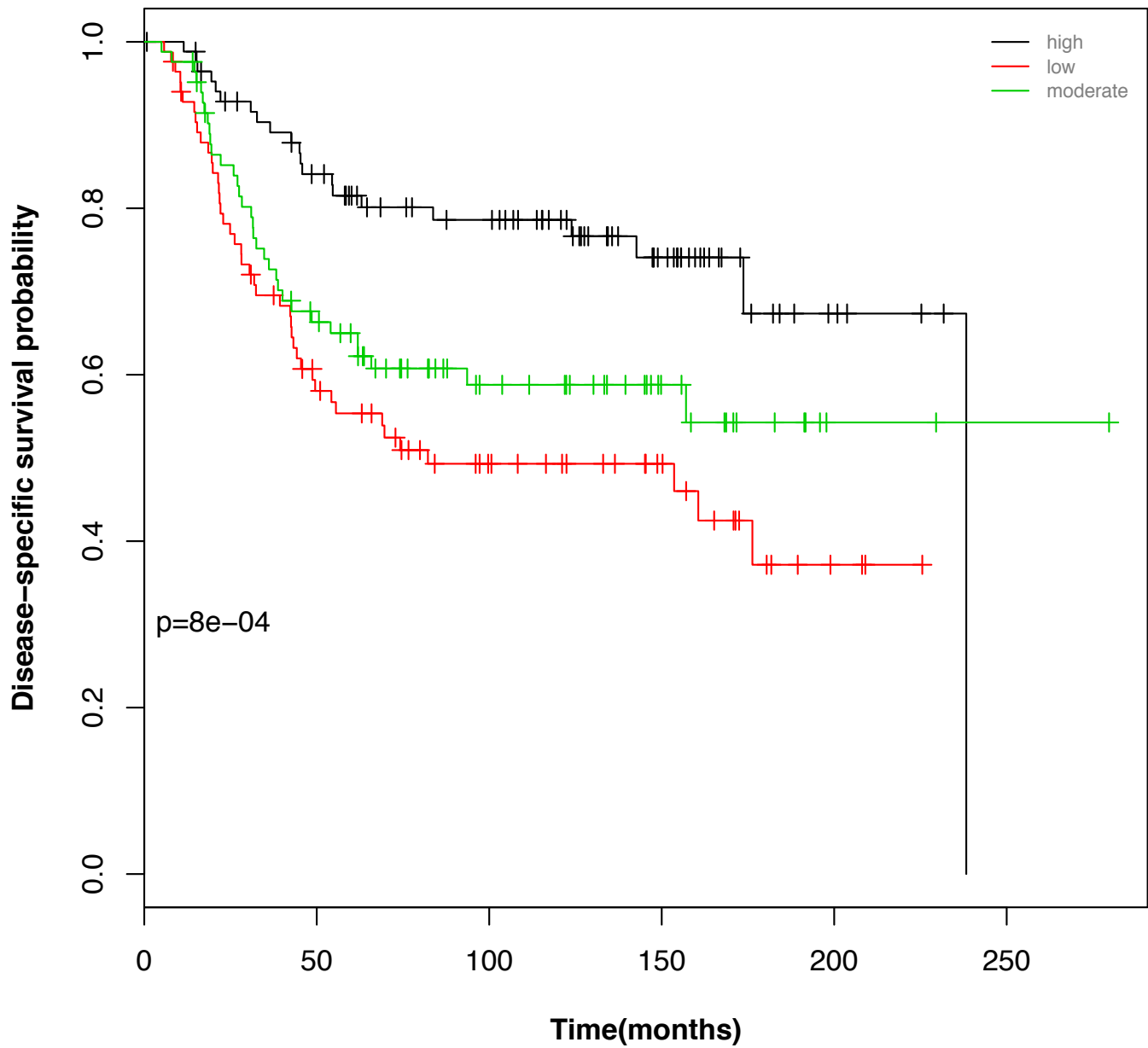
Kaplan Meier plot of disease-specific survival for the Desmedt metagene



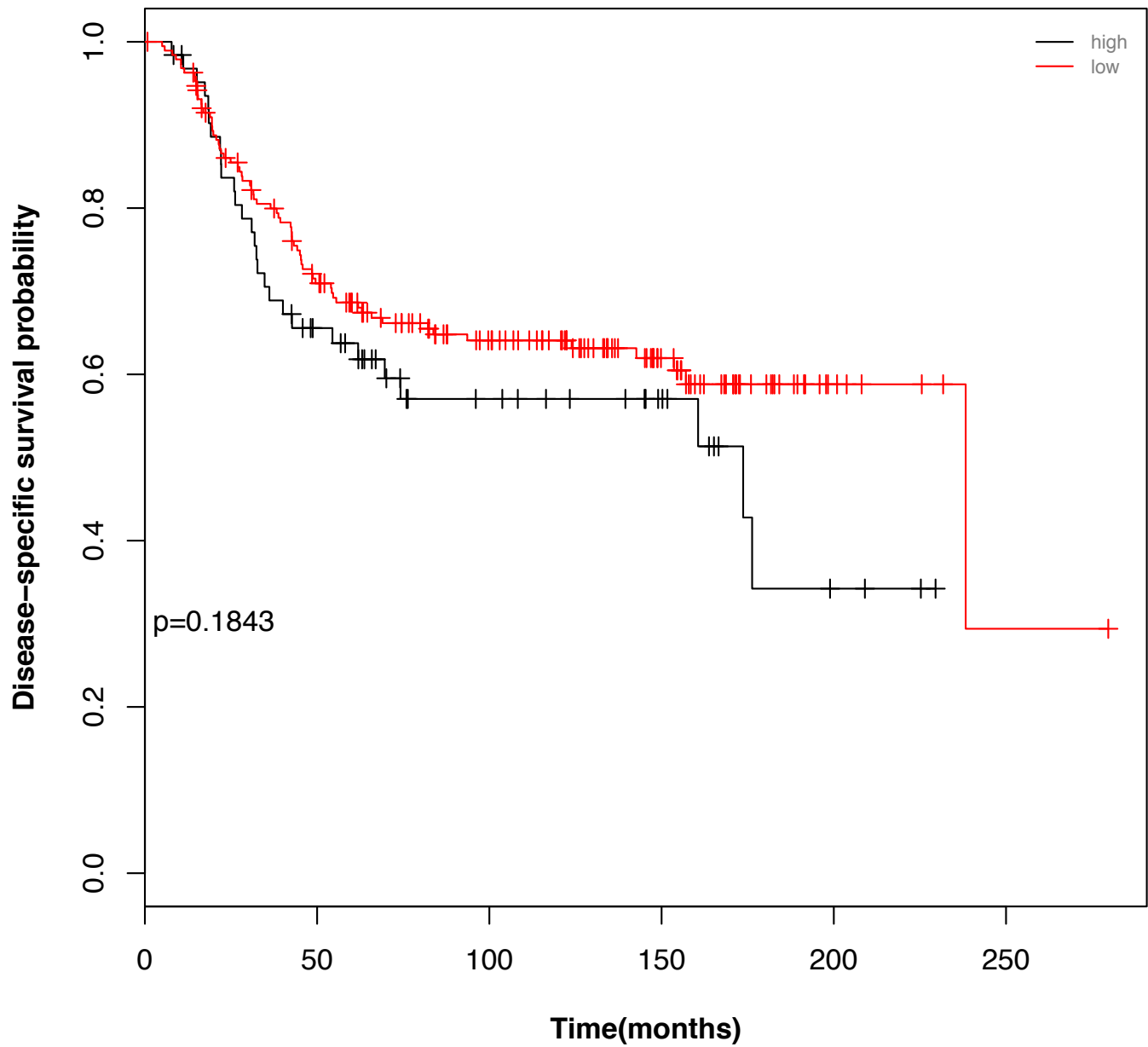
Kaplan Meier plot of disease-specific survival for the Tfh_Gu.Trantien metagene



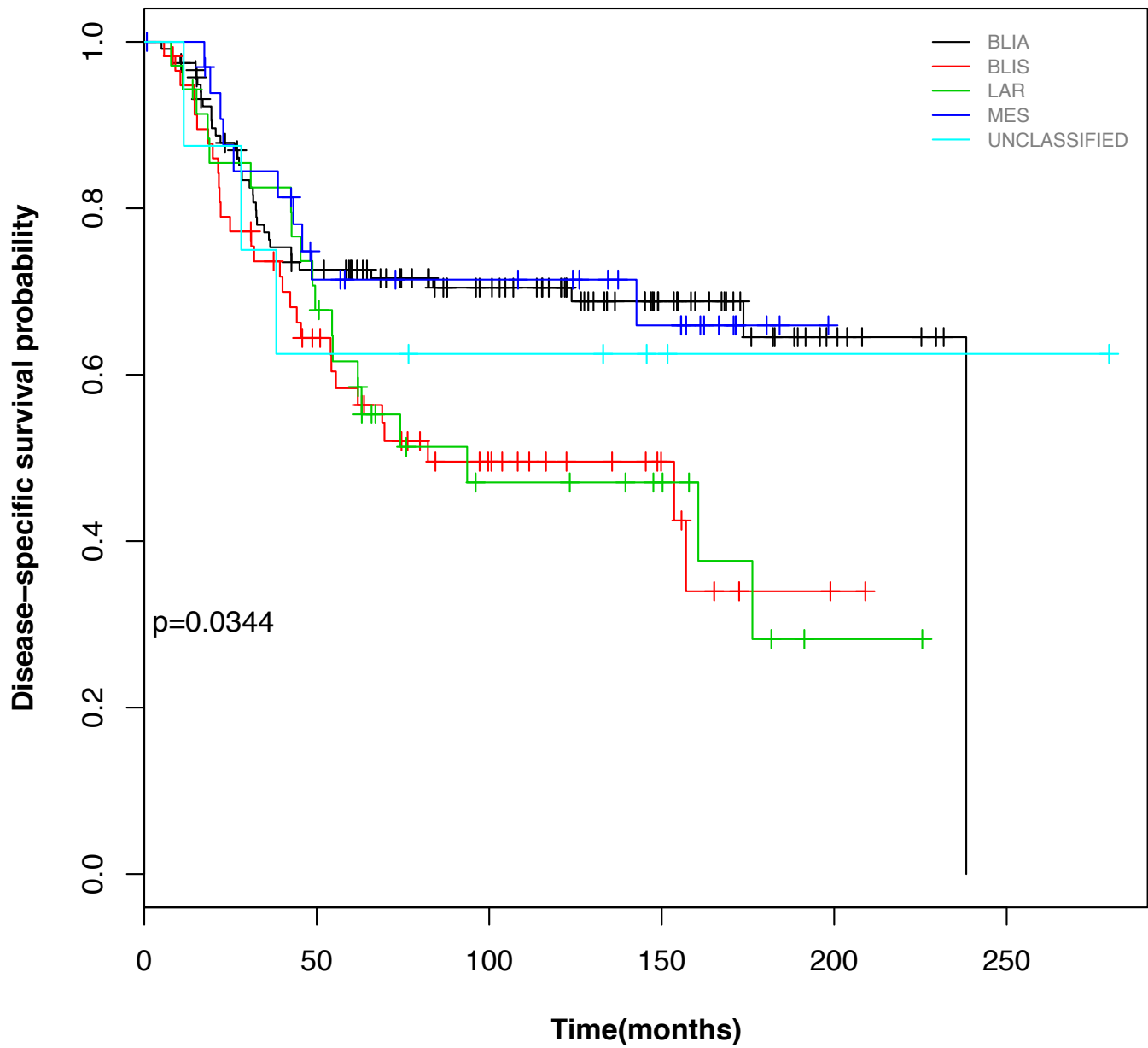
Kaplan Meier plot of disease-specific survival for the Th1_Gu.Trantien metagene

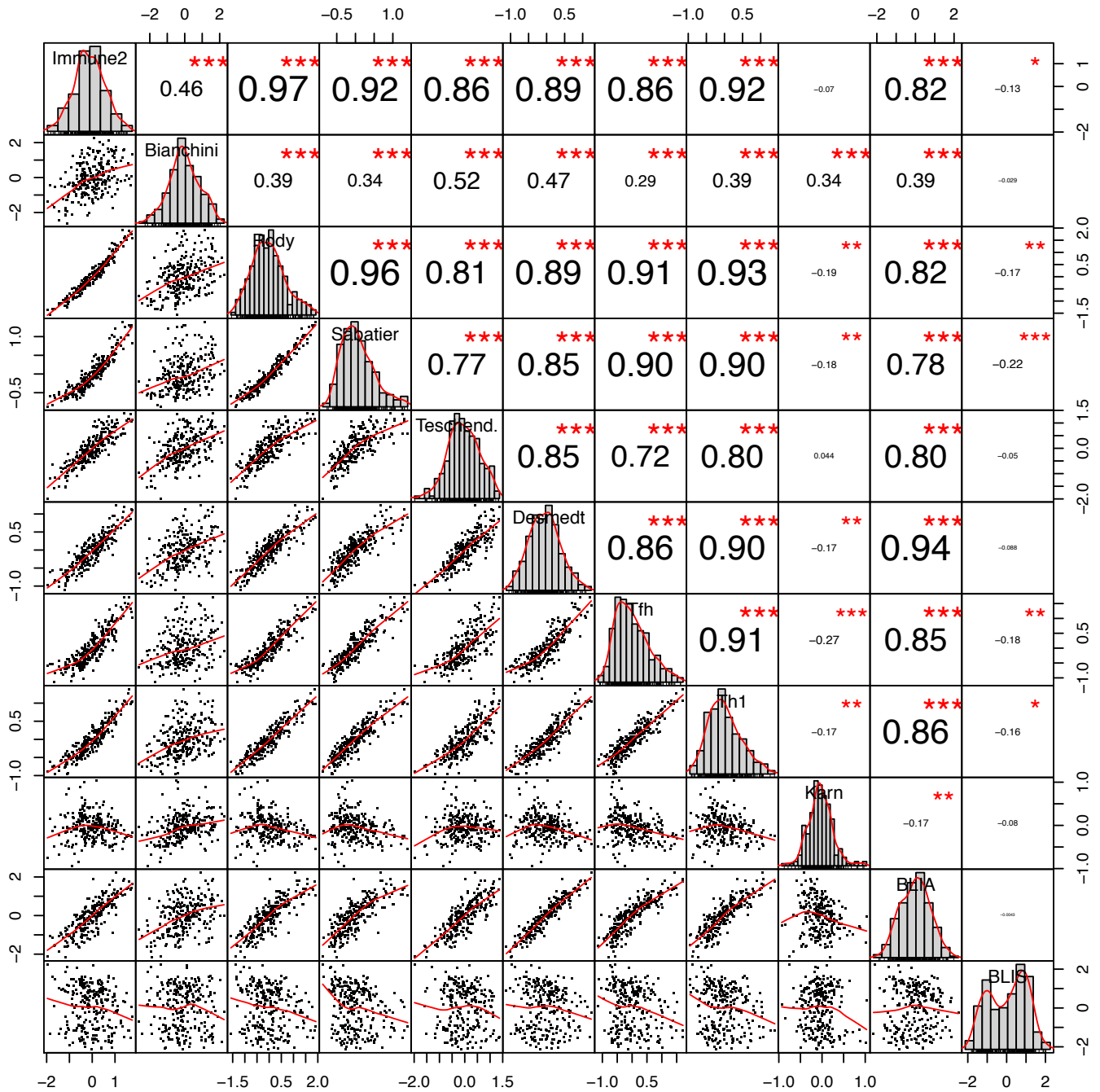


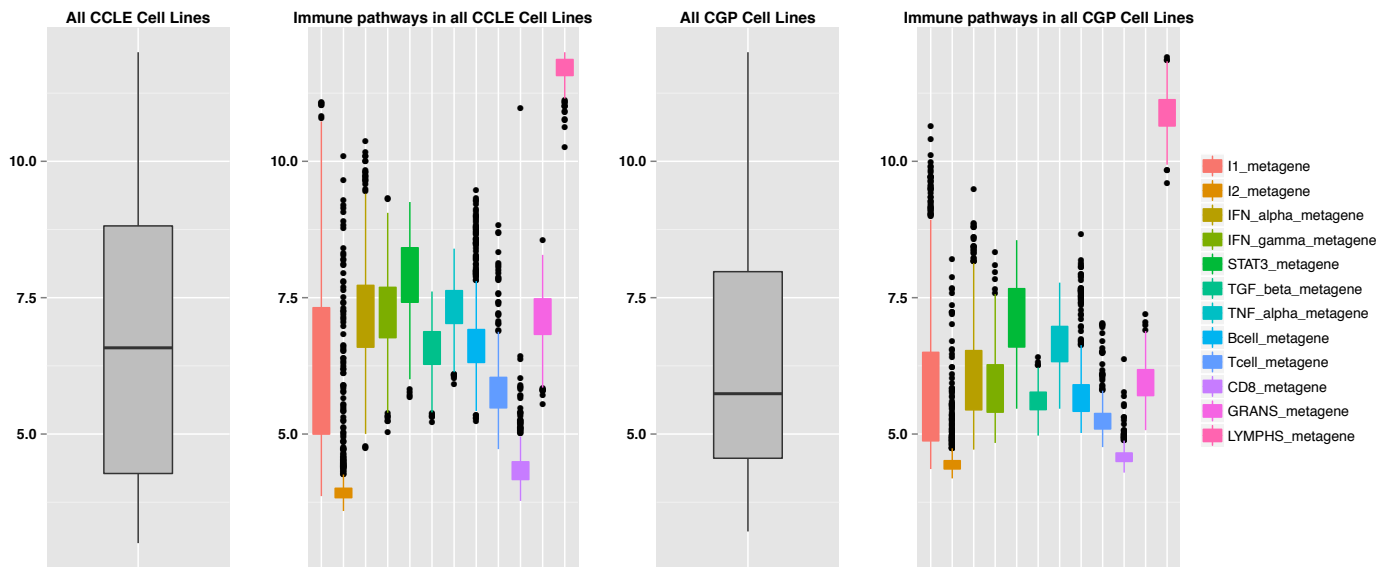
Kaplan Meier plot of disease-specific survival for the Karn metagene

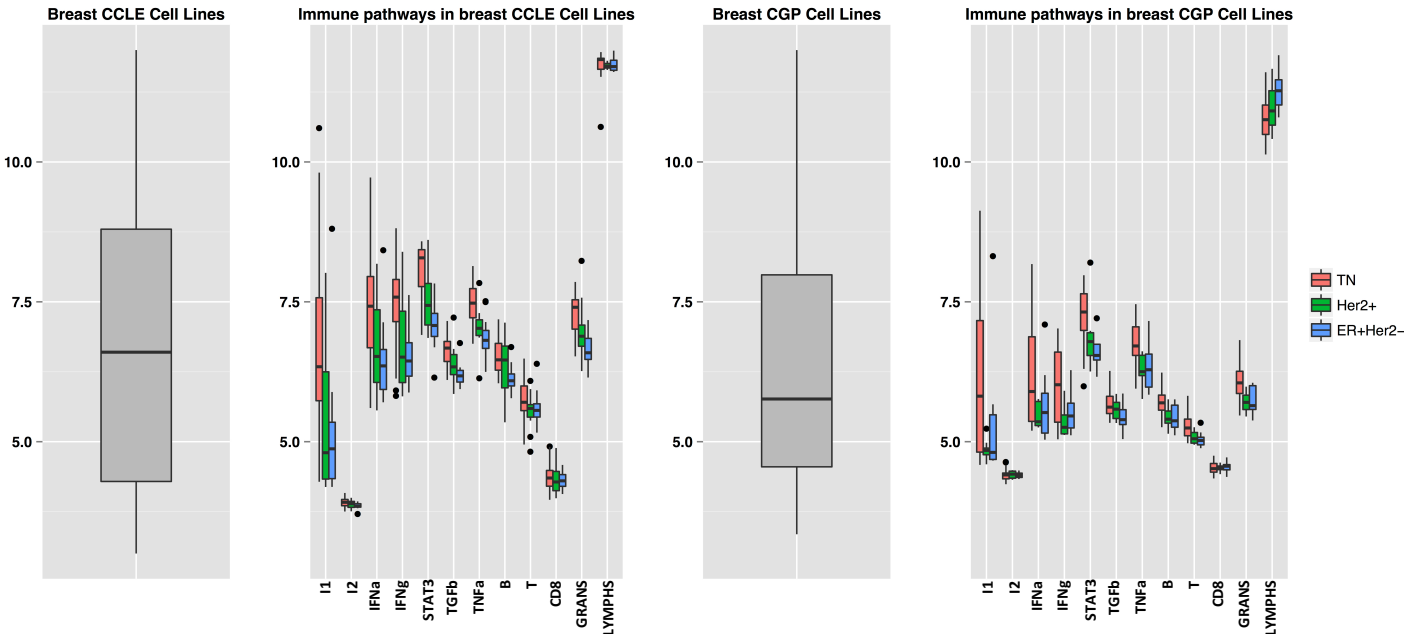


Kaplan Meier plot of disease-specific survival for the Burstein centroids

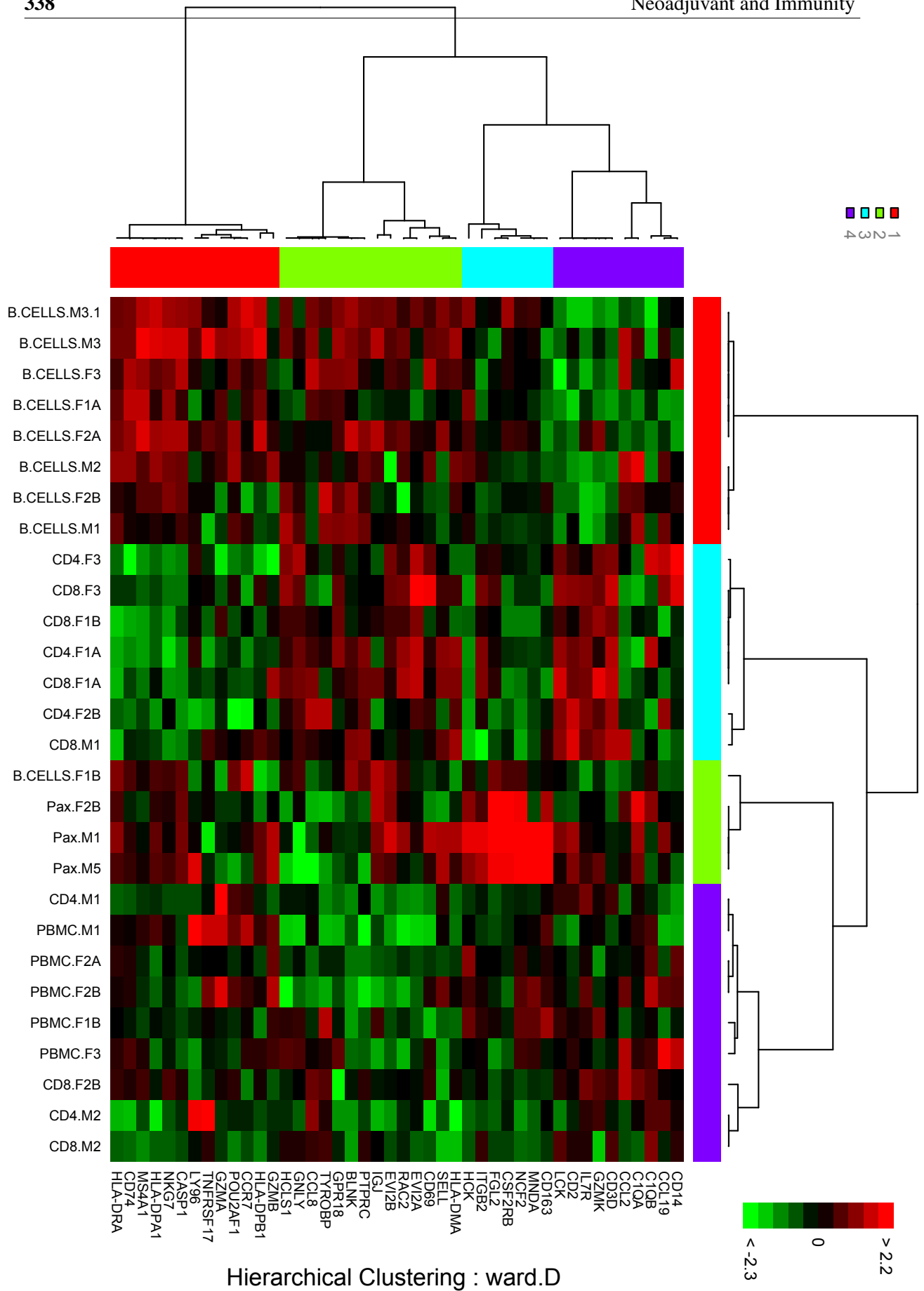




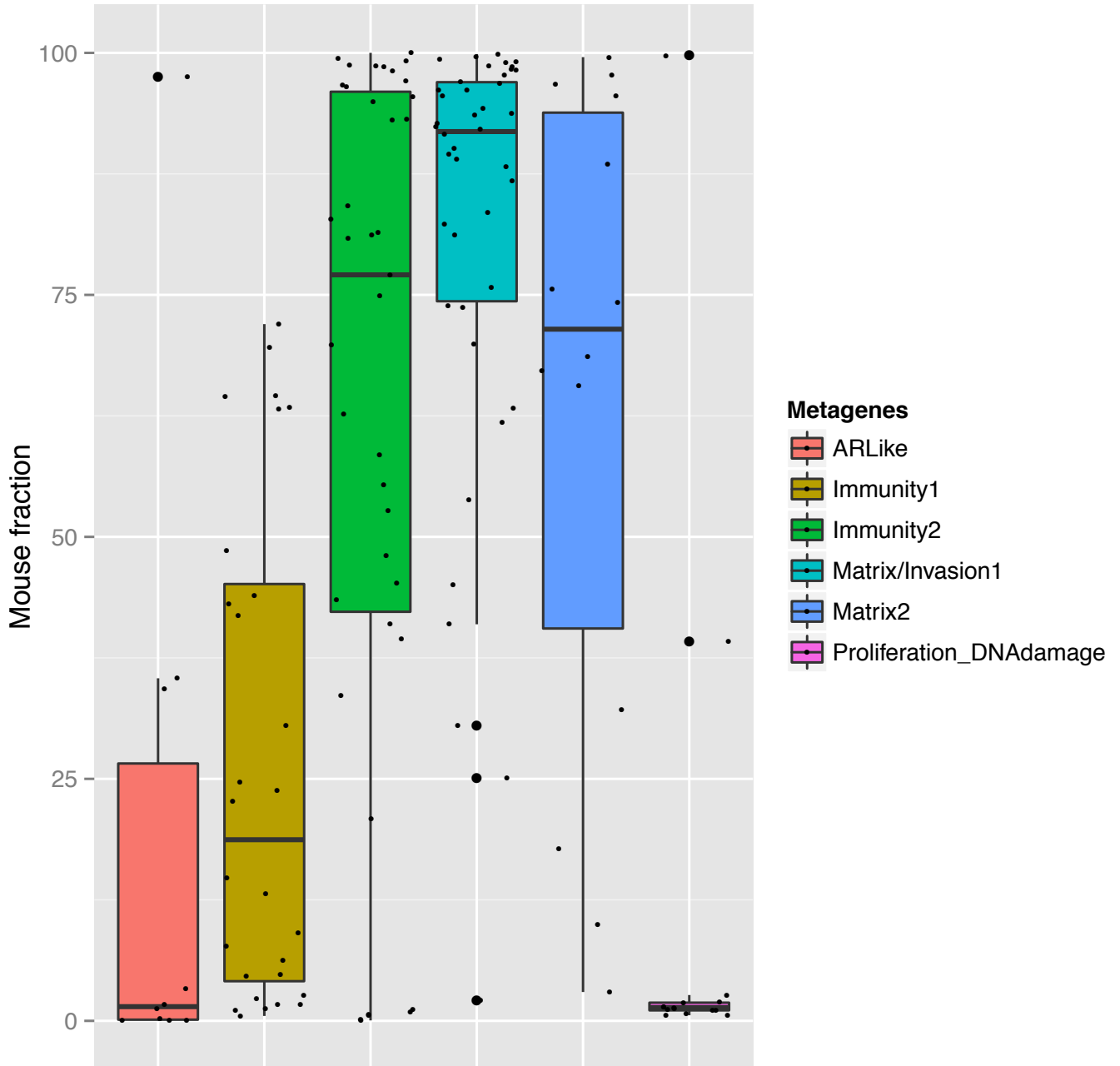


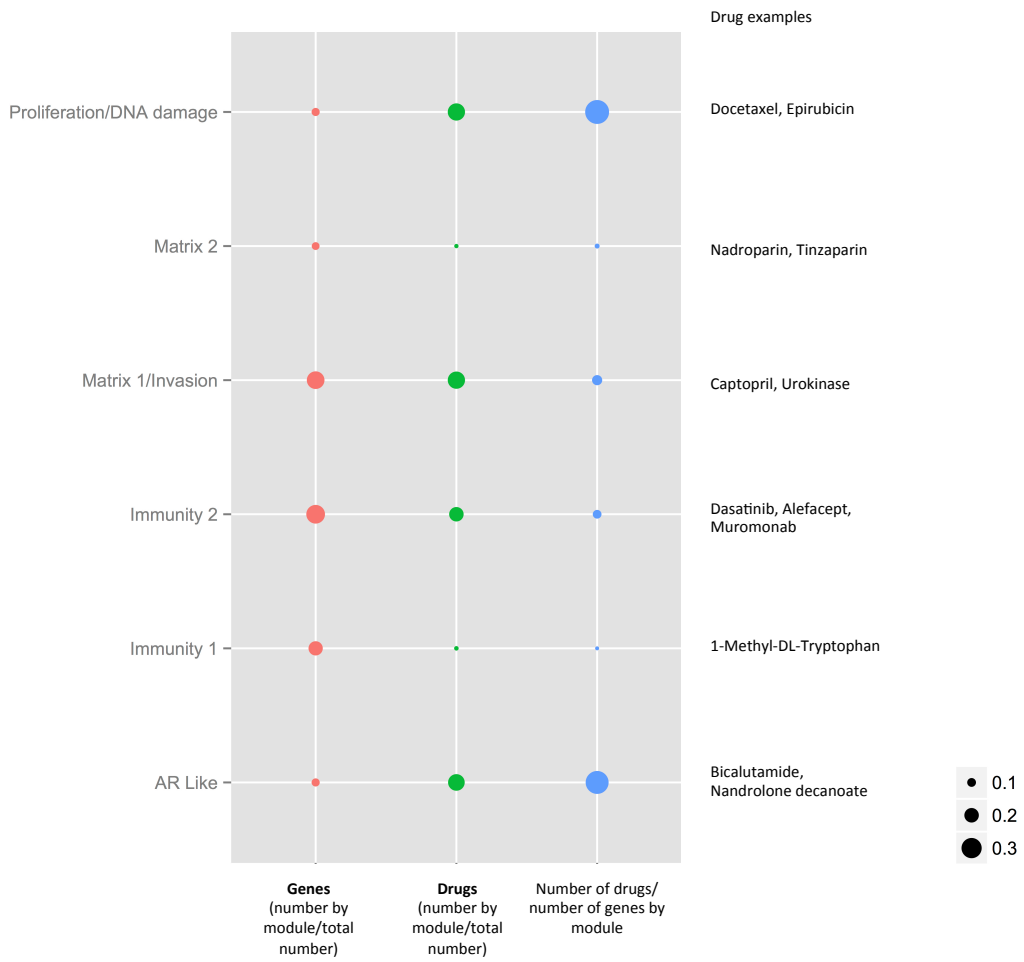


Hierarchical Clustering : ward.D

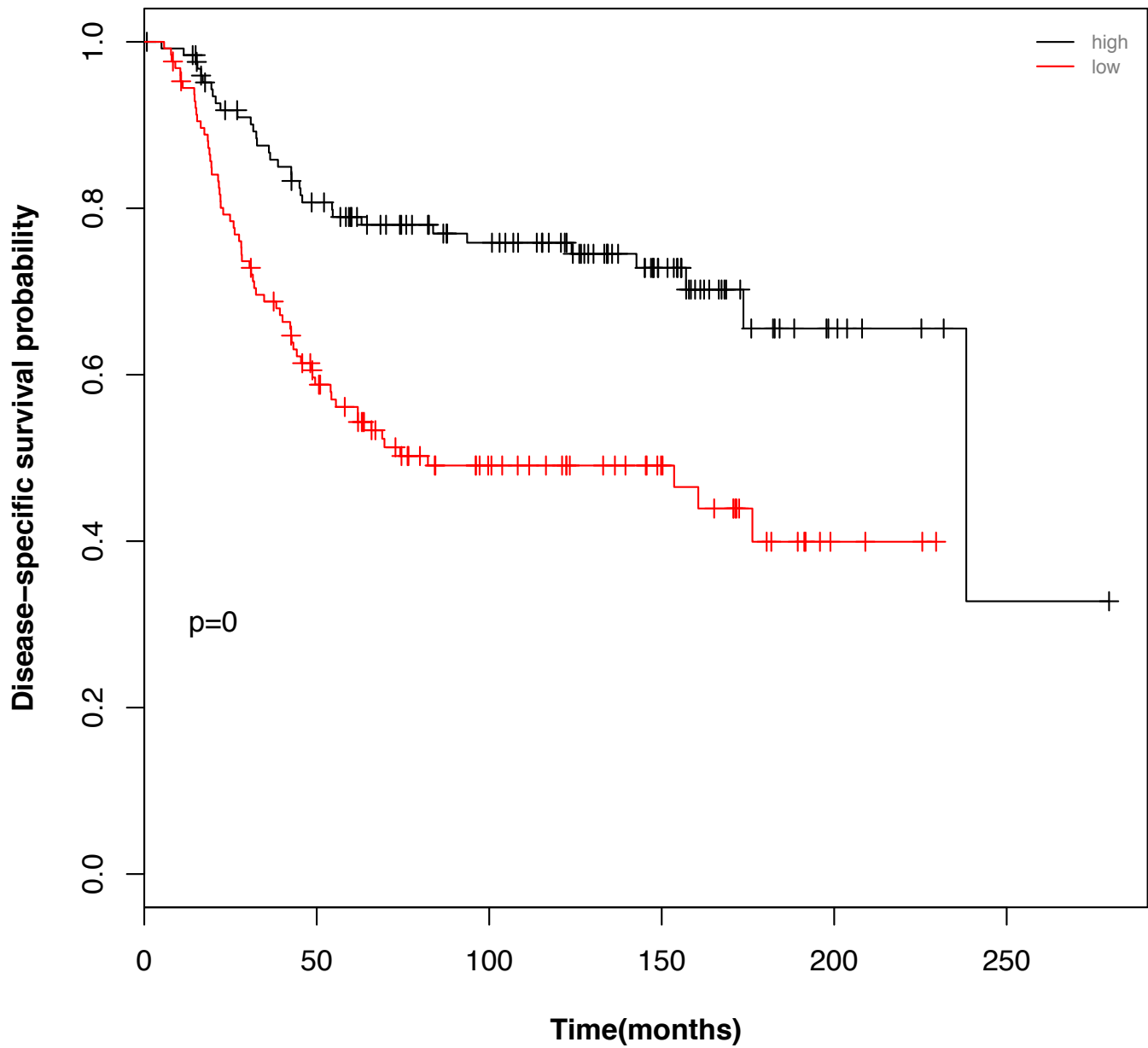


Hierarchical Clustering : ward.D

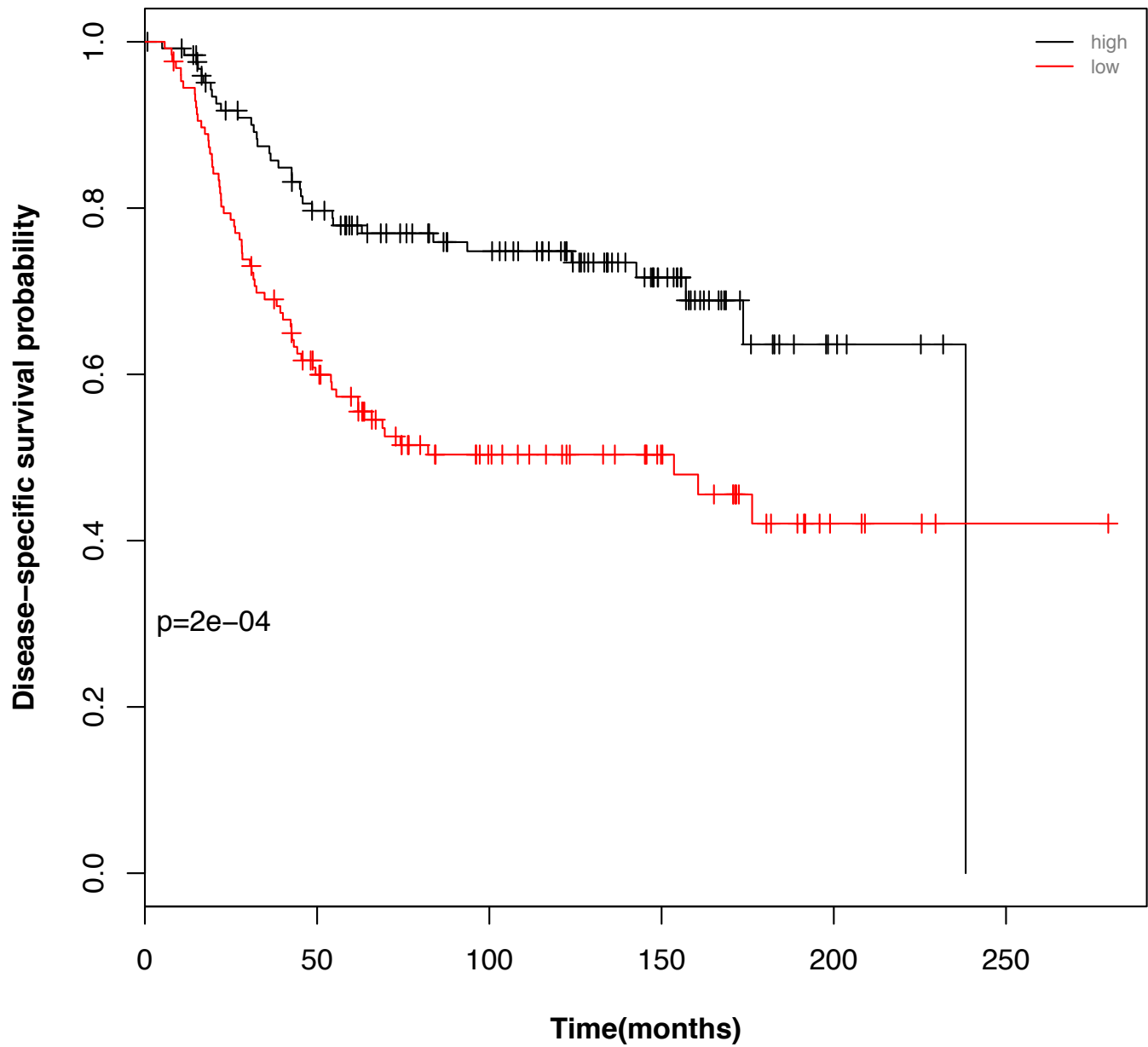




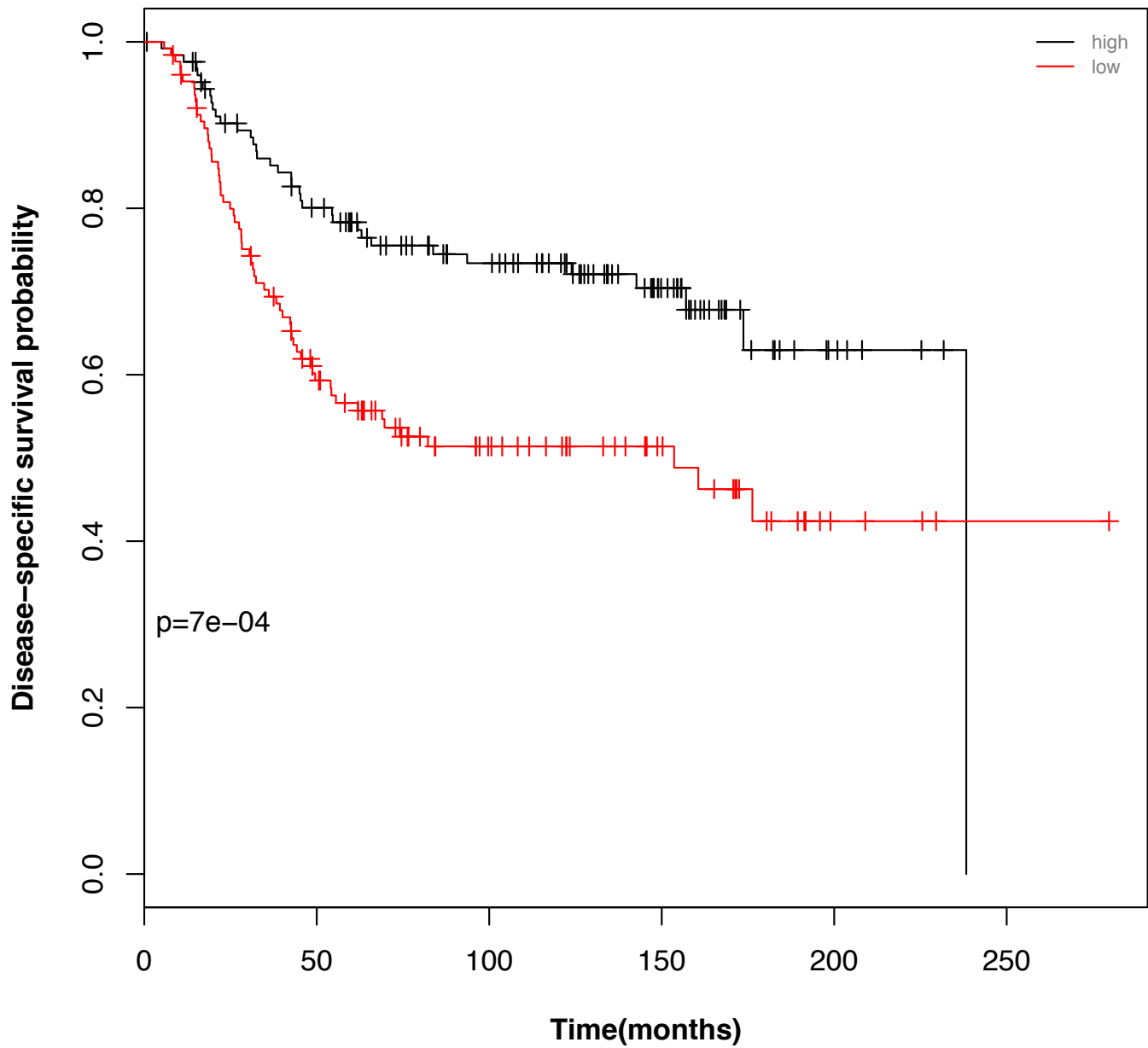
Kaplan Meier plot of disease-specific survival for the PD1 metagene



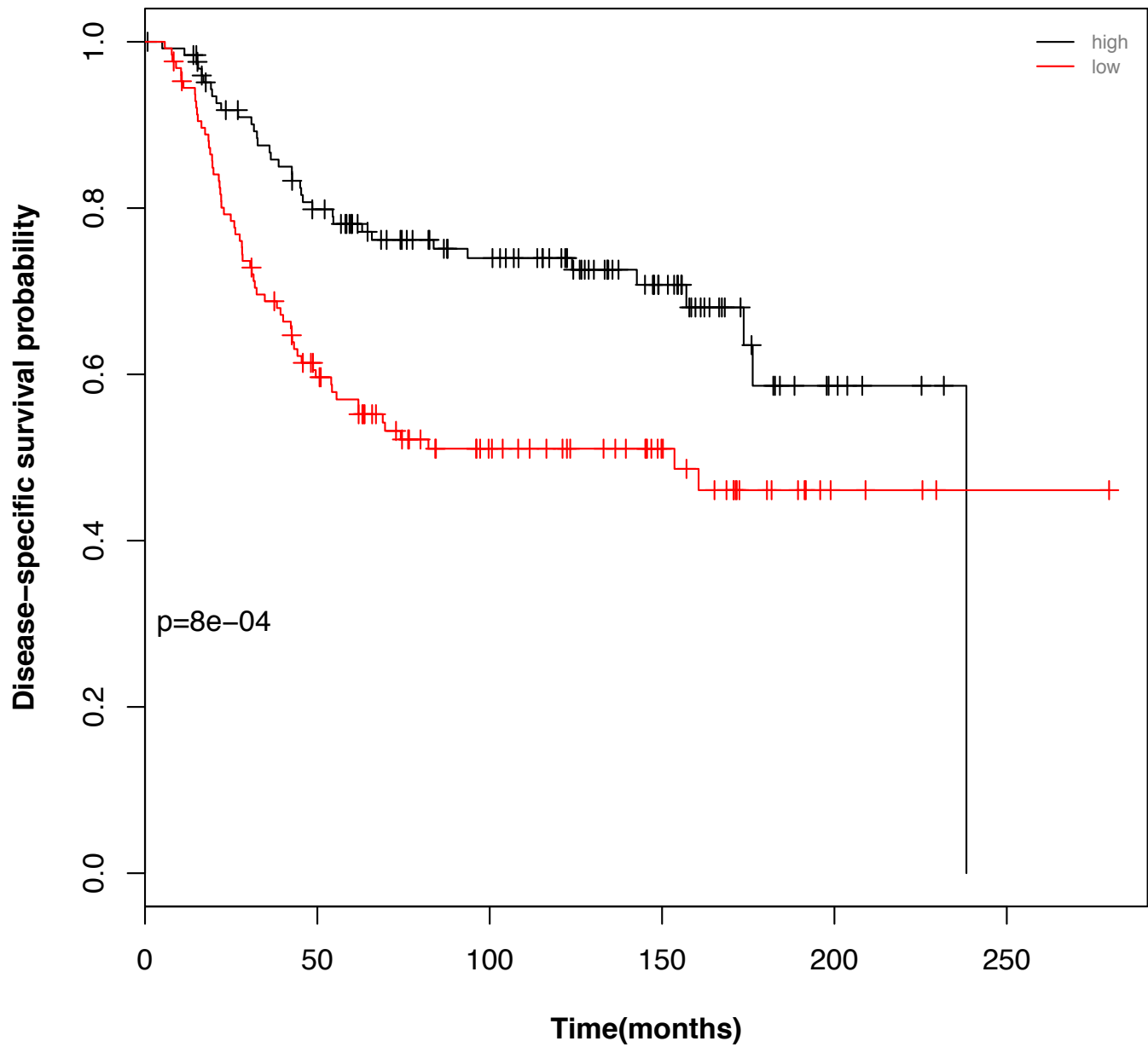
Kaplan Meier plot of disease-specific survival for the PDL1 metagene

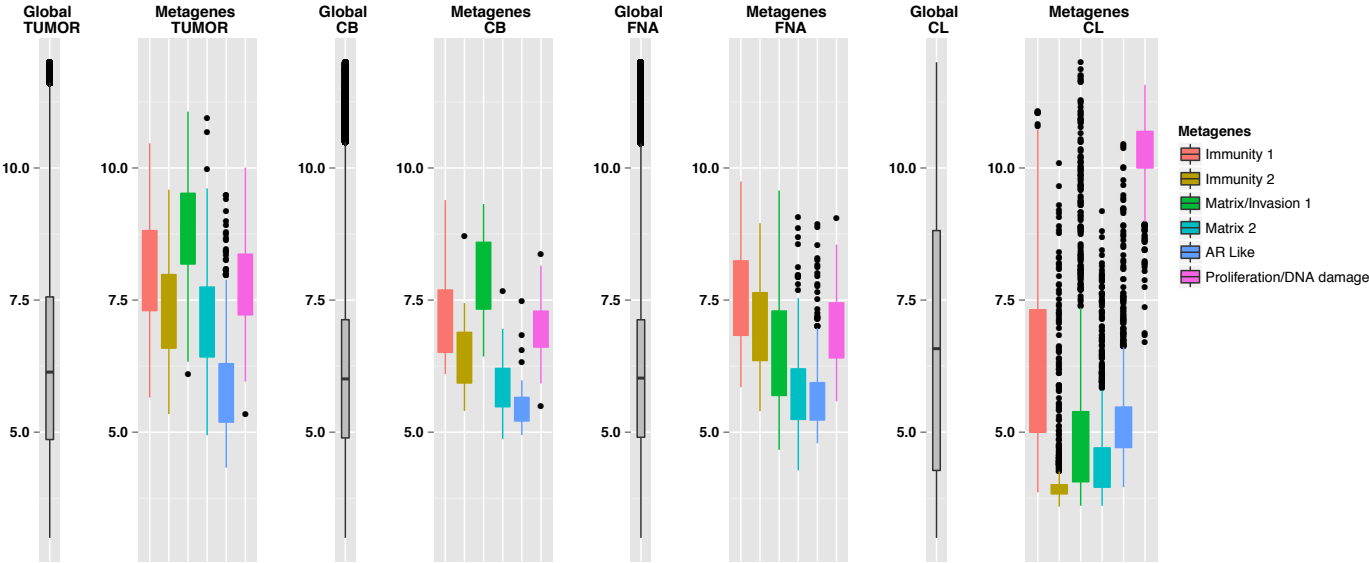


Kaplan Meier plot of disease-specific survival for the CTLA4 metagene

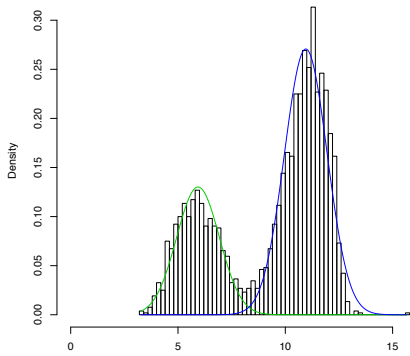


Kaplan Meier plot of disease-specific survival for the TILs metagene

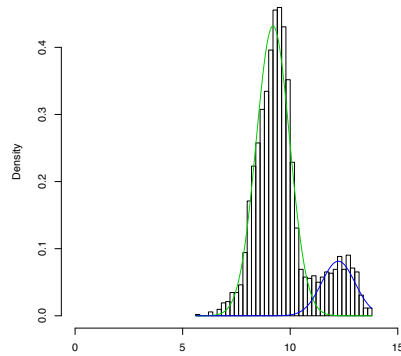




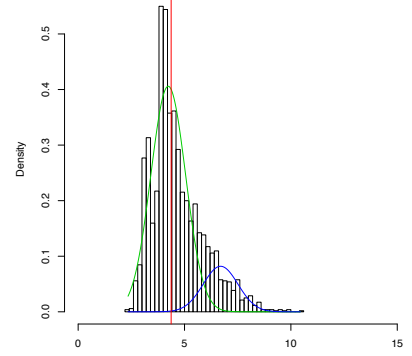
Histogram of ER distribution in Training and Validation Sets



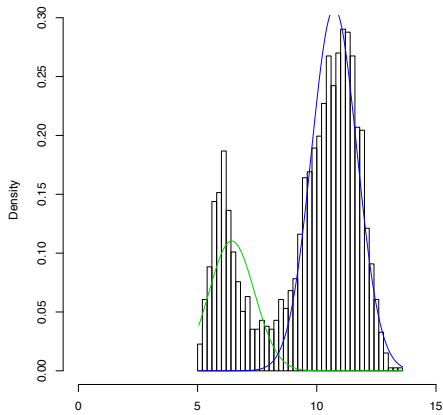
Histogram of HER distribution in Training and Validation Sets



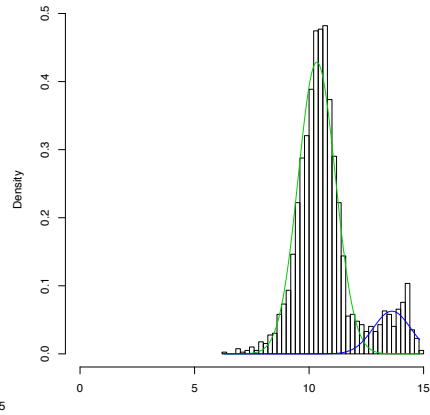
Histogram of PR distribution in Training and Validation Sets



Histogram of ER distribution in Metabric Set



Histogram of HER distribution in Metabric Set



Histogram of PR distribution in Metabric Set

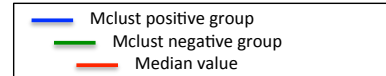
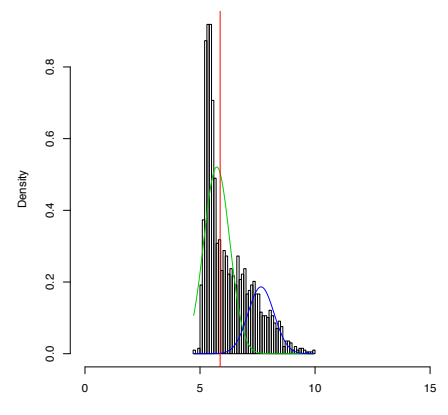


Table S1. Comparison of the chemotherapy-naive and chemotherapy-treated populations in the METABRIC dataset (characteristics of patients and tumors).

Triple-negative breast cancer patients		Chemotherapy-naive population 115 samples	Chemotherapy-treated population 139 samples	
Characteristics		N (%)- Median (range), Mean	N (%)- Median (range), Mean	p value
Age	Median (range)	64.0 (31-96)	49.6 (28-83)	1.2 ¹⁰⁻¹¹
	Mean	61.5	50.1	
Menopausal status	Pre	27 (0.23)	72 (0.52)	5.38 ¹⁰⁻⁵
	Post	88 (0.77)	66 (0.47)	
	Missing data	0	1 (0.01)	
Tumor size (mm)	Median (range)	22 (0-80)	25 (1-182)	0.25
	Mean	24.8	31.3	
	<20 mm	40 (0.35)	36 (0.26)	
	>20 mm	75 (0.65)	101 (0.73)	
	Missing data	0	2 (0.01)	
Tumor grade	II	22 (0.19)	9 (0.07)	0.01
	III	88 (0.77)	128 (0.92)	
	Missing data	5 (0.04)	2 (0.01)	
Lymph node status	0	93 (0.81)	23 (0.17)	< 2.2 ¹⁰⁻¹⁶
	1	18 (0.16)	116 (0.83)	
	Missing data	4 (0.03)	0	
NPI score	<3.4	20 (0.17)	3 (0.02)	2.57 ¹⁰⁻⁵
	3.4-5.4	88 (0.77)	105 (0.76)	
	>5.4	7 (0.06)	31 (0.22)	
Cellularity	Low	16 (0.14)	9 (0.06)	0.03
	Moderate	40 (0.35)	34 (0.24)	
	High	58 (0.50)	93 (0.67)	
	Missing data	1 (0.01)	3 (0.02)	
P53 status	Wild-type	24 (0.21)	40 (0.29)	0.66
	Mutant	10 (0.09)	20 (0.14)	
	Missing data	81 (0.70)	79 (0.57)	
Status at end point	Alive	80 (0.73)	73 (0.53)	0.004
	Dead from cancer	31 (0.27)	66 (0.47)	

Abbreviations: NPI, Nottingham Prognostic Index.

Table S2. Survival analysis (disease-specific survival) with immune prognosis signatures previously published. Univariate and multivariate analysis.

254 triple-negative breast cancer patients		Univariate analysis		Multivariate analysis*		Multivariate analysis**	
		DS-survival HR [95% CI]	p value	DS-survival HR [95% CI]	p value	DS-survival HR [95% CI]	p value
Immunity2 metagene expression	High	1				1	
	Low	2.39 [1.57-3.64]	5.1 ^{10⁻⁵}			3.01 [1.08-8.39]	0.03
Bianchini metagene expression	High	1					
	Moderate	0.83 [0.51-1.36]	0.46				
	Low	1.01 [0.63-1.64]	0.97				
Rody metagene expression	High	1		1		1	
	Low	2.07 [1.21-3.54]	0.008	1.13 [0.57-2.23]	0.73	1.11 [0.41-3.00]	0.84
Sabatier metagene expression	High	1		1		1	
	Low	2.06 [1.36-3.12]	0.0006	0.89 [0.39-2.04]	0.79	0.71 [0.27-1.83]	0.47
Teschendorff metagene expression	High	1		1		1	
	Low	0.70 [0.46-1.06]	0.09	0.84 [0.54-1.30]	0.44	0.89 [0.55-1.46]	0.66
Desmedt metagene expression	High	1		1		1	
	Moderate	2.24 [1.31-3.82]	0.003	1.27 [0.66-2.44]	0.47	1.36 [0.57-3.26]	0.49
	Low	2.33 [1.37-3.96]	0.002	0.94 [0.43-2.01]	0.87	0.83 [0.29-2.34]	0.72
Tfh_Gu.Trantien metagene expression	High	1		1		1	
	Moderate	1.47 [0.85-2.55]	0.17	0.96 [0.46-2.01]	0.91	0.64 [0.23-1.78]	0.40
	Low	2.66 [1.61-4.40]	0.0001	1.30 [0.54-3.16]	0.56	1.04 [0.32-3.41]	0.94
Th1_Gu.Trantien metagene expression	High	1		1		1	
	Moderate	1.91 [1.10-3.30]	0.02	1.17 [0.58-2.39]	0.66	1.16 [0.38-3.51]	0.80
	Low	2.66 [1.58-4.49]	0.0003	1.30 [0.55-2.93]	0.57	1.14 [0.33-3.93]	0.84
Karn metagene expression	High	1					
	Low	0.74 [0.48-1.15]	0.19				
Burstein groups	BLIA	1		1		1	
	BLIS	1.92 [1.17-3.13]	0.01	1.19 [0.68-2.07]	0.55	1.41 [0.75-2.62]	0.28
	LAR	1.87 [1.07-2.27]	0.03	0.98 [0.53-1.80]	0.94	1.04 [0.53-2.06]	0.91
	MES	0.95 [0.47-1.92]	0.89	0.91 [0.45-1.84]	0.79	1.03 [0.49-2.15]	0.94
	UNCLASSIFIED	1.06 [0.31-3.60]	0.92	0.56 [0.16-1.96]	0.36	0.52 [0.14-1.97]	0.33

* 9 multivariate models including NPI score, the Immunity2 metagene and each of the Rody, Sabatier, Teschendorff, Desmedt, Gu-Trantien Tfh, Gu-Trantien Th1, and Burstein signatures, one-by-one, in the model.

HR of the 'Immunity2' metagene in each multivariate model:

With Rody metagene: Immunity2 low expression= 2.44 [1.42-4.19], p=0.001

With Sabatier metagene: Immunity2 low expression= 2.86 [1.23-6.65], p=0.01

With Teschendorff metagene: Immunity2 low expression= 2.45 [1.57-3.83], p=8.64 ^{10⁻⁵}

With Desmedt metagene: Immunity2 low expression= 2.69 [1.46-4.96], p=0.002

With Tfh_Gu.Trantien metagene: Immunity2 low expression= 2.19 [1.04-4.61],

p=0.04

With Th1_Gu.Trantien metagene: Immunity2 low expression= 2.22 [1.13-4.37],

p=0.02

With Burstein centroid: Immunity2 low expression= 2.49 [1.53-4.07], p=0.0003

** global multivariate model with NPI score, the Immunity2 metagene and the Rody, Sabatier, Teschendorff, Desmedt, Gu-Trantien Tfh, Gu-Trantien Th1, and Burstein signatures.

Table S3. Survival analysis (disease-specific survival) with PD1, PDL1, CTLA4 and TILs metagenes. Univariate and multivariate analysis.

254 triple-negative breast cancer patients		Univariate analysis		Multivariate analysis*		Multivariate analysis**	
		DS-survival HR [95% CI]	p value	DS-survival HR [95% CI]	p value	DS-survival HR [95% CI]	p value
Immunity2 metagene expression	High	1				1	
	Low	2.39 [1.57-3.64]	5.1 ^{10⁻⁵}			4.67 [1.51-14.44]	0.007
PD1 metagene expression	High	1		1		1	
	Low	2.42 [1.58-3.71]	4.6 ^{10⁻⁵}	1.48 [0.71-3.09]	0.29	5.99 [1.64-21.92]	0.007
PDL1 metagene expression	High	1		1		1	
	Low	2.15 [1.42-3.27]	0.0003	0.78 [0.31-1.98]	0.60	0.33 [0.08-1.32]	0.12
CTLA4 metagene expression	High	1		1		1	
	Low	2.01 [1.33-3.04]	0.0009	0.85 [0.43-1.68]	0.63	0.54 [0.19-1.57]	0.26
TILs metagene expression	High	1		1		1	
	Low	2.00 [1.33-3.03]	0.001	0.67 [0.27-1.64]	0.38	0.53 [0.17-1.66]	0.28

* 4 multivariate models including NPI score, the Immunity2 metagene and each of the PD1, PDL1 and CTLA4 metagenes, one-by-one, in the model.

HR of the 'Immunity2' metagene in each multivariate model:

With PD1 metagene: Immunity2 low expression= 1.88 [0.90-3.90], p=0.09

With PDL1 metagene: Immunity2 low expression= 3.25 [1.27-8.34], p=0.01

With CTLA4 metagene: Immunity2 low expression= 2.98 [1.47-6.02], p=0.002

With TILs metagene: Immunity2 low expression= 3.75 [1.49-9.42], p=0.005

** global multivariate model with NPI score, the Immunity2 metagene and the PD1, PDL1 and CTLA4 metagenes.

RESEARCH ARTICLE

A Stromal Immune Module Correlated with the Response to Neoadjuvant Chemotherapy, Prognosis and Lymphocyte Infiltration in *HER2*-Positive Breast Carcinoma Is Inversely Correlated with Hormonal Pathways

Anne-Sophie Hamy¹*, Hélène Bonsang-Kitzis^{1,2}*, Marick Lae³, Matahi Moarii^{4,5}, Benjamin Sadacca^{1,6}, Alice Pinheiro¹, Marion Galliot¹, Judith Abecassis^{1,4,5}, Cecile Laurent¹, Fabien Reyal^{1,2*}

1 Institut Curie, PSL Research University, Translational Research Department, INSERM, U932 Immunity and Cancer, Residual Tumor & Response to Treatment Laboratory (RT2Lab), Paris, France, **2** Department of Surgery, Institut Curie, Paris, France, **3** Department of Tumor Biology, Institut Curie, Paris, France, **4** Mines Paristech, PSL-Research University, CBIO-Centre for Computational Biology, Mines ParisTech, Fontainebleau, France, **5** U900, INSERM, Institut Curie, Paris, France, **6** Laboratoire de Mathématiques et Modélisation d'Evry, Université d'Evry Val d'Essonne, Evry, France

* These authors contributed equally to this work.

* fabien.reyal@curie.fr



CrossMark
click for updates

 OPEN ACCESS

Citation: Hamy A-S, Bonsang-Kitzis H, Lae M, Moarii M, Sadacca B, Pinheiro A, et al. (2016) A Stromal Immune Module Correlated with the Response to Neoadjuvant Chemotherapy, Prognosis and Lymphocyte Infiltration in *HER2*-Positive Breast Carcinoma Is Inversely Correlated with Hormonal Pathways. PLoS ONE 11(12): e0167397. doi:10.1371/journal.pone.0167397

Editor: William B. Coleman, University of North Carolina at Chapel Hill School of Medicine, UNITED STATES

Received: September 19, 2016

Accepted: November 14, 2016

Published: December 22, 2016

Copyright: © 2016 Hamy et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: A-S Hamy-Petit was supported by an ITMO-INSERM-AVIESAN cancer translational research grant. Funding was also obtained from the Site de Recherche Intégrée en Cancérologie/ Institut National du Cancer (InCa-DGOS-4654). The funders had no role in study design, data

Abstract

Introduction

HER2-positive breast cancer (BC) is a heterogeneous group of aggressive breast cancers, the prognosis of which has greatly improved since the introduction of treatments targeting *HER2*. However, these tumors may display intrinsic or acquired resistance to treatment, and classifiers of *HER2*-positive tumors are required to improve the prediction of prognosis and to develop novel therapeutic interventions.

Methods

We analyzed 2893 primary human breast cancer samples from 21 publicly available datasets and developed a six-metagenes signature on a training set of 448 *HER2*-positive BC. We then used external public datasets to assess the ability of these metagenes to predict the response to chemotherapy (Ignatiadis dataset), and prognosis (METABRIC dataset).

Results

We identified a six-metagenes signature (138 genes) containing metagenes enriched in different gene ontologies. The gene clusters were named as follows: Immunity, Tumor suppressors/proliferation, Interferon, Signal transduction, Hormone/survival and Matrix clusters. In all datasets, the Immunity metagenes was less strongly expressed in ER-positive than in ER-negative tumors, and was inversely correlated with the Hormonal/survival metagenes. Within the signature, multivariate analyses showed that strong expression of the

collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

Abbreviations: BC, breast cancer; BCSS, breast cancer-specific survival; BC_CL, breast cancer cell lines; CCLE, Cancer Cell Line Encyclopedia; CGP, Cancer Genome Project; ER, estrogen receptor; GE, gene expression; *HER2*-positive BC, *HER2*-positive breast cancer; *HER2*, human epidermal growth factor receptor 2; PR, progesterone receptor; pCR, pathological complete response; RMA, robust multichip average; TILs, tumor-infiltrating lymphocytes.

“Immunity” metagene was associated with higher pCR rates after NAC (OR = 3.71 [1.28–11.91], $p = 0.019$) than weak expression, and with a better prognosis in *HER2*-positive/ER-negative breast cancers (HR = 0.58 [0.36–0.94], $p = 0.026$). Immunity metagene expression was associated with the presence of tumor-infiltrating lymphocytes (TILs).

Conclusion

The identification of a predictive and prognostic immune module in *HER2*-positive BC confirms the need for clinical testing for immune checkpoint modulators and vaccines for this specific subtype. The inverse correlation between Immunity and hormone pathways opens research perspectives and deserves further investigation.

Introduction

HER2-positive breast carcinomas (BCs) are defined by amplification and overexpression of the *HER2* tyrosine kinase receptor gene (17q12). The tumors of this subgroup have aggressive pathological features and a high rate of early distant metastatic events. They are routinely treated with a combination of docetaxel plus a monoclonal antibody targeting the *HER2* receptor (trastuzumab). Other drugs also appear to be of major interest and will probably be made available for routine treatment in the near future (lapatinib, pertuzumab and T-DM1).

HER2-positive BCs constitute a heterogeneous group of tumors differing in histological features, gene expression profiles, clinical behavior, overall prognosis, and response to conventional systemic cytotoxic therapy. Trastuzumab-based treatments have been used for the last decade and have substantially improved outcomes in patients with early or metastatic *HER2*-positive BC. However, some *HER2*-positive tumors display intrinsic or acquired resistance to trastuzumab. Robust classifiers are required, both to improve our understanding of the molecular basis of *HER2*-positive BC and to develop novel therapeutic interventions.

We developed a two-step biological network-driven gene selection process: 1) identification of the most variable genes displaying highly correlated patterns of expression, 2) direct connection of these genes within known biological networks. This method has been shown to construct molecular signatures efficiently [1–3]. We defined a *HER2*-positive molecular subtype classification and identified a stromal immune module gene expression profile strongly correlated with predicted response to chemotherapy, prognosis and lymphocytic infiltration. This classification provides considerable biological insight, and has potential for use in the development of therapeutic interventions, such as novel immunotherapies in particular.

Material and methods

Data normalization and quality control

Training, validation and Ignatiadis datasets. We collected 21 publicly available datasets (described in the [S1 File](#)) containing raw gene expression data for 2893 primary human breast cancer samples. The data were normalized by the robust multichip average (RMA) procedure from the EMA R package [4]. The datasets were split into training (HGU-133A Affymetrix* arrays, 12 datasets, $n = 1921$) and validation (HGU-133Plus2 Affymetrix* arrays (9 datasets, $n = 972$) sets. Batch effects were eliminated by the median centering of each probe-set across arrays and by an independent quantile normalization of all arrays for each dataset. We controlled for outliers with the Array Quality Metrics R package. We also collected two large

datasets to validate our classification: The Ignatiadis dataset (Affymetrix data $n = 996$) [5] and the METABRIC dataset (Illumina data $n = 1992$) published by Curtis *et al.* [6].

Determination and preprocessing of *HER2*-positive breast cancer samples

We identified the *HER2*-positive samples in the training and validation datasets, on the basis of transformed ERBB2 mRNA expression, as described by Gong *et al.* [7], and using the bimodal distribution of ERBB2 expression for the Ignatiadis and the METABRIC dataset.

Gene selection process

Consensus clustering with the ConsensusClusterPlus R Package was applied to the training set with a ward inner, final linkage and Pearson distance, to determine the optimal number of robust gene clusters for the most variable genes (standard deviation >0.8). We investigated the enrichment of each gene cluster in particular types of genes, and categorized and labeled genes clusters according to the different gene ontologies. We then identified known biological networks, for each gene cluster separately, using String* database software version 9.1 (<http://string-db.org/>) [8]. We then applied a two-step selection process: 1) we selected strong biological networks by retaining only genes for which connection scores of at least 0.7 were obtained with String* database software, 2) within each biological network, we selected groups of genes with correlated expression patterns and a correlation coefficient of at least 0.5.

For each dataset (the training, validation, Ignatiadis and METABRIC sets), we applied a hierarchical clustering procedure with a ward inner, final linkage and Pearson distance to the *HER2*-positive gene expression (GE) profiles, using the selected genes to visualize the optimal number of stable *HER2*-positive subtypes.

Metagene construction

We defined a metagene as an aggregate patterns of gene expression. Metagene expression was assessed by calculating the median normalized expression values of all probe sets in the respective gene clusters for each sample. The metagene value for each sample was then discretized on the basis of the median value, as “high” or “low”.

Association between expression of the Immunity metagene and that of ESR1, PGR, and AR

All the analyses were performed on all four datasets (training, validation, Ignatiadis, METABRIC). The levels of expression of ESR1, PGR and AR were compared between “Immunity low” and “Immunity high” samples, by ANOVA. Levels of Immunity metagene expression were compared between samples positive and negative for ER, PR, and AR, by ANOVA. We also performed ANOVA for each gene of the Immunity metagene as a function of ER status.

Analysis of the predicted response to NAC

We analyzed the predicted response to chemotherapy in the datasets published by Ignatiadis *et al.* [5]. Expression data were summarized by defining a metagene for each gene cluster. The clinical and pathological variables available for each dataset are described in [S1 File](#). Qualitative variables were compared with logistic regression models.

Prognostic analysis

Prognostic analysis was performed on the METABRIC set. Expression data were summarized by defining a metagene for each gene cluster. The clinical and pathological variables available for each dataset are described in [S1 File](#). Survival analyses were performed for the whole population, and separately for ER-positive and ER-negative patients, by calculating Kaplan-Meier estimates of the survival function. The endpoint of these analyses was breast cancer-specific survival (BCSS). Survival curves were compared in log-rank tests. Hazard ratios were estimated with Cox's proportional hazard model. Predictive and prognostic analyses were performed with the R survival package. Variables associated with pCR or BCSS with a *P*-value <0.10 in univariate analysis were included in the multivariate model. Variables with *P*-values <0.05 in multivariate analysis were considered statistically significant.

Correlation with tumor-infiltrating lymphocyte levels

We downloaded the gene expression data from the REMAGUS 02 trial [9] and retrieved 27 samples for which paraffin-embedded tissue sections were available at our institution. All patients enrolled in this study gave their informed written consent. Histologic microbiopsy specimens were evaluated independently for the presence of a lymphocytic infiltrate (intratumoral TILs and stromal TILs) by one BC pathologist (ML) and one breast physician (ASH) unaware of the gene expression classification. Percentages of TILs and StrL were compared, as a function of Immunity metagene status, in ANOVA. The correlations between Immunity metagene expression and the percentages of TILs and StrL were assessed by calculating Pearson's correlation coefficient.

Expression of the gene signature in human breast cancer cell lines

We downloaded the gene expression profiles of the human cancer cell lines from the Cancer Cell Line Encyclopedia (CCLE) [10] of Novartis/the Broad Institute and the Cancer Genome Project (CGP) [11] of the Sanger Institute. We normalized the data for all the cell lines from different tissues together.

Statistical analysis

Data were processed and statistical analyses were carried out with R software version 3.1.2 [12] (www.cran.r-project.org).

Results

HER2-positive gene expression profiles identify six main gene clusters

HER2-positive BC samples were selected from 21 publicly available datasets ($n = 3,247$ breast cancer samples) and separated into a training set and a validation set ([S1 File](#) and [S1 Fig](#)). In the training set, we applied a gene selection process based on biological networks ([Fig 1A to 1C](#)), to decrease the instability intrinsic to molecular classification methods (see [S1 File](#)), as previously described for triple-negative breast cancers (TNBCs) [3]. We selected a final set of 138 genes ([S1 Table](#)), composed of six gene clusters enriched in different gene ontologies: Immunity ($n = 28$), Interferon ($n = 11$), Signal transduction ($n = 20$), Hormonal/survival ($n = 22$), Tumor suppressors/Proliferation ($n = 36$), Matrix ($n = 21$) ([Fig 1D](#)). We defined a metagene for each of the six gene clusters identified in this way ([S1 File](#)). The Immunity and Interferon metagenes displayed similar patterns of expression. The Immunity and Hormonal/survival metagenes displayed the strongest inverse correlation for expression (coefficient of -0.46) ([Fig 1E and 1F](#)). The correlations between the 138 genes and the metagenes are

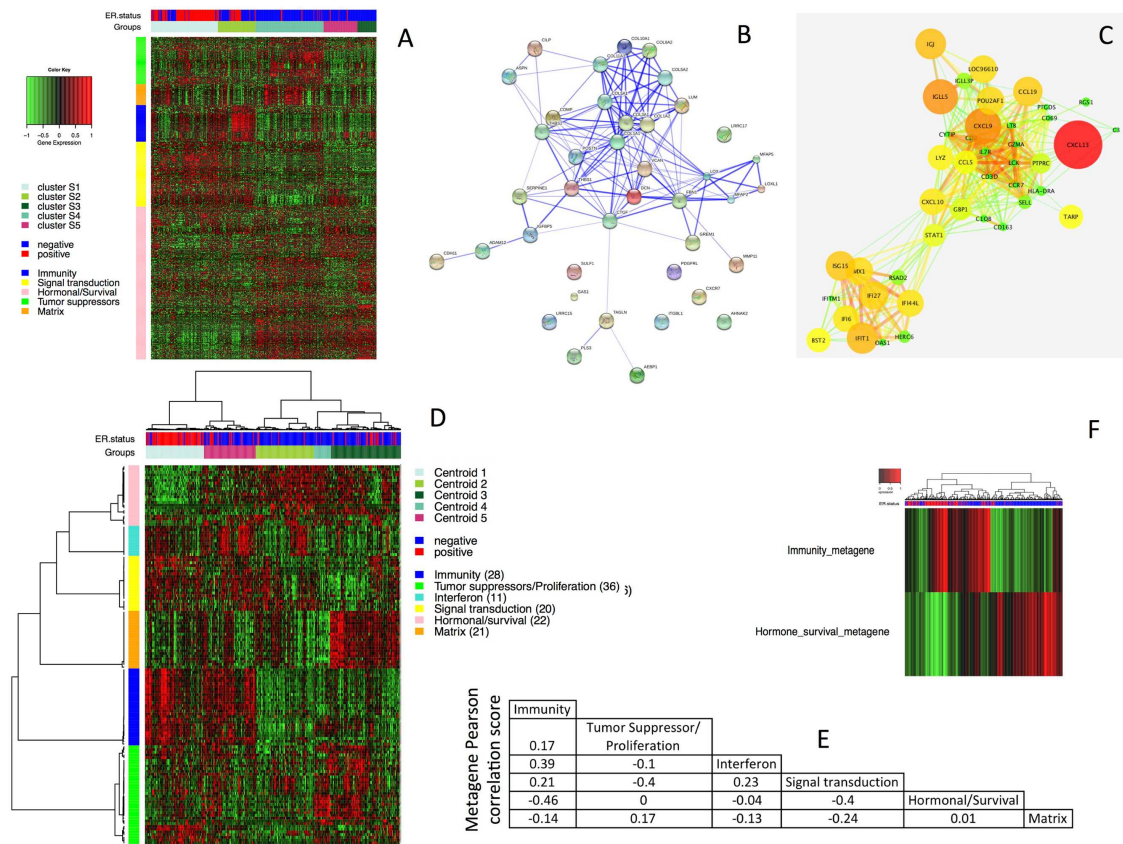


Fig 1. Gene selection process. **A** Heatmap showing the 616 most variable genes in the 448 *HER2*-positive samples (training set). **B** String database software confidence view of the Matrix genes cluster. Stronger associations between genes are represented by thicker lines. **C** Cytoscape View for the Immunity gene cluster. GE correlations between genes are indicated by edges (edge color varies from green to red and edge size increases with increasing correlation) and gene expression variance is represented by node color (node color varies from green to red and node size increases with increasing variance). **D** Heatmap showing the relative expression of 138 selected genes in 448 *HER2*-positive samples from the training set. **E** Table of Pearson's correlation coefficient values for the correlations between the 6 metagenes. **F** Heatmap showing the anticorrelation between the Immunity and the Hormone/Survival metagene.

doi:10.1371/journal.pone.0167397.g001

described in more detail in [S1 File](#). For validation, we applied hierarchical clustering methods to three additional independent *HER2*-positive datasets; a validation set ($n = 194$), the Ignatidis dataset ($n = 82$) and the METABRIC dataset ($n = 248$) ([S1 File](#) and [S2 Fig](#)).

The expression of the Immunity metagene is strongly associated with ER status, PR, and AR status

Given the inverse correlation between Immunity metagene and the Hormonal/survival metagene expression ([Fig 2A](#)) and with the strong correlation of Hormonal/survival metagene expression with ESR1 expression (Pearson correlation coefficient = 0.77), we compared levels of ESR1, PGR and AR expression as a function of Immunity metagene status ([Fig 2B](#)). These three genes were consistently more strongly expressed in the “Immunity low” subgroup than in the “Immunity high” subgroup ($p < 10^{-16}$, $p < 10^{-8}$, $p = 0.002$ respectively). Similar results were obtained with the other three datasets, although less consistently for PR and AR ([S1 File](#)).

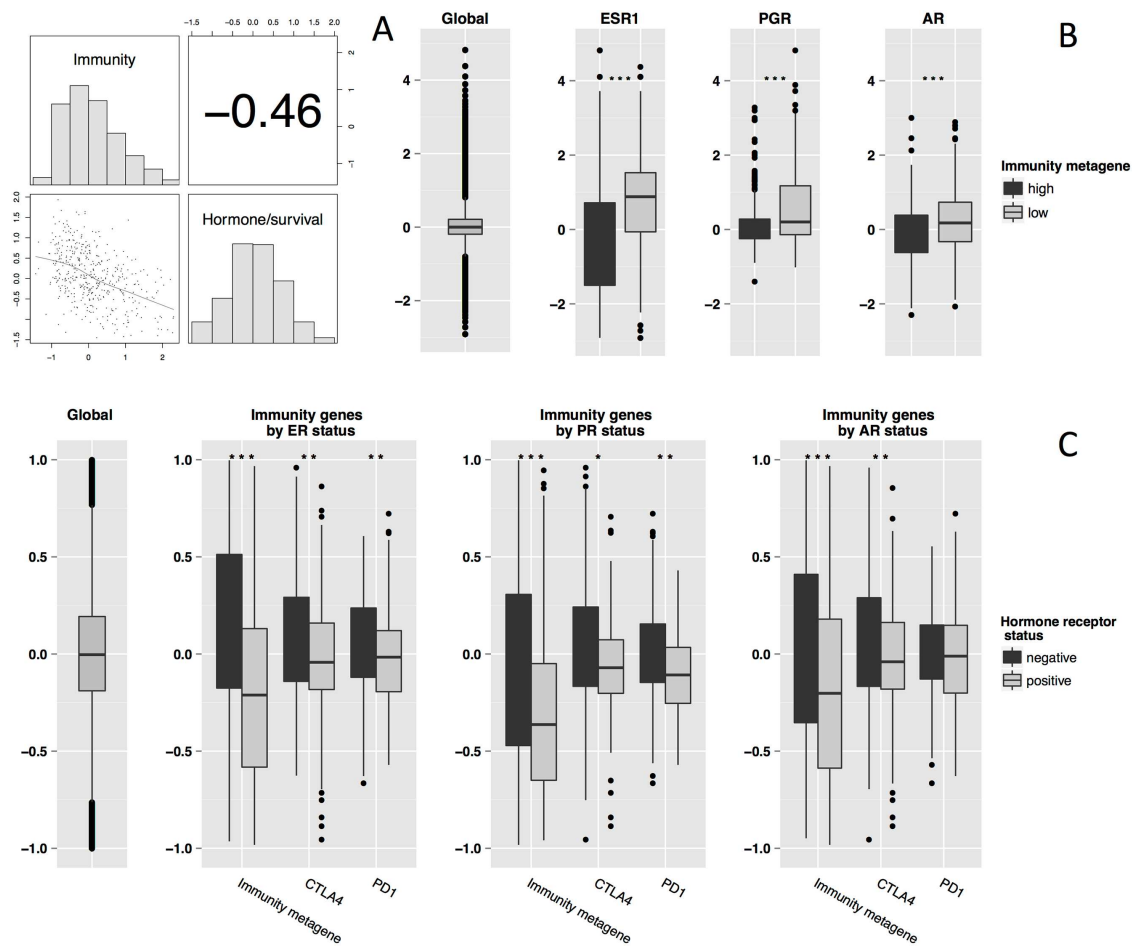


Fig 2. Association between Hormone genes expression and Immunity genes expression. **A** Correlation of Immunity metagene and Hormone/Survival metagene expression (training set). Pearson's correlation coefficient is -0.46 (95% CI [-52.7–38.0], $p < 10^{-16}$). **B** Boxplots of global gene expression and ESR1, PGR and AR expression by Immunity metagene status, "low" versus "high" in the training set (A). P-values for ANOVA are $p = 10^{-16}$, $p = 10^{-6}$ and $p = 0.0002$, respectively. **C** Boxplots of Immunity metagene and immune gene (CTLA4 and PD1) expression levels by ER, PR and AR status in the training set (A). The p values for ANOVA were $p < 10^{-16}$, $p = 0.002$ and $p = 0.008$ for the Immunity metagene, CTLA4 and PD1 by ER status, respectively; $p = 0.0001$, $p = 0.05$ and $p = 0.001$ by PR status, respectively; and $p < 10^{-6}$, $p = 0.006$ and $p = 0.23$ by AR status, respectively. The statistical significance (p -value) of the difference between gene expression values is indicated by black stars (p -value ≤ 0.05 : *; p -value ≤ 0.01 : **; p -value ≤ 0.001 : ***).

doi:10.1371/journal.pone.0167397.g002

We then compared the levels of expression of our Immunity metagene with those of two other immune genes (CTLA4 and PD1; PDL1 was not available on the HGU133a Chip) as a function of ER, PR, and AR status. The Immunity metagene and CTLA4 were significantly more strongly expressed in the ER-negative, PR-negative, and AR-negative subgroups (Fig 2C). PD1 was significantly more strongly expressed in ER-negative and PR-negative tumors, but the difference in expression levels according to AR status was not significant for this gene. Similar findings were obtained when we compared each of the genes of the Immunity metagene separately as a function of ER status, and across the three other datasets. The results were less consistent for PR and AR (see S1 File). The proportions of tumors in the Immunity

metagene “low” and “high” subgroups as a function of ER status differed significantly in three of the four datasets. ER-positive samples were more likely to be in the Immunity metagene “low” group, whereas ER-negative samples were more likely to be in the Immunity metagene “high” group (S1 File).

These findings suggest that there are strong inverse interactions between immune pathways that are captured by the Immunity metagene and ER, PR, and AR hormonal pathways in *HER2*-positive breast cancer tumors.

Predictive value of the Immunity metagene in *HER2*-positive breast cancers

We assessed the value of the six metagenes for predicting the response to neoadjuvant chemotherapy (NAC) on 82 *HER2*-positive samples from the Ignatiadis dataset. Univariate analysis identified four factors (ER status, tumor grade, and Immunity and Hormone/survival metagene expression) correlated with pathological complete response (pCR) (Table 1). In multivariate analysis, both ER status and the Immunity metagene were significantly associated with pCR (ER-positive: OR = 0.29 [0.09–0.82] versus ER-negative (reference class), $p = 0.02$; Immunity metagene “high” expression: OR = 3.71, 95% CI [1.28–11.91], versus “low” expression

Table 1. Association of clinical factors and gene cluster expression with pathological response rates after neoadjuvant chemotherapy in the Ignatiadis dataset, univariate and multivariate analysis.

		n	Univariate analysis			Multivariate analysis		
			OR	IC	<i>p</i> val	OR	IC	<i>p</i> val
Age	<50 y.o.	39	1					
	> = 50 y.o	43	1.1	[0.42–2.9]	0.84			
ER status	ER negative	38	1			1		
	ER positive	44	0.23	[0.08–0.63]	0.006	0.29	[0.09–0.82]	0.023
PR status	PR negative	78	1					
	PR positive	4	NA	NA*	0.99			
Tumoral size	T1 and T2	34	1					
	T3	21	0.34	[0.08–1.14]	0.096			
	T4	27	0.41	[0.12–1.23]	0.122			
Nodal status	N0	12	1					
	N1,N2 or N3	55	1.02	[0.26–5.1]	0.974			
Tumor grade	Grade I or II	24	1					
	Grade III	51	4.16	[1.22–19.26]	0.037			
Immunity metagene expression	low	41	1			1		
	high	41	4.57	[1.65–14.2]	0.005	3.71	[1.28–11.91]	0.019
Tumor suppressor/proliferation metagene	low	41	1					
	high	41	1.61	[0.62–4.3]	0.333			
Interferon metagene expression	low	41	1					
	high	41	0.49	[0.18–1.27]	0.149			
Signal transduction metagene expression	low	41	1					
	high	41	1.27	[0.49–3.33]	0.628			
Hormone/survival metagene expression	low	41	1					
	high	41	0.22	[0.07–0.61]	0.005			
Matrix metagene expression	low	41	1					
	high	41	1.27	[0.49–3.33]	0.628			

*: OR not available, no pCR in the PR-positive group

doi:10.1371/journal.pone.0167397.t001

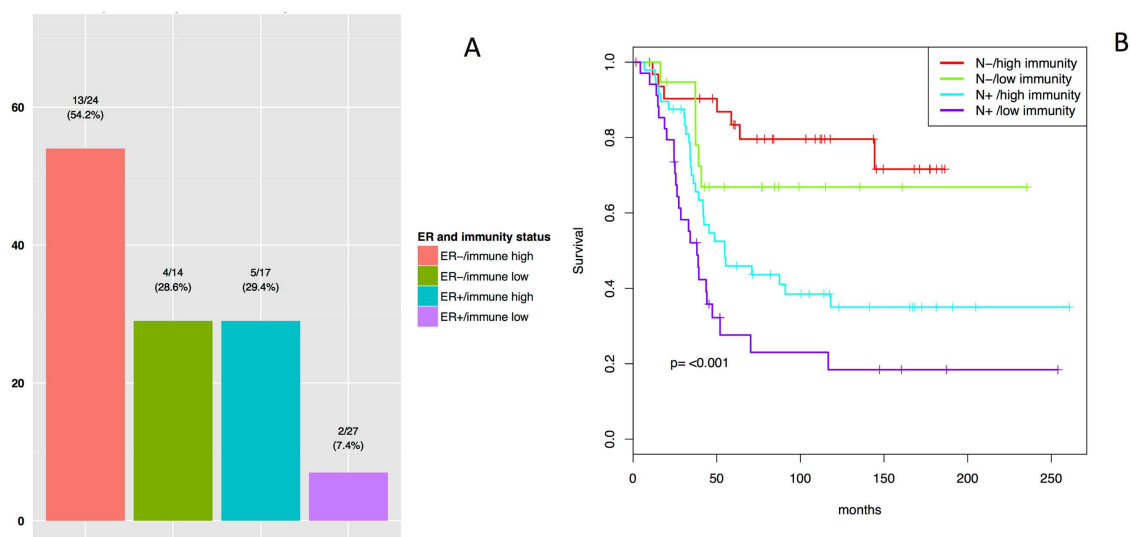


Fig 3. pCR and DSS outcomes in the Ignatiadis and the METABRIC dataset. **A:** pCR rates by ER and Immunity metagene status (low versus high in the Ignatiadis dataset). **B:** Kaplan-Meier plots. Disease-specific survival of the ER-negative population ($n = 138$) according to Immunity metagene expression (low/high) and nodal status in the METABRIC dataset.

doi:10.1371/journal.pone.0167397.g003

(reference class), $p = 0.02$) (Fig 3A). Analyses in the subset of patients that did not receive trastuzumab ($n = 75$) yielded similar results (S1 File).

We compared the predictive value of the Immunity metagene with that of nine immune signatures or metagenes already validated as predictors of the response to chemotherapy for breast cancer, notably in *HER2*-positive BCs [13–18]. In multivariate analysis, the Immunity metagene and six of the other signatures or metagenes tested were identified as predictive of the response to chemotherapy. The smallest p -value obtained was that for our Immunity metagene ($p = 0.019$), OR = 3.71, 95% CI [1.28–11.91] (S2 Table).

We then investigated the reasons for which the Immunity metagene (28 genes) was predictive of pCR in *HER2*-positive BCs, whereas the Immunity2 metagene (47 genes) published by Bonsang *et al.* [3] was not in a TNBC population [3], despite the strong correlation between these two signatures in three independent datasets (correlation coefficients: 0.96; 0.94 and 0.96 in the training set, METABRIC and Ignatiadis dataset, respectively). We applied both signatures to the whole population for the Ignatiadis dataset, and analyzed pCR as a function of breast cancer subtype and Immunity metagene status. We found that pCR rates were significantly higher in the “Immunity high” subgroup in *HER2*-negative/*ER*-positive (16.7% versus 8.4%, OR = 2.17, $p = 0.05$), *HER2*-positive (43.6% versus 16.7%, OR = 3.84, $p = 0.01$), and TNBC breast cancers (37.3 versus 22.6%, OR = 2.08, $p = 0.03$) (S3A Fig). A similar pattern was observed for the Immunity2 metagene (*HER2*-negative-*ER* positive: 16.5% versus 8.1%, OR = 2.22, $p = 0.05$), *HER2*-positive (45.5% versus 18.7%, OR = 3.57, $p = 0.01$), and TNBC breast cancers (36.3 versus 24.6%, OR = 1.75, $p = 0.08$; S3B Fig), but the difference was not statistically significant ($p = 0.08$) in the TNBC subgroup. Interestingly, Immunity metagene status appeared to have a larger effect on pCR rates in the *HER2*-positive subgroup (OR = 3.84 and 3.57, respectively) than in the *ER*-positive (OR = 2.17 and 2.22, respectively) and TNBC (OR = 2.08 and 1.75, respectively) subgroups. The Immunity metagene therefore seems to be

associated with the response to NAC in all breast cancer subtypes, with a marked effect in terms of both the strength and magnitude of the association in the *HER2*-positive subgroup.

Prognostic value of the Immunity metagene in *HER2*-positive breast cancers

The prognostic value of the 138-gene *HER2*-positive signature was assessed with 248 *HER2*-positive samples from the METABRIC dataset. Univariate analysis identified five factors (menopausal status, tumor size, nodal status, Immunity and Signal transduction metagene expression) significantly correlated with a poor outcome (disease-specific survival) (Table 2).

In multivariate analysis, nodal status (node-negative versus node-positive) was significantly associated with a poor outcome (HR = 3.29 [2.14–5.06], $p < 0.001$), and there was a trend towards association between high levels of Immunity metagene expression and better disease-free survival (DFS; HR = 0.70 [0.48–1.01], $p = 0.054$). In the ER-negative population, the Immunity metagene was found to be of significant prognostic value in multivariate analysis ($n = 138$) (HR = 0.58 [0.36–0.94], $p = 0.026$; Fig 3B), but was not associated with DFS in the ER-positive population ($n = 110$) ($p = 0.43$). We compared the prognostic value of the Immunity metagene with that of nine previously published immune signatures or metagenes known to predict survival in several breast cancer subtypes [14,17–22]. None of the signatures or metagenes described above was significantly associated with prognosis (S2F Table).

The Immunity metagene is correlated with tumor-infiltrating lymphocytes (TILs) in *HER2*-positive breast cancer

We then investigated the correlation between Immunity metagene expression and lymphocyte infiltration. We analyzed an independent set of *HER2*-positive tumors for which both histology and gene expression data were available ($n = 27$). Intratumoral TILs (TLs) and stromal TILs (StrL) were evaluated separately. Intratumoral TIL percentages were significantly higher in patients with strong Immunity metagene expression than in those with weak Immunity metagene expression (24% and 9%, respectively, $p = 0.001$) (Fig 4A). The same pattern was observed for the percentage of stromal TILs (36% versus 16.6%, $p = 0.009$) (Fig 4B). The coefficients of correlation between Immunity metagene expression level on the one hand and the percentage of intratumoral TILs (Fig 4C) or stromal TILs (Fig 4D) on the other hand were high ($r = 0.60$, $p < 0.001$ and $r = 0.69$, $p < 0.00001$ respectively). Lymphocyte infiltration is shown for two specimens, one with weak (Fig 5A and 5B), and the other with strong lymphocyte infiltration (Fig 5C and 5D). The Immunity metagene was therefore strongly correlated with the amount of lymphocyte infiltration in both the stromal compartment and the tumor bed.

The Immunity metagene corresponds to the B-cell, T-cell and CD8 cell pathways

The Immunity metagene was strongly correlated with several published immune signatures (S4 Fig and S1 File), suggesting the use of similar immune pathways (see S1 File). We analyzed the correlation between expression of the Immunity and Interferon metagenes and expression of the metagenes defined by Gatzka *et al.* [23] (IFN-alpha, IFN-gamma, STAT3, TGF-beta, TNF-alpha) and Palmer *et al.* [24] (LB, LT, CD8, GRANS, LYMPHS). This analysis was performed on the METABRIC dataset. The Immunity metagene was highly correlated with the B-cell, T-cell and CD8 cell metagenes (Pearson correlation coefficients: 0.89, 0.86, and 0.90, respectively; S5 Fig). We also assessed the correlations between the expression of PD1, PDL1,

Table 2. Survival analysis (disease-specific survival) in the METABRIC dataset (univariate and multivariate analysis); whole population and ER-negative population.

		Whole population (n = 248)							ER negative population (n = 138)								
		n	Univariate analysis			Multivariate analysis			n	Univariate analysis			Multivariate analysis				
			HR	IC	pval	HR	IC	pval		HR	IC	pval	HR	IC	pval		
Age at diagnosis	<= 45 y. o.	52	1		-				25	1							
	45–55	59	0.67	[0.4–1.14]	0.142				19	0.65	[0.36–1.18]	0.153					
	>55	130	0.66	[0.43–1.04]	0.071				23	0.62	[0.35–1.1]	0.103					
Menopausal status	Pre	74	1		-				32	1							
	Post	167	0.68	[0.46–1]	0.051				33	0.67	[0.41–1.09]	0.11					
Tumoral size	< 20 mm	68	1		-				15	1							
	>= 20 mm	173	1.87	[1.18–2.96]	0.008				52	1.51	[0.85–2.69]	0.159					
Tumor grade	I	3	1		-				10	1	-	-					
	II	53	1.66	[0.22–12.19]	0.621				55	0.942	[0.48–1.85]	0.863					
	III	178	1.81	[0.25–13.05]	0.554				10	NA	NA	NA					
ER status	negative	135	1		-												
	positive	108	0.74	[0.51–1.07]	0.108												
PR status	negative	193	1		-				65	1							
	positive	50	0.84	[0.53–1.34]	0.46				2	2.3	[0.56–9.49]	0.25					
Nodal status	N-	105	1		-	1			13	1			1				
	N+	138	3.26	[2.13–5.01]	<0.001	3.29	[2.14–5.06]	<0.001	54	3.55	[1.93–6.51]	<0.001	3.57	[1.94–6.55]	<0.001		
NPI	GP	38	1		-				6	1							
	IP	155	1.26	[0.71–2.25]	0.433				35	1.01	[0.42–2.4]	0.988					
	PP	50	3.32	[1.78–6.19]	<0.001				26	2.81	[1.15–6.84]	0.023					
Metagene expression																	
Immunity	low	122	1		-	1			54	1							
	high	121	0.71	[0.49–1.03]	0.073	0.70	[0.48–1.01]	0.054	81	0.58	[0.36–0.94]	0.028	0.58	[0.36–0.94]	0.026		
TS/proliferation	low	121	1		-				50	1							
	high	122	1.04	[0.72–1.51]	0.828				85	0.84	[0.51–1.38]	0.491					
Interferon	low	122	1		-				78	1							
	high	121	1.23	[0.85–1.78]	0.278				57	1.28	[0.79–2.07]	0.316					
Signal transduction	low	121	1		-				72	1							
	high	122	1.48	[1.02–2.14]	0.04				63	1.34	[0.83–2.17]	0.232					
Hormone/survival	low	122	1		-				114	1							
	high	121	0.94	[0.65–1.36]	0.751				21	1.35	[0.72–2.52]	0.351					
Matrix	low	121	1		-				69	1							
	high	122	1.05	[0.73–1.52]	0.785				66	1.03	[0.64–1.67]	0.889					

Abbreviations: GP: good prognosis, IP: intermediate prognosis, PP: poor prognosis; TS: tumor suppressor

doi:10.1371/journal.pone.0167397.t002

CTLA4, and that of their respective metagenes. The PD1 and CTLA-4 metagenes were constructed from the genes most strongly correlated with the PD1 and CTLA-4 genes, respectively (Pearson's correlation coefficient > 0.8). The PDL1 metagene was defined by Sabatier *et al.* [25]. Pearson's correlation coefficients for the relationships between the Immunity metagene and each individual gene were strong for PD1 and CTLA-4 (Pearson's correlation coefficient: 0.75 and 0.84, respectively), and weaker for PDL1 (0.36), but the expression of all three metagenes was strongly correlated with that of the Immunity metagene (Pearson's correlation

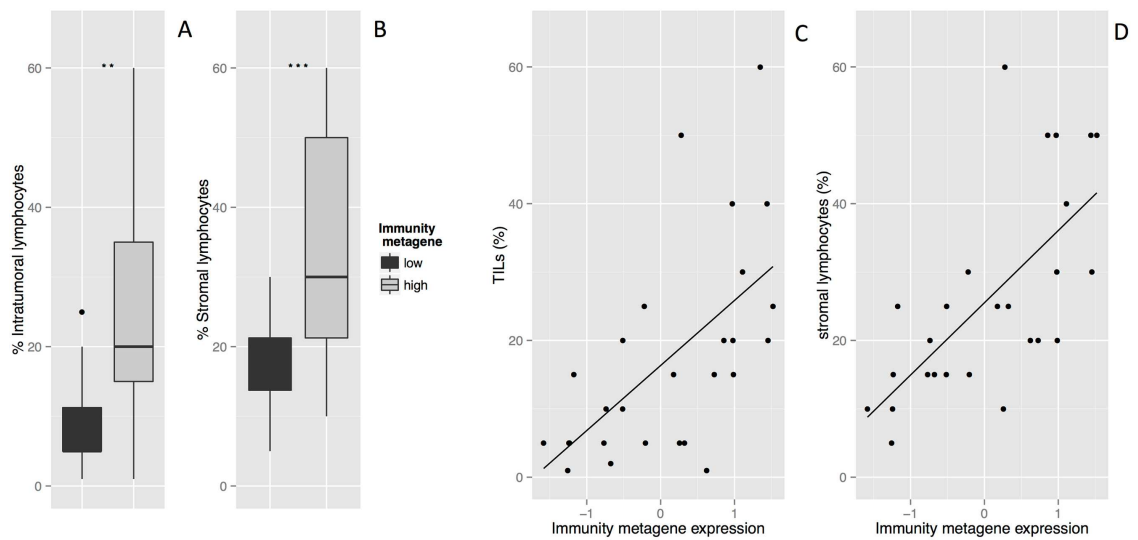


Fig 4. Association between tumor-infiltrating lymphocyte levels and Immunity metagene expression in the REMAGUS dataset. A: Percentage of intratumoral TILs according to Immunity metagene status (low versus high). **B** Percentage of stromal TILs according to Immunity metagene status (low versus high). **C:** Correlation between metagene expression and the percentages of intratumoral TILs. **D:** Correlation between metagene expression and the percentage of stromal TILs.

doi:10.1371/journal.pone.0167397.g004

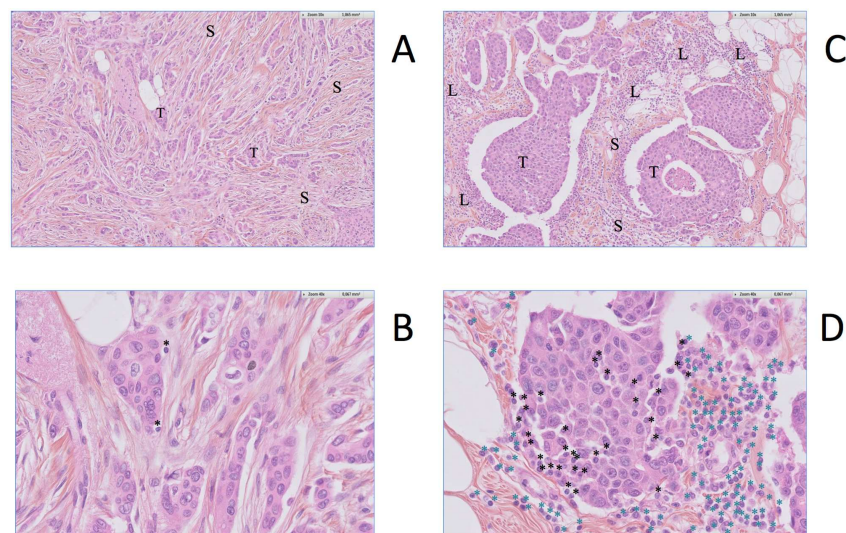


Fig 5. Lymphocytic infiltration in breast tumors. A and B: Tumor specimen with weak lymphocytic infiltration (A: zoom x10 B: zoom x 40). Abbreviations: S = stroma, T = tumor, L = lymphocytes. Intratumoral TILs are indicated by a black star. **C and D:** Tumor specimen with prominent lymphocytic infiltration. (C: zoom x10 D: zoom x 40). Abbreviations: S = stroma, T = tumor, L = lymphocytes. Intratumoral TILs are indicated by a black star; stromal TILs are indicated by a blue star.

doi:10.1371/journal.pone.0167397.g005

coefficient: PD1: 0.89, PDL1: 0.95, CTLA-4: 0.93), opening up new possibilities for therapeutic intervention.

The Immunity metagene is probably expressed by stromal cells

In breast cancer cell lines (CCLE and CGP datasets), the Immunity metagene displayed very low levels of expression, similar to those of the CD8 metagene (S6A and S6B Fig), consistent with expression only in the tumor stromal compartment. This pattern was observed for all cell lines and breast cancer cell lines tested. The Interferon module genes had higher median expression levels and a broader range of expression than those of the Immunity metagene in breast cancer cell lines, consistent with their expression by tumor cells. We also explored the contributions of stromal and cancer cells to the expression of the Immunity and Interferon metagenes in detail, by comparing our gene lists with the “stromal contribution to global gene expression evaluated in PDX RNAseq data”, as defined by Isella *et al.* [26]. The stromal fraction of the Immunity metagene was high, although lower than those of the Matrix and the Tumor suppressor/proliferation metagenes. The Interferon metagene had a low stromal fraction, like the Hormone/survival and Signal transduction metagenes (S6C Fig). Although these data relate to the colon cancer PDX model, they provide support for the stromal expression of the Immunity metagene.

Discussion

By analyzing the gene expression profiles of 448 *HER2*-positive breast cancers, we identified a six-metagene signature (138 genes) in which each of the various metagenes was enriched in a different gene ontology. Within these metagenes, we identified an immune stromal module inversely correlated with the ER and hormonal pathways and strongly associated with the predicted response to chemotherapy, prognosis, and tumor lymphocyte infiltration. We report here one of the first immune signatures identified as both predictive and prognostic, reflecting histological immune infiltration in *HER2*-positive breast cancers. We also provide a relevant analysis by HR status.

We previously developed a strategy for defining gene expression signatures based on the analysis of biological networks for the most variable genes [3]. Since the early 2000s, a molecular classification of breast cancers has emerged that is continually being refined. Several authors have proposed TNBC subclassifications [3,27,28] but, to our knowledge, only one classifier has been published, but was not subsequently validated in *HER2*-positive BC [18]. The various metagenes in our signature were enriched in different gene ontologies: two clusters were enriched in immunity genes, one in signal transduction genes, one in hormonal/survival genes, one in tumor suppressor/proliferation genes and one in matrix genes. Unlike several other teams [29–31], we did not identify a subgroup to tumors overexpressing androgen receptor pathways in *HER2*-positive BCs by our biology-driven approach. The expression of the Immunity and Hormone/survival metagenes accurately predicted the response to NAC, but the expression of the Hormone/survival metagene had no significant effect in multivariate analysis, because the information it provided largely overlapped with ER status. Moreover, only the Immunity metagene was found to be of significant prognostic value.

Several authors have previously identified immunity patterns in *HER2*-positive BC. The Immunity module identified in our study had many biological connections with other predictive or prognostic immune signatures published for *HER2*-positive breast cancers [13–21], but it outperformed previous classifiers. This module includes genes encoding chemokines for T cells (CXCL10, CXCL9, CCL5), B cells (CXCL13), both B and T cells (CCL19) or other immune cells (CXCL13, CCL5); chemokine receptors (CCR7); cytokines (LTB); adhesion

molecule-associated genes (SELL), and genes encoding proteins involved antigen processing and presentation (HLA-DRA), B-lymphocyte cell surface molecules (PTPRC, HLA-DRA), complement pathway proteins (C1QB), and proteins involved in CTL-mediated immune responses to target cells (CD3D), dendritic cell regulation of Th1 and Th2 development (CD2, IL7R), granzyme-mediated apoptosis (GZMA), IL12-mediated signaling events (CD3D, HLA-DRA, GZMA, LCK), the IL2 signaling pathway (LCK), T-cell surface molecules (PTPRC, CD3D, CD2), and molecules of the T-cell receptor signaling pathway (PTPRC, CD3D, HLA-DRA, LCK). It was also strongly correlated with the B-cell, T-cell and CD8 cell pathways.

There was a marked significant inverse association between *ESR1* expression and that of the Immunity metagene. Similar inverse associations were found between *PGR*, *AR* and immunity, but these associations were weaker and less consistent. There is growing evidence for sex-based differences in the innate and adaptive immune responses underlying susceptibility to infectious diseases and the prevalence of autoimmune diseases. A higher proportion of men than of women display infectious diseases and their severity is also greater in men than in women [32]. By contrast, many autoimmune diseases predominantly affect women [33]. There are also difference between men in terms of humoral and cellular responses to infection and vaccination, with women often displaying higher response rates and mounting stronger humoral responses [34]. Estrogen receptors are expressed in most of the cells of the innate and adaptive immune system, including T cells, B cells, neutrophils, macrophages, dendritic cells (DC), and natural killer (NK) cells [35]. The effects of major sex steroid hormones were reviewed by Giefing-Kröll [36]. Estradiol and testosterone have opposite effects on the cells of the adaptive and innate immune systems, with estradiol having mostly enhancing and testosterone mostly suppressive effects. Estrogens affect the expression of some chemokine receptors (*CCR1* and *CCR5*) by T cells [37]. They also affect B-cell development [38], decrease the cytotoxicity of NK cells [39] and regulate DC development [40]. TReg-cell frequencies within the $CD4^+$ population change considerably during the ovarian cycle, with potential effects on immunoregulation [41]. Unlike the differences between the sexes in terms of infection and auto-immunity, the relationships between tumor immunology, sex and steroid hormones have remained largely unexplored. In two phase III trials, immunotherapy had a significant beneficial effect on survival only in male patients [42,43]. However, it remains unclear whether there is a true “sex” effect on the efficacy of immunotherapy or whether these findings are purely incidental.

The interaction between the ER, immunity and *HER2* pathways is complex. There is increasing evidence to suggest that interactions between *HER2* and hormone-receptor pathways play an important role in disease progression and that there is extensive, complex, bidirectional, crosstalk between the *HER2* and ER pathways [44]. Immune signatures have been reported to have a predictive or prognostic role mostly in ER-negative breast cancers [45–48]. In *HER2*-positive breast cancer subtypes, Rody found that an immune T-cell metagene was of predictive value in both ER-positive and ER-negative *HER2*-positive BC [49]. The prognostic value of HDDP was demonstrated in both subgroups (11), but its value for predicting the response to NAC was not evaluated as a function of ER status. Conversely, the IRSN-23 [15] was not predictive in the ER-positive subpopulation. However, few authors determined the predictive [18] or prognostic value of their metagene or signature as a function of ER status within *HER2*-positive breast cancers [5,13,14,19–21]. The inverse association observed between *ESR1* expression and immunity genes may be an important piece of the puzzle, and merits further investigation.

Consistent with previous reports [13,15,16], we found that the Immunity metagene was predictive of the response to NAC in *HER2*-positive BC. However, despite the similar gene

module identification methods used and the strong correlation between the Immunity metagene and the Immunity2 metagene previously described by our team for TNBC [3], the Immunity metagene was predictive of the response to chemotherapy in *HER2*-positive BC, whereas the Immunity2 metagene was not predictive of the response to chemotherapy in TNBC. This finding was reported in the princeps report by Ignatiadis, in which high immune module scores were strongly and independently associated with a higher probability of pCR probability in *HER2*-positive tumors, whereas this association, although still significant, was weaker in TNBC [5]. ER-positive tumors have long been described as chemoresistant, with low pCR rates after NAC. Taking Immunity metagene expression into account, pCR rates ranged from 7.4 to 29.4%, with the highest rates close to those of ER-negative tumors.

The Immunity metagene was also prognostic in *HER2*-positive ER-negative breast cancer. The impact of immunity on prognosis has been reported before [21] (Alexe et al., 2007) [21]¹⁴[18,20,21]. Together with our work, these findings suggest that immunity gene expression is highly predictive and of prognostic value in *HER2*-positive breast cancer. Nevertheless, the *HER2*-positive patients of the METABRIC dataset did not receive targeted anti-*HER2* therapies, and our results would probably be influenced by adjuvant trastuzumab treatment.

We also demonstrated a correlation between Immunity metagene expression and stromal and intratumoral lymphocyte infiltration. The significance of TILs has recently become apparent, with advances in tumor immunology and the availability of cancer immunotherapies. TIL levels are strongly correlated with breast cancer subtype, and are higher in *HER2*-positive BCs than in ER-positive BCs, but lower than in TNBCs [50]. TIL levels are consistently higher in ER-negative tumors than in ER-positive tumors [51]. This was also found to be the case when the analysis was limited to *HER2*-positive BC only [52], [50]. The value of TIL levels for predicting pCR after NAC is less clear in *HER2*-positive BC than in TNBC. Stromal TILs and the lymphocyte-predominant breast cancer phenotype (LPBC) were strongly associated with treatment response in the GeparSixto trial [13]. However, this effect was found to be nonlinear in the NeoALTTO trial, and the optimal cutoff value remains unclear [52]. Two large studies in the adjuvant setting gave conflicting results. A positive association between higher levels of TILs and greater benefit from trastuzumab in *HER2*-positive disease was found in a retrospective analysis of the FinHER trial [50], whereas the opposite result was reported in the ALLIANCE N9831 study [53]. No difference in DFS between chemotherapy and chemotherapy plus trastuzumab was found in LPBC, whereas benefits of trastuzumab in addition to chemotherapy were observed only in non-LPBC. Thus, the prognostic impact of TILs on survival remains a matter of debate in *HER2*-positive BC. A few authors have reported a correlation between TIL and stromal lymphocyte levels and gene expression in *HER2*-positive breast cancers [13,15,21]. If this correlation is further validated, TIL levels could be used as a surrogate marker for the Immunity metagene, as TIL assessment is carried out in routine practice and is currently undergoing standardization [54].

Conclusion

Our work opens up a number of exciting therapeutic perspectives in *HER2*-positive breast cancers. Due to the high immunogenicity of *HER2*-positive breast cancers and the considerable predictive and prognostic impact of immunity in this subtype, immunotherapies may soon become part of the therapeutic arsenal for such cancers. Preclinical models have suggested that there is synergy between anti-*HER2* monoclonal antibody and anti-PD-1 [55] or anti-CTLA4 antibodies [56]. The PANACEA phase Ib/II trial is currently investigating the use of pembrolizumab (KEYTRUDA[®]) in combination with trastuzumab, to determine whether the addition of an anti-PD-1 treatment can overcome trastuzumab resistance in patients with *HER2*-

positive breast cancer whose cancer spread whilst they were on trastuzumab. Future challenges in the field of immunity and *HER2*-positive breast cancers include:

1. The public accessibility of large sets of gene expression data for tumors from patients treated with *HER2*-targeting treatments. As treatments are constantly changing for this breast cancer subtype, it is important for expression data to be shared promptly, to facilitate comprehensive research and the identification of predictive and prognostic markers in patients treated with cutting edge care.
2. Improvements in our understanding of hormone and immunity pathways in *HER2*-positive breast cancers. In particular, it would be very useful to determine whether a subset of patients with *HER2*-positive ER-positive cancers could be effectively treated by a combination of endocrine therapy/immune checkpoint blockade/ targeted therapy, without the need for chemotherapy.
3. Drug positioning strategies in *HER2*-positive BC, because, by contrast to other breast cancer subtypes, the *HER2*-targeting drug pipeline contains many candidates despite the comparative rarity of this particular disease.
4. The selection criteria for the candidates most likely to benefit from immune checkpoint blockade is a key point. The use of PD-L1 as a surrogate marker of anti-PD-1 efficacy remains controversial, even in cancers for which immunotherapy treatments have proved effective, and few data are available for breast cancer. The standardization and demonstrations of the reproducibility of published immune signatures would be useful, as would improvements in our understanding of the prognostic value of TILs in *HER2*-positive breast cancers. Moreover, it remains to be determined whether and how the immunogenic power of tumors with low expression of immunity genes could be enhanced.

Once these challenges have been overcome, given the outstanding results of immunotherapy for other cancers (e.g. melanoma, lung cancer) and the expected efficacy of such treatment for *HER2*-positive disease, such therapies could revolutionize the course of *HER2*-positive breast cancer in the near future.

Supporting Information

S1 Fig. Methodology flow chart.

(PDF)

S2 Fig. Heatmaps of the selected genes in the *HER2*-positive datasets. Training set (upper left); validation set (upper right), Ignatiadis (lower left), METABRIC (lower right).

(JPG)

S3 Fig. pCR rates by breast cancer subtype and Immunity metagene. A: pCR rates by breast cancer subtype by Immunity metagene status (low *versus* high). B: pCR rates by breast cancer subtype by Immunity2 metagene status (low *versus* high) as previously published by Bonsang et al [3].

(PDF)

S4 Fig. Heatmaps of the gene expression profiles of published immune signatures and connections between all immune genes. Fig A. Heatmap of the gene expression profiles of the nine immune predictive signatures or metagenes previously published, applied to the Ignatiadis dataset. The samples were ordered according to our classification of Low/High 'Immunity' metagene expression. B: Heatmap of the gene expression profiles of the immune prognostic signatures or metagenes previously published, applied to the METABRIC dataset. The samples

were ordered according to our classification of Low/High 'Immunity' metagene expression. C: String Software connections between genes of our Immunity metagenes and the genes of previously published predictive or prognostic immune signatures or metagenes. Stronger associations between genes are represented by thicker lines. Associations between genes with a coefficient < 0.9 are shown in green. Associations between genes with a coefficient ≥ 0.9 are shown in red. Associations between genes with a coefficient between 0.4 to 0.7 are not shown. (PDF)

S5 Fig. Distribution histograms for our Immune metagenes and immune pathways. Distribution histograms for our Immune metagenes (Immunity and Interferon) and the immune pathway metagenes published by Gatzka *et al.* (Interferon alpha, Interferon gamma, STAT3, TGF beta, TNF alpha) and Palmer *et al.* (B Cell, T Cell, CD8 T Cells, Granulocytes, Lymphocytes), Pearson correlation coefficient values and pairwise scatter plots. (PDF)

S6 Fig. Gene expression for the Immune metagenes and pathway, in cell lines and xenografts. A. Boxplots of gene expression for the Immune metagenes, the immune pathway metagenes (published by Gatzka *et al.* and Palmer *et al.*) and the PD1, PDL1, CTLA4 metagenes in breast cancer cell lines from the CCLE (A) and the CGP (B). C: Boxplots of the stromal contribution to global gene expression evaluated with PDX RNAseq data (Isella *et al.*), for each of the gene clusters for our signature. (PDF)

S1 File. Supplementary methods and results.
(PDF)

S1 Table. 138-gene signature.
(XLS)

S2 Table. Association of published immune signatures or metagenes with response to chemotherapy and prognosis. Response to chemotherapy is assessed in the Ignatiadis dataset (univariate and multivariate analysis) (S2A to S2E Table). The association of published immune signatures or metagenes with prognosis is assessed in the METABRIC dataset (univariate analysis) (S2F Table).
(XLS)

Acknowledgments

The authors thank Vassili Soumelis and Sergio Roman-Roman for reviewing of the study and the manuscript.

Author Contributions

Conceptualization: FR AP MG.

Data curation: BS.

Formal analysis: BS JA.

Investigation: BS CL.

Methodology: CL MM.

Project administration: FR CL.

Resources: ML.

Supervision: FR.

Validation: ML.

Writing – original draft: ASH HBK.

Writing – review & editing: ASH.

References

- Fröhlich H. Network based consensus gene signatures for biomarker discovery in breast cancer. *PLoS One*. 2011; 6: e25364. doi: [10.1371/journal.pone.0025364](https://doi.org/10.1371/journal.pone.0025364) PMID: [22046239](https://pubmed.ncbi.nlm.nih.gov/22046239/)
- Cun Y, Fröhlich HF. Prognostic gene signatures for patient stratification in breast cancer: accuracy, stability and interpretability of gene selection approaches using prior knowledge on protein-protein interactions. *BMC Bioinformatics*. 2012; 13: 69. doi: [10.1186/1471-2105-13-69](https://doi.org/10.1186/1471-2105-13-69) PMID: [22548963](https://pubmed.ncbi.nlm.nih.gov/22548963/)
- Bonsang-Kitzis H, Sadacca B, Hamy-Petit AS, Moarii M, Pinheiro A, Laurent C, et al. Biological network-driven gene selection identifies a stromal immune module as a key determinant of triple-negative breast carcinoma prognosis. *Oncoimmunology*. 2015;
- Servant N, Gravier E, Gestraud P, Laurent C, Paccard C, Biton A, et al. EMA—A R package for Easy Microarray data analysis. *BMC Res Notes*. BioMed Central Ltd; 2010; 3: 277.
- Ignatiadis M, Singhal SK, Desmedt C, Haibe-Kains B, Criscitiello C, Andre F, et al. Gene modules and response to neoadjuvant chemotherapy in breast cancer subtypes: a pooled analysis. *J Clin Oncol*. 2012; 30: 1996–2004. doi: [10.1200/JCO.2011.39.5624](https://doi.org/10.1200/JCO.2011.39.5624) PMID: [22508827](https://pubmed.ncbi.nlm.nih.gov/22508827/)
- Curtis C, Shah SP, Chin S-F, Turashvili G, Rueda OM, Dunning MJ, et al. The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups. *Nature*. 2012; 486: 346–352. doi: [10.1038/nature10983](https://doi.org/10.1038/nature10983) PMID: [22522925](https://pubmed.ncbi.nlm.nih.gov/22522925/)
- Gong Y, Yan K, Lin F, Anderson K, Sotiriou C, Andre F, et al. Determination of oestrogen-receptor status and ERBB2 status of breast carcinoma: a gene-expression profiling study. *Lancet Oncol*. 2007; 8: 203–211. doi: [10.1016/S1470-2045\(07\)70042-6](https://doi.org/10.1016/S1470-2045(07)70042-6) PMID: [17329190](https://pubmed.ncbi.nlm.nih.gov/17329190/)
- Jensen LJ, Kuhn M, Stark M, Chaffron S, Creevey C, Muller J, et al. STRING 8—a global view on proteins and their functional interactions in 630 organisms. *Nucleic Acids Res*. 2009; 37: D412–6. doi: [10.1093/nar/gkn760](https://doi.org/10.1093/nar/gkn760) PMID: [18940858](https://pubmed.ncbi.nlm.nih.gov/18940858/)
- De Cremoux P, Valet F, Gentien D, Lehmann-Che J, Scott V, Tran-Perennou C, et al. Importance of pre-analytical steps for transcriptome and RT-qPCR analyses in the context of the phase II randomised multicentre trial REMAGUS02 of neoadjuvant chemotherapy in breast cancer patients. *BMC Cancer*. 2011; 11: 215. doi: [10.1186/1471-2407-11-215](https://doi.org/10.1186/1471-2407-11-215) PMID: [21631949](https://pubmed.ncbi.nlm.nih.gov/21631949/)
- Barretina J, Caponigro G, Stransky N, Venkatesan K, Margolin AA, Kim S, et al. NIH Public Access of anticancer drug sensitivity. 2012; 483: 603–607.
- Garnett MJ, Edelman EJ, Heidorn SJ, Greenman CD, Dastur A, Lau KW, et al. Europe PMC Funders Group Systematic identification of genomic markers of drug sensitivity in cancer cells. *Nature*. 2012; 483: 570–575.
- R Development Core Team. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing. Vienna, Austria 2009. [Internet]. <http://www.R-project.org>
- Denkert C, von Minckwitz G, Brase JC, Sinn BV, Gade S, Kronenwett R, et al. Tumor-infiltrating lymphocytes and response to neoadjuvant chemotherapy with or without Carboplatin in human epidermal growth factor receptor 2-positive and triple-negative primary breast cancers. *J Clin Oncol Off J Am Soc Clin Oncol*. 2015; 33: 983–991.
- Gu-Trantien C, Loi S, Garaud S, Equeter C, Libin M, Wind De A, et al. CD4+ follicular helper T cell infiltration predicts breast cancer survival. *J Clin Invest*. 2013; 123: 1–20.
- Sota Y, Naoi Y, Tsunashima R, Kagara N, Shimazu K, Maruyama N, et al. Construction of novel immune-related signature for prediction of pathological complete response to neoadjuvant chemotherapy in human breast cancer. *Ann Oncol Off J Eur Soc Med Oncol ESMO*. 2014; 25: 100–106.
- Stoll G, Enot D, Mlecnik B, Galon J, Zitvogel L, Kroemer G. Immune-related gene signatures predict the outcome of neoadjuvant chemotherapy. *Oncoimmunology*. 2014; 3: e27884. doi: [10.4161/onci.27884](https://doi.org/10.4161/onci.27884) PMID: [24790795](https://pubmed.ncbi.nlm.nih.gov/24790795/)
- Rody A, Karn T, Liedtke C, Pusztai L, Ruckhaeberle E, Hanker L, et al. A clinically relevant gene signature in triple negative and basal-like breast cancer. *Breast Cancer Res BCR*. 2011; 13: R97. doi: [10.1186/bcr3035](https://doi.org/10.1186/bcr3035) PMID: [21978456](https://pubmed.ncbi.nlm.nih.gov/21978456/)

18. Staaf J, Ringnér M, Vallon-Christersson J, Jönsson G, Bendahl P-O, Holm K, et al. Identification of subtypes in human epidermal growth factor receptor 2—positive breast cancer reveals a gene signature prognostic of outcome. *J Clin Oncol Off J Am Soc Clin Oncol*. 2010; 28: 1813–1820.
19. 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. doi: [10.1158/1078-0432.CCR-07-4756](https://doi.org/10.1158/1078-0432.CCR-07-4756) PMID: [18698033](https://pubmed.ncbi.nlm.nih.gov/18698033/)
20. Finak G, Bertos N, Pepin F, Sadekova S, Souleimanova M, Zhao H, et al. Stromal gene expression predicts clinical outcome in breast cancer. *Nat Med*. 2008; 14: 518–527. doi: [10.1038/nm1764](https://doi.org/10.1038/nm1764) PMID: [18438415](https://pubmed.ncbi.nlm.nih.gov/18438415/)
21. Alexe G, Dalgin GS, Scandfield D, Tamayo P, Mesirov JP, DeLisi C, et al. High expression of lymphocyte-associated genes in node-negative *HER2+* breast cancers correlates with lower recurrence rates. *Cancer Res*. 2007; 67: 10669–10676. doi: [10.1158/0008-5472.CAN-07-0539](https://doi.org/10.1158/0008-5472.CAN-07-0539) PMID: [18006808](https://pubmed.ncbi.nlm.nih.gov/18006808/)
22. Perez EA, Thompson EA, Ballman KV, Anderson SK, Asmann YW, Kalari KR, et al. Genomic Analysis Reveals That Immune Function Genes Are Strongly Linked to Clinical Outcome in the North Central Cancer Treatment Group N9831 Adjuvant Trastuzumab Trial. *J Clin Oncol Off J Am Soc Clin Oncol*. 2015;
23. Gatza ML, Lucas JE, Barry WT, Kim JW, Wang Q, Crawford MD, et al. A pathway-based classification of human breast cancer. *Proc Natl Acad Sci U S A*. 2010; 107: 6994–6999. doi: [10.1073/pnas.0912708107](https://doi.org/10.1073/pnas.0912708107) PMID: [20335537](https://pubmed.ncbi.nlm.nih.gov/20335537/)
24. Palmer C, Diehn M, Alizadeh A a, Brown PO. Cell-type specific gene expression profiles of leukocytes in human peripheral blood. *BMC Genomics*. 2006; 7: 115. doi: [10.1186/1471-2164-7-115](https://doi.org/10.1186/1471-2164-7-115) PMID: [16704732](https://pubmed.ncbi.nlm.nih.gov/16704732/)
25. Sabatier R, Finetti P, Mamessier E, Adelaide J, Chaffanet M, Ali HR, et al. Prognostic and predictive value of PDL1 expression in breast cancer. *Oncotarget*. 2015; 6: 5449–5464. doi: [10.18632/oncotarget.3216](https://doi.org/10.18632/oncotarget.3216) PMID: [25669979](https://pubmed.ncbi.nlm.nih.gov/25669979/)
26. Isella C, Terrasi A, Bellomo SE, Petti C, Galatola G, Muratore A, et al. Stromal contribution to the colorectal cancer transcriptome. *Nat Genet*. Nature Publishing Group; 2015; 1–11.
27. Lehmann BD, Bauer JA, Chen X, Sanders ME, Chakravarthy AB, Shyr Y, et al. Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies. *J Clin Invest*. 2011; 121: 2750–2767. doi: [10.1172/JCI45014](https://doi.org/10.1172/JCI45014) PMID: [21633166](https://pubmed.ncbi.nlm.nih.gov/21633166/)
28. Burstein MD, Tsimelzon A, Poage GM, Covington KR, Contreras A, Fuqua S, et al. Comprehensive Genomic Analysis Identifies Novel Subtypes and Targets of Triple-negative Breast Cancer. *Clin Cancer Res Off J Am Assoc Cancer Res*. 2014;
29. Doane AS, Danso M, Lal P, Donaton M, Zhang L, Hudis C, et al. An estrogen receptor-negative breast cancer subset characterized by a hormonally regulated transcriptional program and response to androgen. *Oncogene*. 2006; 25: 3994–4008. doi: [10.1038/sj.onc.1209415](https://doi.org/10.1038/sj.onc.1209415) PMID: [16491124](https://pubmed.ncbi.nlm.nih.gov/16491124/)
30. Farmer P, Bonnefoi H, Becette V, Tubiana-Hulin M, Fumoleau P, Larsimont D, et al. Identification of molecular apocrine breast tumours by microarray analysis. *Oncogene*. 2005; 24: 4660–4671. doi: [10.1038/sj.onc.1208561](https://doi.org/10.1038/sj.onc.1208561) PMID: [15897907](https://pubmed.ncbi.nlm.nih.gov/15897907/)
31. Lehmann-Che J, Hamy A-S, Porcher R, Barritault M, Bouhidel F, Habuella H, et al. Molecular apocrine breast cancers are aggressive estrogen receptor negative tumors overexpressing either *HER2* or *GCDFP15*. *Breast Cancer Res*. 2013; 15: R37. doi: [10.1186/bcr3421](https://doi.org/10.1186/bcr3421) PMID: [23663520](https://pubmed.ncbi.nlm.nih.gov/23663520/)
32. Klein SL. The effects of hormones on sex differences in infection: from genes to behavior. *Neurosci Biobehav Rev*. 2000; 24: 627–638. PMID: [10940438](https://pubmed.ncbi.nlm.nih.gov/10940438/)
33. Lockshin MD. Sex differences in autoimmune disease. *Lupus*. 2006; 15: 753–756. PMID: [17153846](https://pubmed.ncbi.nlm.nih.gov/17153846/)
34. Cook IF. Sexual dimorphism of humoral immunity with human vaccines. *Vaccine*. 2008; 26: 3551–3555. doi: [10.1016/j.vaccine.2008.04.054](https://doi.org/10.1016/j.vaccine.2008.04.054) PMID: [18524433](https://pubmed.ncbi.nlm.nih.gov/18524433/)
35. Fish EN. The X-files in immunity: sex-based differences predispose immune responses. *Nat Rev Immunol*. 2008; 8: 737–744. doi: [10.1038/nri2394](https://doi.org/10.1038/nri2394) PMID: [18728636](https://pubmed.ncbi.nlm.nih.gov/18728636/)
36. Giefing-Kröll C, Berger P, Lepperdinger G, Grubeck-Loebenstien B. How sex and age affect immune responses, susceptibility to infections, and response to vaccination. *Aging Cell*. 2015; 14: 309–321. doi: [10.1111/acer.12326](https://doi.org/10.1111/acer.12326) PMID: [25720438](https://pubmed.ncbi.nlm.nih.gov/25720438/)
37. Mo R, Chen J, Grolleau-Julius A, Murphy HS, Richardson BC, Yung RL. Estrogen regulates *CCR* gene expression and function in T lymphocytes. *J Immunol Baltim Md 1950*. 2005; 174: 6023–6029.
38. Sakiani S, Olsen NJ, Kovacs WJ. Gonadal steroids and humoral immunity. *Nat Rev Endocrinol*. 2013; 9: 56–62. doi: [10.1038/nrendo.2012.206](https://doi.org/10.1038/nrendo.2012.206) PMID: [23183675](https://pubmed.ncbi.nlm.nih.gov/23183675/)
39. Hao S, Zhao J, Zhou J, Zhao S, Hu Y, Hou Y. Modulation of 17beta-estradiol on the number and cytotoxicity of NK cells in vivo related to MCM and activating receptors. *Int Immunopharmacol*. 2007; 7: 1765–1775. doi: [10.1016/j.intimp.2007.09.017](https://doi.org/10.1016/j.intimp.2007.09.017) PMID: [17996687](https://pubmed.ncbi.nlm.nih.gov/17996687/)

40. Siracusa MC, Overstreet MG, Housseau F, Scott AL, Klein SL. 17beta-estradiol alters the activity of conventional and IFN-producing killer dendritic cells. *J Immunol Baltim Md 1950*. 2008; 180: 1423–1431.
41. Arruvito L, Sanz M, Banham AH, Fainboim L. Expansion of CD4+CD25+and FOXP3+ regulatory T cells during the follicular phase of the menstrual cycle: implications for human reproduction. *J Immunol Baltim Md 1950*. 2007; 178: 2572–2578.
42. Robert C, Thomas L, Bondarenko I, O'Day S, Weber J, Garbe C, et al. Ipilimumab plus dacarbazine for previously untreated metastatic melanoma. *N Engl J Med*. 2011; 364: 2517–2526. doi: [10.1056/NEJMoa1104621](https://doi.org/10.1056/NEJMoa1104621) PMID: [21639810](https://pubmed.ncbi.nlm.nih.gov/21639810/)
43. Brahmer J, Reckamp KL, Baas P, Crinò L, Eberhardt WEE, Poddubska E, et al. Nivolumab versus Docetaxel in Advanced Squamous-Cell Non-Small-Cell Lung Cancer. *N Engl J Med*. 2015; 373: 123–135. doi: [10.1056/NEJMoa1504627](https://doi.org/10.1056/NEJMoa1504627) PMID: [26028407](https://pubmed.ncbi.nlm.nih.gov/26028407/)
44. Giuliano M, Trivedi MV, Schiff R. Bidirectional Crosstalk between the Estrogen Receptor and Human Epidermal Growth Factor Receptor 2 Signaling Pathways in Breast Cancer: Molecular Basis and Clinical Implications. *Breast Care Basel Switz*. 2013; 8: 256–262.
45. Sabatier R, Finetti P, Mamessier E, Raynaud S, Cervera N, Lambaudie E, et al. Kinome expression profiling and prognosis of basal breast cancers. *Mol Cancer*. 2011; 10: 86. doi: [10.1186/1476-4598-10-86](https://doi.org/10.1186/1476-4598-10-86) PMID: [21777462](https://pubmed.ncbi.nlm.nih.gov/21777462/)
46. Teschendorff AE, Miremadi A, Pinder SE, Ellis IO, Caldas C. An immune response gene expression module identifies a good prognosis subtype in estrogen receptor negative breast cancer. *Genome Biol*. 2007; 8: R157. doi: [10.1186/gb-2007-8-8-r157](https://doi.org/10.1186/gb-2007-8-8-r157) PMID: [17683518](https://pubmed.ncbi.nlm.nih.gov/17683518/)
47. Karn T, Puzstai L, Holtrich U, Iwamoto T, Shiang CY, Schmidt M, et al. Homogeneous datasets of triple negative breast cancers enable the identification of novel prognostic and predictive signatures. *PLoS One*. 2011; 6: e28403. doi: [10.1371/journal.pone.0028403](https://doi.org/10.1371/journal.pone.0028403) PMID: [22220191](https://pubmed.ncbi.nlm.nih.gov/22220191/)
48. West NR, Milne K, Truong PT, Macpherson N, Nelson BH, Watson PH. Tumor-infiltrating lymphocytes predict response to anthracycline-based chemotherapy in estrogen receptor-negative breast cancer. *Breast Cancer Res BCR*. BioMed Central Ltd; 2011; 13: R126.
49. Rody A, Holtrich U, Puzstai L, Liedtke C, Gaetje R, Ruckhaeberle E, et al. T-cell metagene predicts a favorable prognosis in estrogen receptor-negative and HER2-positive breast cancers. *Breast Cancer Res BCR*. 2009; 11: R15. doi: [10.1186/bcr2234](https://doi.org/10.1186/bcr2234) PMID: [19272155](https://pubmed.ncbi.nlm.nih.gov/19272155/)
50. Loi S, Michiels S, Salgado R, Sirtaine N, Jose V, Fumagalli D, et al. Tumor infiltrating lymphocytes are prognostic in triple negative breast cancer and predictive for trastuzumab benefit in early breast cancer: results from the FinHER trial. *Ann Oncol Off J Eur Soc Med Oncol ESMO*. 2014; 25: 1544–1550.
51. Mahmoud SMA, Paish EC, Powe DG, Macmillan RD, Grainge MJ, Lee AHS, et al. Tumor-infiltrating CD8+ lymphocytes predict clinical outcome in breast cancer. *J Clin Oncol Off J Am Soc Clin Oncol*. 2011; 29: 1949–1955.
52. Salgado R, Denkert C, Campbell C, Savas P, Nucifero P, Aura C, et al. Tumor-Infiltrating Lymphocytes and Associations With Pathological Complete Response and Event-Free Survival in HER2-Positive Early-Stage Breast Cancer Treated With Lapatinib and Trastuzumab: A Secondary Analysis of the NeoALTTO Trial. *JAMA Oncol*. 2015; 1: 448–454. doi: [10.1001/jamaoncol.2015.0830](https://doi.org/10.1001/jamaoncol.2015.0830) PMID: [26181252](https://pubmed.ncbi.nlm.nih.gov/26181252/)
53. Perez E. Stromal tumor-infiltrating lymphocytes(S-TILs): In the alliance N9831 trial S-TILs are associated with chemotherapy benefit but not associated with trastuzumab benefit [Internet]. 2014. http://www.abstracts2view.com/sabcs14/view.php?nu=SABCS13L_1455
54. Salgado R, Denkert C, Demaria S, Sirtaine N, Klauschen F, Pruner G, et al. The evaluation of tumor-infiltrating lymphocytes (TILs) in breast cancer: recommendations by an International TILs Working Group 2014. *Ann Oncol Off J Eur Soc Med Oncol ESMO*. 2015; 26: 259–271.
55. Stagg J, Loi S, Divisekera U, Ngiow SF, Duret H, Yagita H, et al. Anti-ErbB-2 mAb therapy requires type I and II interferons and synergizes with anti-PD-1 or anti-CD137 mAb therapy. *Proc Natl Acad Sci U S A*. 2011; 108: 7142–7147. doi: [10.1073/pnas.1016569108](https://doi.org/10.1073/pnas.1016569108) PMID: [21482773](https://pubmed.ncbi.nlm.nih.gov/21482773/)
56. Wang Q, Li S-H, Wang H, Xiao Y, Sahin O, Brady SW, et al. Concomitant targeting of tumor cells and induction of T-cell response synergizes to effectively inhibit trastuzumab-resistant breast cancer. *Cancer Res*. 2012; 72: 4417–4428. doi: [10.1158/0008-5472.CAN-12-1339-T](https://doi.org/10.1158/0008-5472.CAN-12-1339-T) PMID: [22773664](https://pubmed.ncbi.nlm.nih.gov/22773664/)

Supporting information file 1

1 Supplementary Methods

1.1 Data

1.1.1 Training and validation sets

We collected 21 publicly available datasets containing raw gene expression data from microarrays (Affymetrix© GeneChip Human Genome HG-U133A and HG-U133Plus2) for 2893 primary human breast cancer samples. The raw data were downloaded from the NCBI Gene Expression Omnibus or ArrayExpress, with the following identifiers: GSE1456 (Pawitan Y.), GSE1561, GSE2034 (Wang Y), GSE2603 (Minn AJ), GSE2990 (Sotiriou C), GSE3494 (Miller LD), GSE5327, GSE5847 (Boersma BJ), GSE7390 (Desmedt C), GSE11121 (Schmidt M), GSE20194, MDA133, GSE2109, GSE7904 (Richardson AL.), GSE12276 (Bos PD), GSE16446 (Juil N), GSE18864 (Juil N), GSE19615 (Juil N), GSE22513 (Bauer A), GSE28796, GSE28821. Raw GE values for each dataset ($n=21$) were normalized independently, for identification of the *HER2*-positive samples in each dataset. The training set included samples hybridized on HGU-133A Affymetrix© arrays (12 datasets, $n=1921$), to eliminate cross-platform discrepancies and to ensure robust normalization. The validation set included samples hybridized on HGU-133Plus2 Affymetrix© arrays (9 datasets, $n=972$).

1.1.2 The Ignatiadis dataset

We collected eight publicly available datasets from the following studies: EORTC10994, I-SPY-1, LBJ/INEN/GEICAM, the MDACC trial, TOP, MAQCII/MDACC, MAQCIII, USO-02103, all of which used Affymetrix GeneChip Human Genome HG-U133A arrays.

Ignatiadis *et al.*[1] used these datasets to analyze the responses of various molecular subtypes of breast cancer to neoadjuvant chemotherapy (anthracycline, with or without taxane; $n=996$). Raw GE values for each dataset were normalized independently, for identification of the *HER2*-positive samples in each dataset. As different sample types were available for gene expression, we chose to keep only tumors sampled by fine needle aspiration, to ensure that the samples studied were homogeneous, as this was the technique used for the majority of tumors in the Ignatiadis dataset ($n=586$).

1.1.3 The METABRIC set

We used the METABRIC — Molecular Taxonomy of Breast Cancer International Consortium — dataset published by Curtis *et al.*[2], which was established for analysis of the prognosis of various molecular subtypes of breast cancer. We normalized the 1992 samples together, using scripts and Rdata provided by the authors. We fitted a linear model (limma R package) to remove the batch effect and probes were filtered according to three criteria: probe quality [3], GC content and presence in more than 5% of the samples.

1.2 Determination of *HER2*-positive status, ER, PR and AR status

The Affymetrix probe 216836_s_at was chosen to provide information about *HER2* expression for the training, validation and Ignatiadis datasets [4]. For the METABRIC dataset, after quality control, the Illumina probe ILMN_2352131 was chosen to provide information about *HER2* expression. We used a threshold value of 1150 for *ERBB2* mRNA to identify *ERBB2*-positive patients, as described by Gong[5] for the training and validation datasets, and the bimodal distribution of *ERBB2* expression for the Ignatiadis and METABRIC datasets. GE analyses identified 448, 194, 82 and 248 *HER2*-positive samples in the training set, the validation set, the Ignatiadis set and the METABRIC set, respectively. We

identified the ER-, PR- and AR-positive samples in each dataset as follows: the distributions of ER PR and AR expression were analyzed empirically, with a two-component Gaussian mixture model, and parameters for bimodal filtering were estimated with the R Mclust package. A density plot of ER, PR and AR gene expression in each dataset showed a bimodal distribution for ER and PR, but not for AR. The median value was used as the cutoff for AR expression in each dataset.

1.3 Preprocessing of *HER2*-positive samples

1.3.1 Affymetrix© platform

For each dataset (training, validation and prediction), we used the R arrayQualityMetrics package on an Affybatch object to filter out outliers. We excluded samples detected as outliers by at least two of the following methods: distances between arrays, boxplots, relative log expression (RLE), normalized unscaled standard error (NUSE), MA plots, spatial distribution of M. The *HER2*-positive samples were selected and split into training and validation sets. We filtered out 52 outlier samples from the training set and 11 from the validation set. Raw GE values for the *HER2*-positive samples in each dataset (training, validation and prediction) were normalized independently. The optimal microarray probe set to represent a gene was selected with the R JetSet package. This package developed scoring methods for the assessment of each probe set for specificity, coverage, and degradation resistance. For each dataset, batch effects were removed by median centering of each probe set across arrays and the quantile normalization of all arrays separately for each set.

1.3.2 Illumina© platform

We used the Illumina© probe quality score introduced by Barbosa-Morais in 2010 to ensure that all the probes used were of high quality (deleting probes scored as “bad” or “no match”). Probes were filtered on the basis of three criteria: probe quality[3], GC content between 38%

and 64% and presence in more than 5% of the samples ($n= 20,009$ probes). We eliminated any batch effects associated with sample collection sites, by fitting a linear model (R limma package). We then chose the most variable probes as the optimal probe set.

1.4 Statistical analyses

All statistical analyses were performed with R software. Affymetrix© microarrays were normalized with the robust multichip average (RMA) procedure from the EMA R package[6]. Principal component analysis was carried out and heatmaps were generated with the R gplots package.

1.5 Establishment of the *HER2*-positive classifier

1.5.1 Gene selection process

Consensus clustering[7] was applied to the training set, to determine the optimal number of robust gene clusters from the most variable genes (standard deviation >0.8) (ConsensusClusterPlus R package). Cluster robustness was assessed by hierarchical clustering (1,000 iterations) with a ward inner, final linkage and Pearson distance. The optimal number of clusters was determined from the cumulative distribution function (CDF), which plots the corresponding empirical cumulative distribution, defined over the range [0,1], and by calculating the proportional increase in the area under the CDF curve. The number of clusters was set as that at which an increase in cluster number (k) did not lead to a marked increase in CDF area. We calculated Pearson's correlation coefficient for the relationships between genes within the same cluster, to assess the heterogeneity of each gene cluster. The consensus clustering method and hierarchical clustering identified five main gene clusters. Further increases in cluster number yielded no significant increase in the consensus distribution function area. Each gene cluster was tested for gene enrichment (biological process (BP), molecular function (MF), cellular component (CC)) by a conditional test for

overrepresentation, in the R runHyperGO package. The various gene clusters were associated with different gene ontologies). The clusters were named as follows: Immunity cluster (70 genes), Signal/transduction cluster (130 genes), Hormonal/survival cluster (312 genes), Tumor suppressor cluster (65 genes) and Matrix cluster (39 genes). The Matrix and Immunity clusters were the most homogeneous, with strong correlations between the gene expression profiles of most of the genes within each of these clusters (Pearson's correlation coefficients of 0.60 and 0.43, respectively). We then used the String© database (<http://string-db.org/help/index.jsp?topic=/org.string-db.docs/ch04.html>)[8] to identify biological gene networks. String© is a database of known and predicted protein interactions. The interactions include physical and functional associations derived from four sources: genomic context, high-throughput experiments, conserved coexpression, previous knowledge. For each gene cluster, we excluded genes that were not connected to any of the other genes present in the cluster. We then applied a two-step selection process: 1) we selected strong biological networks, by retaining genes with connection scores of at least 0.7 to each other, according to the String database 2) within each biological network, we then selected groups of genes with correlated patterns of expression, with correlation coefficients of at least 0.5. For this step, we used Cytoscape (<http://cytoscapeweb.cytoscape.org>), an open-source software platform for visualizing complex networks and integrating them with any type of attribute data. Attribute data, such as correlations, variance and interquartile range, were calculated from the expression data matrix.

After selection, we checked that the various genes selected from the same cluster clustered together again (R package ConsensusClusterPlus). Following biological network-driven gene selection, it became clear that the original Immunity cluster was more accurately described by splitting into two slightly different subclusters (Immunity ($n=28$), Interferon ($n=11$)). This approach yielded an increase in the area under the consensus distribution function (CDF)

curve. The six clusters were renamed as follows, after reclustering: Immunity (n=28), Interferon (n=11), Signal transduction (n=20), Hormonal/survival (n=22), Tumor suppressors/Proliferation (n=36), Matrix (n=21).

1.5.2 Metagene identification

For each dataset (training, validation, Ignatiadis and METABRIC), each gene cluster was used to define a metagene (for the Affymetrix© platform: from the 138 selected genes; for the prognosis set, we used the 136 genes common to the preprocessed prognosis expression matrix and the selected genes matrix from the Affymetrix© platform). Metagene expression was assessed by calculating the median value for the normalized expression values of all probe sets in the respective gene clusters for each sample. For each dataset, we calculated the correlation between expression levels for the various metagenes, using the R psych package. The metagene value for each sample was then classified as corresponding to “high” or “low” expression according to the median value for the metagene.

1.5.3 Classification of HER2-positive samples

In each dataset, hierarchical clustering was applied to the *HER2*-positive GE profiles, using the selected genes to visualize the optimal number of stable *HER2*-positive subtypes (ConsensusClusterPlus R package). We identified five *HER2*-positive subtypes, using the 138-genes signature. We checked the concordance between each of the validation sets and the training set (Pearson’s correlation coefficient for the relationship between centroids). Centroid expression values were determined by calculating the mean normalized expression values for all samples in the sample cluster, for each probe set (Figure S2).

1.6 Analysis of the predicted response to chemotherapy prognosis, and the correlation with intratumoral and stromal lymphocyte levels

1.6.1 Analysis of the response to chemotherapy

We assessed the predictive value of our metagenes, by collecting the independent dataset described above (the Ignatiadis set), which contained data for gene expression and clinical variables. We selected *HER2*-positive samples and the data were preprocessed as described above. The following clinical and pathological variables were available and were categorized as follows: age (<50 *versus* ≥ 50), pre-chemotherapy clinical tumor size (T1 and T2, T3, T4), pre-chemotherapy tumor nodal status (N0 *versus* N1, N2, N3), tumor grade (Grade I and II *versus* III), treatment type, and response to chemotherapy (pathological complete response *versus* no pathological complete response). ER and PR status were assessed by gene expression (as described above). We assessed the expression of the metagenes in each population. Given their unimodal distribution, the expression of each metagene was classified as “low” or “high”, based on the median value for the six metagenes. Pathological complete response (pCR) was defined as the disappearance of the invasive component of the primary tumor in one study[9] and no residual invasive cancer in the breast and axillary lymph nodes in the other seven studies. Factors predictive of pCR were introduced into a univariate logistic regression model. A multivariate logistic model was then generated. The covariates selected for the multivariate analysis were those with a likelihood ratio test *p*-value lower than 0.10 in univariate analysis. A backward stepwise selection procedure was used.

We also tested the predictive value of nine immune signatures or metagenes validated as predictive of the response to chemotherapy in *HER2*-positive breast cancer patients: 12 immune-gene signatures [10], Th1 and T-fh metagenes [11], an immune-related 23-gene signature for NAC (IRSN-23) [12], the CXCL13, CCL8 and CXCL9 metagenes [13], the

LCK and IgG combined metagene [14], and the *HER2*-derived prognostic predictor (HDPP)[15]. We performed several multivariate analyses with each signature or metagene independently and the clinical variables significantly associated with pCR in univariate analysis (tumor grade, tumor stage, ER status). We generated a heatmap of the gene expression profiles of each of the above predictive signatures (Figure S4A). The samples were ordered according to our classification of low/high levels of Immunity metagene expression. Expression patterns were similar for the Immunity metagene and for the other predictive gene expression signatures or metagenes, with the exception of Staaf's signature genes.

1.6.2 Prognosis

We assessed the prognostic classification, by collecting the independent dataset described above (METABRIC set), which contained data for gene expression and clinical variables. We selected *HER2*-positive samples and the data were preprocessed as described above. The following clinical and pathological variables were available and were categorized as follows: age (≤ 45 , [45-55], > 55), menopausal status, tumor size, tumor grade according to the Elston and Ellis grading system, number of lymph nodes involved (0 (N-, node negative) versus 1 or more lymph nodes involved (N+, node positive)), the Nottingham Prognostic Index score (good prognosis, intermediate prognosis, poor prognosis), treatment type, last follow-up status and the time at which last-follow-up occurred. ER and PR statuses were assessed on the basis of gene expression (as described above). We assessed the expression of the metagenes in each population and the expression of each metagene was classified as “low” or “high”, relative to the median value for the six metagenes. Survival analyses were performed by calculating Kaplan-Meier estimates of the survival function. The endpoint of these analyses was breast cancer-specific survival (BCSS) (death from breast cancer). Time-censoring analyses were performed with a right censoring of events from 1 to 20 years. Log-rank tests were used to compare survival curves. Hazard ratios and their associated 95% confidence intervals were

calculated with the Cox proportional hazard model. Variables with a p -value for the likelihood ratio test lower than 0.10 in univariate analysis were included in the multivariate model. Backward selection was used to establish the final multivariate model. However, NPI was excluded due to redundancy with the variables size, grade and lymph node status.

Analyses were performed in the whole population, and in the ER-positive, and ER-negative populations. We also assessed the prognostic value of nine immune signatures or metagenes previously validated as predictive of prognosis in *HER2*-positive breast cancer patients: the LCK metagene[14], an immune response module described by Desmedt [16], the T-fh and Th1 metagenes, and CXCL13 alone[11], the stroma-derived prognosis predictor (SDDP)[17] or signatures developed specifically for *HER2*-positive breast cancers (HDPP [18]¹⁸(18)(17)(17)[15], the 105 lymphocyte-associated gene signature [19], the 14-immune gene signature [20]). We generated a heatmap of the gene expression profiles of each of the above prognostic signatures (Figure S4B). The samples were ordered according to our classification of low/high Immunity metagene expression. The expression patterns were similar among signatures, except for the Staaf signature genes, which were associated with poor outcome, and the Finak signature genes associated with a mixed or poor outcome.

1.6.3 Assessment of tumor and stromal infiltrating lymphocytes and Immunity metagene expression in the REMAGUS dataset.

We selected 27 *HER2*-positive breast cancer patients treated by NAC with or without neoadjuvant trastuzumab at our institution during the REMAGUS 02 trial [21]. Core biopsies were obtained before treatment, and separate cores were processed for histology and for RNA extraction, amplification, and hybridization to Affymetrix U133P2 arrays. We selected *HER2*-positive patients on the basis of the expression of the “216836_s_at” probeset. According to the bimodal distribution of ERBB2 expression, 74 of the 226 samples for which

transcriptomic data were available were considered *HER2*-positive. Gene expression data were normalized with the RMA package and batch effects were removed by the median centering of each probe set across arrays and the quantile normalization of all arrays separately for each set. Thirty of these patients were treated at our institution, and pretreatment microbiopsies corresponding to the gene expression chips were retrieved for 27 of them. Histologic microbiopsy specimens were evaluated independently for the presence of a lymphocytic infiltrate by one BCA pathologist (ML) and one physician (ASHP) unaware of the gene expression classification. Intratumoral TILs and stromal TILs were quantified in a semi-quantitative manner as percentages, as previously recommended [22]. Samples were split into Immunity “low” and “high” expression, relative to the median value for the metagene, as described for the other datasets. Intratumoral TIL and stromal TIL percentages were compared between the Immune “high” and Immune “low” subgroups by ANOVA. The correlation between gene expression and intratumoral TIL and stromal TIL percentages was assessed by calculating Pearson’s correlation coefficient.

1.6.4 Expression of our gene signature in human breast cancer cell lines

We assessed the expression of our signature in “*in vitro*” models, as a means of validating our classification and its prognostic value. We used the gene expression profiles of the human cancer cell lines from the Cancer Cell Line Encyclopedia (CCLE)[23] of Novartis/the Broad Institute and from the Cancer Genome Project (CGP)[24] of the Sanger Institute. All the cell lines from different tissues were normalized together. The global gene expression signal is shown for breast cancer cell lines from the CCLE (58) and CGP (39). Box plots were generated for the expression of the Immunity and Interferon metagenes, and the metagenes defined by Gatza *et al.* (16) (IFN-alpha, IFN-gamma, STAT3, TGF-beta, TNF-alpha) and Palmer *et al.* (17) (LB, LT, CD8, GRANS, LYMPHS) in the CCLE and CGP.

2 Supplementary results

2.1 Correlations between 138 genes and metagenes

We assessed the correlation of each gene of the 138-gene signature with each of the six metagenes. For the Immunity, Interferon and Matrix metagenes, the expression of several genes was correlated with that of the metagene with correlation coefficients greater than 0.90 (Immunity metagene: CCL5, LCK, CD3D, CD2; Interferon metagene: IFI44L, IFIT1, ISG15, MX1; Matrix metagene: CDH11, FNBI, COL5A1, COL5A2, THBS2). The ESR1 gene was the gene most strongly correlated with the Hormone survival metagene, with a correlation coefficient of 0.77. The ESR1 gene was also inversely correlated with the Immunity metagene, with a correlation coefficient of -0.43.

2.2 Validation of the 138-gene signature

For Affymetrix© arrays, we used the 138 selected genes. For the Illumina© platform, we used 136 common genes. The corresponding heatmaps and the correlation coefficients for the relationships between each sample subgroup centroid in the three validation sets and the corresponding subgroup centroid in the training set are shown in Supplementary Figure 2.

Sample clustering was moderately consistent between the training and validation gene sets. Concordance was high for centroid 1 (high Immunity, low Matrix, low Hormone/survival, concordance from 69 to 79%), but reproducibility was not high for the other centroids in the validation datasets.

2.3 Correlation of Immunity metagene expression with hormonal pathways

The levels of expression of ESR1, PGR and AR as a function of Immunity metagene expression status were compared in the four datasets (see the results section for the training set). The results below are presented with *p*-values for the differences in the Immunity “low”

versus the Immunity “high” group, in the training, validation, Ignatiadis and METABRIC datasets. ESR1 expression was consistently higher in the “Immunity low” subgroup than in the “Immunity high” subgroup ($p < 10^{-16}$, $p = 0.008$, $p = 0.003$, $p < 0.00001$, respectively). PGR expression was also stronger in the Immunity metagene “low” expression group, in three of the four datasets ($p < 10^{-6}$, $p = 0.003$, $p = 0.07$ and $p = 0.02$, respectively). Similarly, AR expression was stronger in the Immunity metagene “low” expression group, in two of the four datasets ($p = 0.0002$, $p = 0.43$, $p = 0.003$ and $p = 0.17$, respectively).

We also compared the level of Immunity metagene expression across the four datasets as a function of ER, PR and AR status, as previously described. Immunity metagene expression was significantly stronger in the ER-negative subgroup than in the ER-positive subgroup, in all four datasets ($p < 10^{-9}$, $p = 0.03$, $p < 0.0001$ and $p = 0.02$, respectively). PR negativity was associated with higher levels of Immunity metagene expression in all but the Ignatiadis dataset ($p = 0.0001$, $p = 0.05$, $p = 0.24$ and $p = 0.04$, respectively), and AR negativity was associated with stronger Immunity metagene expression in the training and METABRIC datasets ($p = 10^{-6}$ and $p = 0.04$, respectively), whereas no such association was observed in the validation and Ignatiadis datasets ($p = 0.75$ and 0.80 , respectively).

We also compared the expression levels of each gene of the Immunity metagene separately as a function of ER status. Most of the genes of the Immunity metagene were significantly less strongly expressed in ER-positive than in ER-negative tumors in all four datasets (training set: 27/28 genes, validation set: 19/28, METABRIC: 23/26 genes, and Ignatiadis dataset: 16/28).

We then assessed the proportion of Immunity “low” and Immunity “high” samples across the four datasets as a function of ER status. The samples of ER-positive patients were more likely to be classified as Immunity “low” in all but the validation set (81% versus 19%, $p < 10^{-9}$; 47%

versus 53%, $p=0.54$; 63% versus 37%, $p<0.001$; 61% versus 39%, $p=0.03$). Samples from ER-negative patients were more likely to be classified as Immunity “high” in all but the validation set (71% versus 29%, $p<10^{-9}$; 48% versus 52%, $p=0.54$; 60% versus 40%, $p<0.001$; 63% versus 37%, $p=0.03$).

2.4 Prediction of the response to chemotherapy

We also performed similar analyses in the subset of patients that did not receive trastuzumab ($n=75$). The rates of pCR as a function of ER status were 16.3% (7/43) for ER-positive tumors and 40.6% (13/32) for ER-negative tumors ($p=0.02$). After stratification for Immunity metagene status, the pCR rates obtained were significantly different ($p=0.003$): 7.4% (2/27 Immunity low) v 31.3% (5/16 Immunity high) for ER-positive tumors ($p=0.08$) and 16.7% (2/12 Immunity low) versus 55.0% (11/20 Immunity high) for ER-negative tumors ($p=0.08$).

2.5 Correlations between the Immunity metagene and published signatures

String database connections between the Immunity genes and published predictive and prognostic metagenes or immune signatures are provided in Figure S4. The gene intersection was poor, but our immune signature nevertheless appears to be strongly correlated with other published signatures, consistent with the use of similar immune pathways. Pearson’s correlation coefficients for the signatures are shown in brackets. The Immunity metagene was strongly correlated with the T-fh metagene ($r=0.89$), the CXCL13 metagene ($r=0.74$), and the LCK metagene ($r=0.96$). In comparisons with prognostic signatures, it was found to be correlated with the Denkert signature ($r=0.73$), the Th1 metagene ($r=0.75$), the IRSN-23 predictor ($r=0.62$), the CXCL9 metagene ($r=0.70$), and the IgG metagene ($r=0.78$), although the coefficients were lower.

References

1. Ignatiadis M, Singhal SK, Desmedt C, Haibe-Kains B, Criscitiello C, Andre F, Loi S, Piccart M, Michiels S, Sotiriou C: Gene modules and response to neoadjuvant chemotherapy in breast cancer subtypes: a pooled analysis. *J Clin Oncol* 2012, 30:1996–2004.
2. Curtis C, Shah SP, Chin S-F, Turashvili G, Rueda OM, Dunning MJ, Speed D, Lynch AG, Samarajiwa S, Yuan Y, Gräf S, Ha G, Haffari G, Bashashati A, Russell R, McKinney S, Langerød A, Green A, Provenzano E, Wishart G, Pinder S, Watson P, Markowitz F, Murphy L, Ellis I, Purushotham A, Børresen-Dale A-L, Brenton JD, Tavaré S, Caldas C, et al.: The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups. *Nature* 2012, 486:346–352.
3. Barbosa-Morais NL, Dunning MJ, Samarajiwa S a, Darot JFJ, Ritchie ME, Lynch AG, Tavaré S: A re-annotation pipeline for Illumina BeadArrays: improving the interpretation of gene expression data. *Nucleic Acids Res* 2010, 38:e17.
4. Karn T, Metzler D, Ruckhaberle E, Hanker L, Gatje R, Solbach C, Ahr A, Schmidt M, Holtrich U, Kaufmann M, Rody A: Data-driven derivation of cutoffs from a pool of 3,030 Affymetrix arrays to stratify distinct clinical types of breast cancer. *Breast Cancer Res Treat* 2010, 120:567–579.
5. Gong Y, Yan K, Lin F, Anderson K, Sotiriou C, Andre F, Holmes FA, Valero V, Booser D, Pippin JE, Vukelja S, Gomez H, Mejia J, Barajas LJ, Hess KR, Sneige N, Hortobagyi GN, Pusztai L, Symmans WF: Determination of oestrogen-receptor status and ERBB2 status of breast carcinoma: a gene-expression profiling study. *Lancet Oncol* 2007, 8:203–211.
6. Servant N, Gravier E, Gestraud P, Laurent C, Paccard C, Biton A, Brito I, Mandel J, Asselain B, Barillot E, Hupé P: EMA - A R package for Easy Microarray data analysis. *BMC Res Notes* 2010, 3:277.
7. Monti S, et al.: Consensus clustering: a resampling-based method for class discovery and visualization of gene expression microarray data. *Mach. Learn* 2003, 52:91–118.
8. Jensen LJ, Kuhn M, Stark M, Chaffron S, Creevey C, Muller J, Doerks T, Julien P, Roth A, Simonovic M, Bork P, von Mering C: STRING 8--a global view on proteins and their functional interactions in 630 organisms. *Nucleic Acids Res* 2009, 37(Database issue):D412–6.
9. Bonnefoi H, Potti A, Delorenzi M, Mauriac L, Campone M, Tubiana-Hulin M, Petit T, Rouanet P, Jassem J, Blot E, Becette V, Farmer P, André S, Acharya CR, Mukherjee S, Cameron D, Bergh J, Nevins JR, Iggo RD: Validation of gene signatures that predict the response of breast cancer to neoadjuvant chemotherapy: a substudy of the EORTC 10994/BIG 00-01 clinical trial. *Lancet Oncol* 2007, 8:1071–1078.
10. Denkert C, von Minckwitz G, Brase JC, Sinn BV, Gade S, Kronenwett R, Pfitzner BM, Salat C, Loi S, Schmitt WD, Schem C, Fisch K, Darb-Esfahani S, Mehta K, Sotiriou C, Wienert S, Klare P, André F, Klauschen F, Blohmer J-U, Krappmann K, Schmidt M, Tesch H, Kümmel S, Sinn P, Jackisch C, Dietel M, Reimer T, Untch M, Loibl S: Tumor-infiltrating

lymphocytes and response to neoadjuvant chemotherapy with or without Carboplatin in human epidermal growth factor receptor 2-positive and triple-negative primary breast cancers. *J Clin Oncol Off J Am Soc Clin Oncol* 2015, 33:983–991.

11. Gu-Trantien C, Loi S, Garaud S, Equeter C, Libin M, Wind A De, Ravoet M, Buanec H Le, Sibille C, Manfouo-Foutsop G, Veys I, Haibe-Kains B, Singhal SK, Michiels S, Rothé F, Salgado R, Duvillier H, Ignatiadis M, Desmedt C, Bron D, Larsimont D, Piccart M, Sotiriou C, Willard-Gallo K: CD4+ follicular helper T cell infiltration predicts breast cancer survival. *J Clin Invest* 2013, 123:1–20.

12. Sota Y, Naoi Y, Tsunashima R, Kagara N, Shimazu K, Maruyama N, Shimomura A, Shimoda M, Kishi K, Baba Y, Kim SJ, Noguchi S: Construction of novel immune-related signature for prediction of pathological complete response to neoadjuvant chemotherapy in human breast cancer. *Ann Oncol Off J Eur Soc Med Oncol ESMO* 2014, 25:100–106.

13. Stoll G, Enot D, Mlecnik B, Galon J, Zitvogel L, Kroemer G: Immune-related gene signatures predict the outcome of neoadjuvant chemotherapy. *Oncoimmunology* 2014, 3:e27884.

14. Rody A, Karn T, Liedtke C, Pusztai L, Ruckhaeberle E, Hanker L, Gaetje R, Solbach C, Ahr A, Metzler D, Schmidt M, Müller V, Holtrich U, Kaufmann M: A clinically relevant gene signature in triple negative and basal-like breast cancer. *Breast Cancer Res BCR* 2011, 13:R97.

15. Staaf J, Ringnér M, Vallon-Christersson J, Jönsson G, Bendahl P-O, Holm K, Arason A, Gunnarsson H, Hegardt C, Agnarsson BA, Luts L, Grabau D, Fernö M, Malmström P-O, Johannsson OT, Loman N, Barkardottir RB, Borg A: Identification of subtypes in human epidermal growth factor receptor 2--positive breast cancer reveals a gene signature prognostic of outcome. *J Clin Oncol Off J Am Soc Clin Oncol* 2010, 28:1813–1820.

16. Desmedt C, Haibe-Kains B, Wirapati P, Buyse M, Larsimont D, Bontempi G, Delorenzi M, Piccart M, Sotiriou C: Biological processes associated with breast cancer clinical outcome depend on the molecular subtypes. *Clin Cancer Res* 2008, 14:5158–5165.

17. Finak G, Bertos N, Pepin F, Sadekova S, Souleimanova M, Zhao H, Chen H, Omeroglu G, Meterissian S, Omeroglu A, Hallett M, Park M: Stromal gene expression predicts clinical outcome in breast cancer. *Nat Med* 2008, 14:518–527.

18. Teschendorff AE, Miremadi A, Pinder SE, Ellis IO, Caldas C: An immune response gene expression module identifies a good prognosis subtype in estrogen receptor negative breast cancer. *Genome Biol* 2007, 8:R157.

19. Alexe G, Dalgin GS, Scandfeld D, Tamayo P, Mesirov JP, DeLisi C, Harris L, Barnard N, Martel M, Levine AJ, Ganesan S, Bhanot G: High expression of lymphocyte-associated genes in node-negative HER2+ breast cancers correlates with lower recurrence rates. *Cancer Res* 2007, 67:10669–10676.

20. Perez EA, Thompson EA, Ballman KV, Anderson SK, Asmann YW, Kalari KR, Eckel-Passow JE, Dueck AC, Tenner KS, Jen J, Fan J-B, Geiger XJ, McCullough AE, Chen B, Jenkins RB, Sledge GW, Winer EP, Gralow JR, Reinholz MM: Genomic Analysis Reveals That Immune Function Genes Are Strongly Linked to Clinical Outcome in the North Central

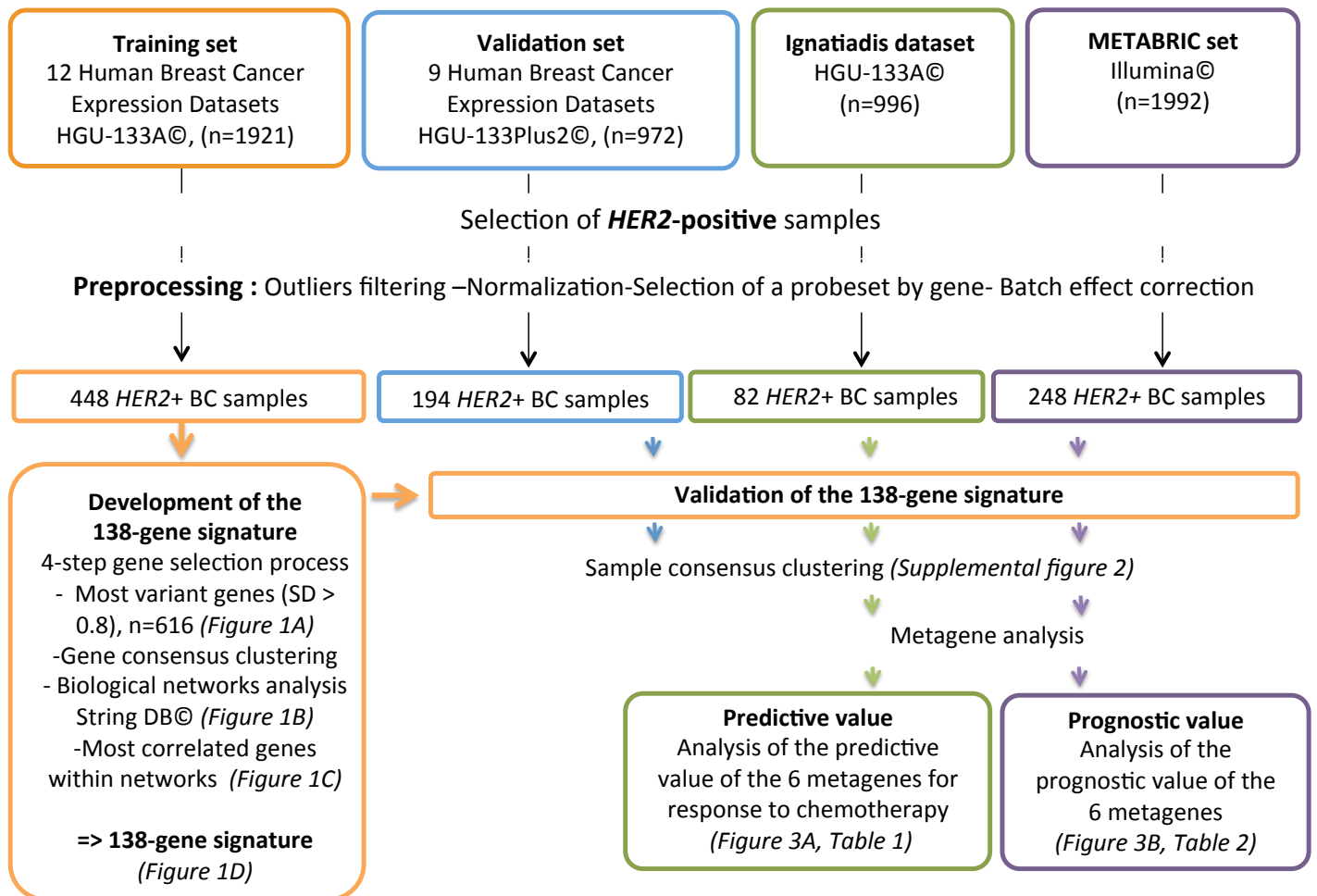
Cancer Treatment Group N9831 Adjuvant Trastuzumab Trial. *J Clin Oncol Off J Am Soc Clin Oncol* 2015.

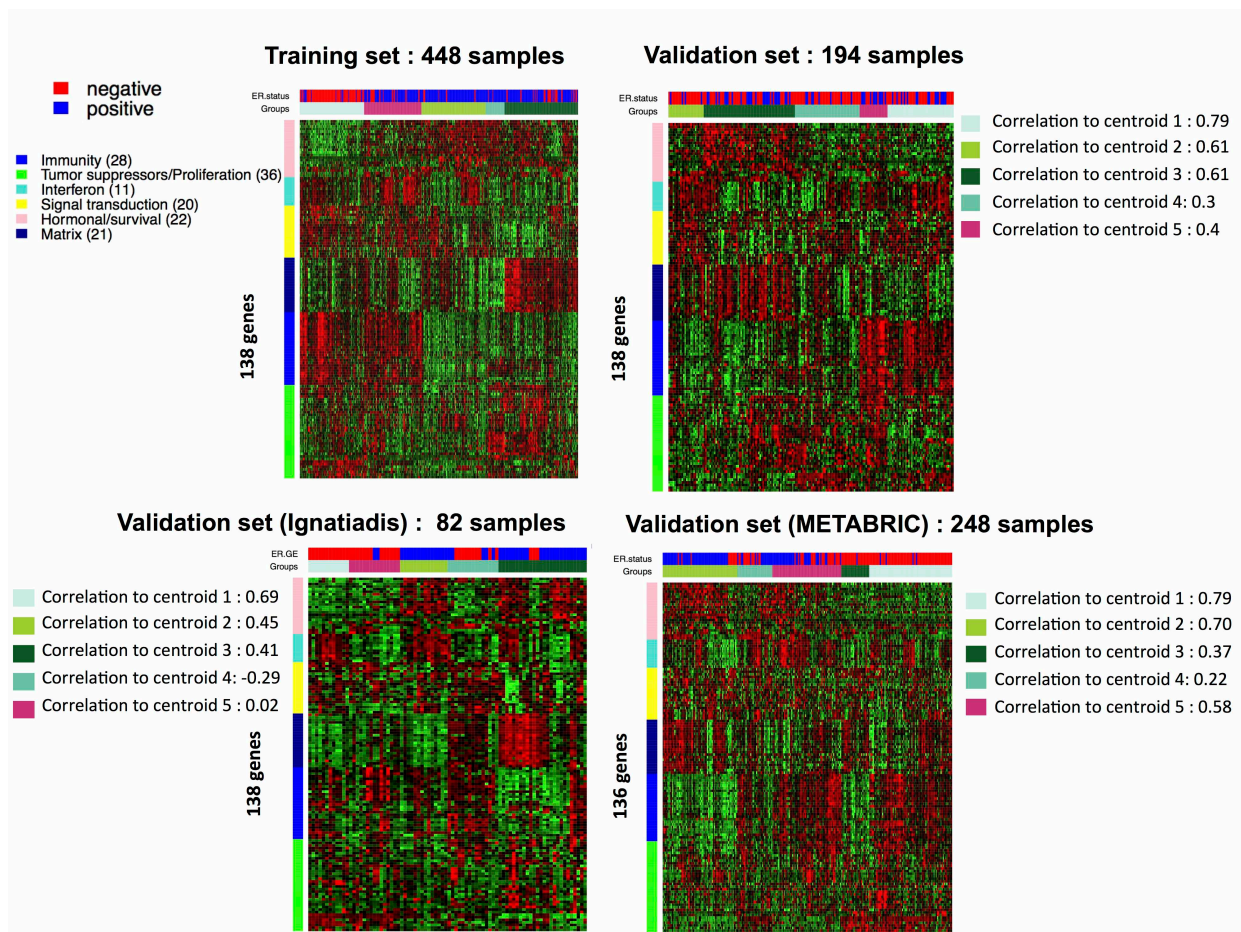
21. De Cremoux P, Valet F, Gentien D, Lehmann-Che J, Scott V, Tran-Perennou C, Barbaroux C, Servant N, Vacher S, Sigal-Zafrani B, Mathieu M-C, Bertheau P, Guinebretière J-M, Asselain B, Marty M, Spyrtos F: Importance of pre-analytical steps for transcriptome and RT-qPCR analyses in the context of the phase II randomised multicentre trial REMAGUS02 of neoadjuvant chemotherapy in breast cancer patients. *BMC Cancer* 2011, 11:215.

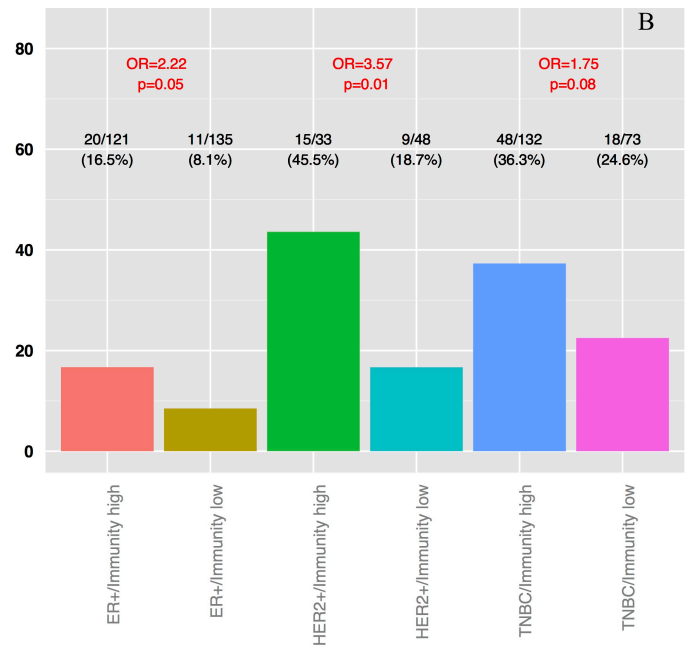
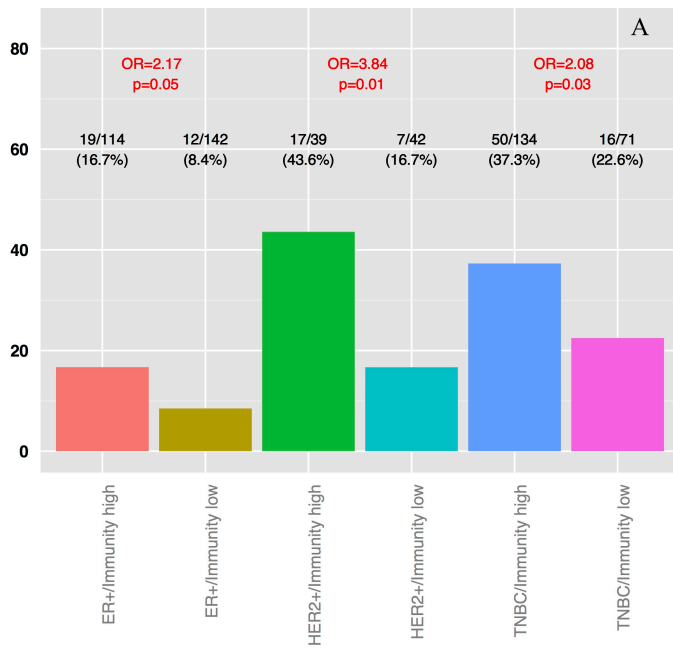
22. Salgado R, Denkert C, Demaria S, Sirtaine N, Klauschen F, Pruneri G, Wienert S, Van den Eynden G, Baehner FL, Penault-Llorca F, Perez EA, Thompson EA, Symmans WF, Richardson AL, Brock J, Criscitiello C, Bailey H, Ignatiadis M, Floris G, Sparano J, Kos Z, Nielsen T, Rimm DL, Allison KH, Reis-Filho JS, Loibl S, Sotiriou C, Viale G, Badve S, Adams S, et al.: The evaluation of tumor-infiltrating lymphocytes (TILs) in breast cancer: recommendations by an International TILs Working Group 2014. *Ann Oncol Off J Eur Soc Med Oncol ESMO* 2015, 26:259–271.

23. Barretina J, Caponigro G, Stransky N, Venkatesan K, Margolin AA, Kim S, Wilson CJ, Lehár J, Gregory V, Sonkin D, Reddy A, Liu M, Murray L, Michael F, Monahan JE, Morais P, Meltzer J, Korejwa A, Jané- J, Mapa FA, Thibault J, Bric-furlong E, Raman P, Engels IH, Cheng J, Yu GK, Yu J, Jr PA, Silva M De, Jagtap K, et al.: NIH Public Access of anticancer drug sensitivity. 2012, 483:603–607.

24. Garnett MJ, Edelman EJ, Heidorn SJ, Greenman CD, Dastur A, Lau KW, Greninger P, Thompson IR, Luo X, Liu Q, Iorio F, Surdez D, Chen L, Milano RJ, Bignell GR, Tam AT, Davies H, Stevenson J a, Barthorpe S, Lutz SR, Kogera F, Lawrence K, McLaren-douglas A, Mironenko T, Thi H, Richardson L, Zhou W, Hur W, Yang W, Deng X, et al.: Europe PMC Funders Group Systematic identification of genomic markers of drug sensitivity in cancer cells. *Nature* 2012, 483:570–575.











ORIGINAL ARTICLE

Stromal lymphocyte infiltration after neoadjuvant chemotherapy is associated with aggressive residual disease and lower disease-free survival in *HER2*-positive breast cancer

A.-S. Hamy^{1,2}, J.-Y. Pierga^{3,4}, A. Sabaila⁵, E. Laas⁵, H. Bonsang-Kitzis^{1,2,5}, C. Laurent^{1,2}, A. Vincent-Salomon⁶, P. Cottu³, F. Lerebours³, R. Rouzier⁵, M. Lae^{6†} & F. Reyat^{1,2,5*}

¹Residual Tumor & Response to Treatment Laboratory, RT2Lab, Translational Research Department, PSL Research University, Institut Curie, Paris; ²INSERM, U932 Immunity and Cancer, Paris; ³Department of Medical Oncology, Institut Curie, Paris; ⁴Faculty of Medicine, Paris Descartes University, Paris; ⁵Departments of Surgery; ⁶Tumor Biology, Institut Curie, Paris, France

*Correspondence to: Dr Fabien Reyat, Department of Surgery, Institut Curie, 26 rue d'Ulm, 75005 Paris, France. Tel: +33-1-44-32-40-87; Fax: +33-6-15-27-19-80; E-mail: fabien.reyat@curie.fr

[†]These authors contributed equally to this work.

Background: The role of tumor-infiltrating lymphocytes (TILs) in breast cancer has been extensively studied over the last decade. High TILs levels have been associated with pathological response rate in the neoadjuvant setting and with better outcomes in the adjuvant setting. However, little attention has been paid to changes in TILs and residual TIL levels after neoadjuvant chemotherapy (NAC). We investigated TIL levels before, after chemotherapy, and their dynamics during treatment; and we assessed the correlation of these levels with response to NAC and prognosis.

Materials and methods: We identified 175 patients with primary *HER2*-positive breast cancers receiving NAC+/- trastuzumab between 2002 and 2011. Microbiopsy specimens and paired surgical samples were evaluated for stromal lymphocyte infiltration. Univariate and multivariate analyses were carried out to assess the association of clinical and pathological factors with pathological complete response (pCR) and disease-free survival.

Results: Baseline TIL levels were not significantly associated with pCR. TIL levels decreased during treatment in 78% of the patients. The magnitude of the decrease was strongly associated with pCR. After chemotherapy, TIL levels were high in tumors displaying aggressive patterns (high residual cancer burden score, mitotic index >22, tumor cellularity >5%). In the population with residual disease, TIL levels >25% at the end of NAC were significantly associated with an adverse outcome (TILs >25%, HR = 7.98, *P* = 0.009) after multivariate analyses including BMI, post-NAC mitotic index and tumor grade.

Conclusion: A decrease in TIL levels during chemotherapy was positively associated with response to treatment. In tumor failing to achieve pCR, post-NAC lymphocytic infiltration was associated with higher residual tumor burden and adverse clinical outcome. Further studies are required to characterize immune infiltration in residual disease to identify candidates who could benefit from second-line therapy trials including immune checkpoint inhibitors.

Key words: tumor-infiltrating lymphocytes, *HER2*-positive, prediction, prognosis, neoadjuvant chemotherapy

Introduction

Breast cancer (BC) is the most frequently diagnosed cancer and the leading cause of cancer-related death in women. *HER2*-positive BCs display amplification and overexpression of the *HER2*

tyrosine-kinase receptor gene. This subgroup is defined by aggressive pathological though trastuzumab-based treatments have greatly improved their outcomes over the last decade [1].

Neoadjuvant treatment is currently administered to patients with locally advanced BC. Beyond increasing the rate of

Original article

Annals of Oncology

breast-conserving surgery, it serves as a test of *in vivo* chemosensitivity, and the analysis of residual tumor burden may help understanding resistance to treatments.

AQ7

The role of tumor-infiltrating lymphocytes (TILs) in BC has been studied in detail over the last decade. Many studies have reported associations between high levels of TILs at diagnosis and better response to neoadjuvant chemotherapy [2–4], and better prognosis in both neoadjuvant and adjuvant chemotherapy [5–8], particularly for triple negative BCs (TNBC) and HER2-positive BC. Only few studies investigated TILs following neoadjuvant chemotherapy (NAC) and addressed their prognostic significance.

In this study, we assessed the association between TIL levels (before, during, and after NAC) and response to treatment, and their prognostic values in a real-life cohort of HER2-positive BC patients.

Materials and methods

Patients

We analyzed a cohort of 175 T1-3Nxm0 patients with HER2-positive invasive BC (NEOREP Cohort, CNIL declaration number 1547270) treated at Institut Curie, Paris, between 2002 and 2012. We included unilateral, non-recurrent, non-inflammatory, non-metastatic tumors, excluding T4 tumors. All patients received NAC, followed by surgery and radiotherapy. NAC regimens changed over time (anthracycline-based regimen or sequential anthracycline-taxane regimen), with trastuzumab used in an adjuvant and/or neoadjuvant setting since 2005. Endocrine therapy (tamoxifen or aromatase inhibitor) was prescribed when indicated. The study was approved by the Breast Cancer Study Group of Institut Curie and was conducted according to institutional and ethical rules regarding research on tissue specimens and patients. Considering that neoadjuvant trastuzumab and adjuvant trastuzumab use have been associated with an increase in pathological complete response (pCR) rates and disease-free survival (DFS), respectively [1], this study provides data on the subpopulation of patients who received both neoadjuvant and adjuvant trastuzumab as supplementary Materials and methods, available at *Annals of Oncology online* (supplementary Tables S4–S6 and Figures S6–S10, available at *Annals of Oncology online*).

Tumor samples and pathology review

Pretreatment core needle biopsies and post-NAC surgical specimens were reviewed for the purpose of the study and were evaluated independently for the presence of a mononuclear cells infiltrate (including lymphocytes and plasma cells, excluding polymorphonuclear leukocytes) following the recommendations of the international TILs Working Group [9]. TILs were reviewed by one expert breast pathologist (ML) on hematoxylin and eosin-stained sections without additional staining. They were evaluated in the stroma, within the border of the tumor scar, after excluding areas around ductal carcinoma *in situ*, and tumor zones with necrosis and artifacts, and were scored continuously as the average percentage of stromal area occupied by mononuclear cells. To ensure that the mononuclear cells infiltrate considered as TILs in the analyses indeed corresponded to lymphocytes, we carried out a CD3+ immunostaining on a subset of 20 surgical specimens, which strongly correlated with the levels of unstained TILs (see supplementary Results, Table S3, and Figures S2–S5, available at *Annals of Oncology online*). The scar area was first analyzed and measured at gross examination. Scar appeared as a white aspect of the breast parenchyma representing the previous tumor bed modified by NAC, and is characterized by histiocytes, lymphocytes, macrophages, fibrosis, and elastosis. The whole fibro-inflammatory scar was evaluated on HE sections (size evaluation in millimeters and stromal

TILs evaluation). Cases were considered estrogen receptor (ER) or progesterone receptor (PR) positive if at least 1% of the tumor cells expressed estrogen and/or progesterone receptors (ER/PR). Results using a 10% positivity threshold in accordance with guidelines used in France [10] are provided in the Supplementary Materials and methods, available at *Annals of Oncology online* (supplementary Tables S7–S9 and Figures S11 and S12, available at *Annals of Oncology online*). HER2 expression was determined by immunohistochemistry and scoring was carried out according to the American Society of Clinical Oncology (ASCO)/College of American Pathologists (CAP) guidelines [11]. Scores 3+ were reported as positive, score 1+/0 as negative. Tumors with scores 2+ were further tested by FISH. With regard to HER2 gene amplification, an average of 40 tumor cells per sample was evaluated and the mean HER-2 signals per nuclei was calculated. A HER2/CEN17 ratio ≥ 2 was considered positive, and a ratio < 2 negative [11].

Study end points

ypTN stage was defined according to the American Joint Committee on Cancer.

A pCR was defined as the absence of invasive residual tumor in both breast and axillary nodes (ypT0/is N0). Determination of residual cancer burden (RCB) was realized according to Symmans [12]. DFS was defined as the time from surgery to death, locoregional recurrence or distant recurrence. Patients for whom none of these events were recorded were censored at the date of their last known contact.

Statistical analysis

TIL levels and qualitative variables in classes were compared by ANOVA test with a post hoc Tukey analysis when necessary. Relative changes in TIL levels were calculated by the difference between TIL levels post- and pre-NAC divided by pre-NAC TIL levels. Changes in mean values were investigated in paired *t*-tests. As no cut-off value for TIL levels after NAC has been published, we investigated the prognostic performance of each threshold value concerning the association with DFS and retained the cut-off value yielding the lowest *P*-value.

Factors predictive of pCR were introduced into a univariate logistic regression model. A multivariate logistic model with forward stepwise selection procedure was then implemented with the covariates having a likelihood ratio test *P*-value below 0.10. Survival probabilities were estimated by the Kaplan–Meier method, and survival curves were compared in log-rank tests. Hazard ratios and their 95% confidence intervals were calculated with the Cox proportional hazards model. Variables with a *P*-value for the likelihood ratio test ≤ 0.10 in univariate analysis were included in the multivariate model. Forward selection was used to establish the final multivariate model. The significance threshold was 5%.

Analyses were carried out with R software, version 3.1.2.

Results

A total of 175 patients were included in the cohort. The median age was 47 years (range: 27–78 years), 64% ($n = 109$) were premenopausal, and 29.1% ($n = 51$) were overweight or obese (BMI > 25). Clinically, most patients were classified as having stage T2, node-positive BC (64%, $n = 109$). Most of the tumors were grade 3 tumors (70%, $n = 121$), and 38.3% were ER-negative. Most of them received anthracycline-taxane based regimen (88%, $n = 154$) as neoadjuvant treatment, 82% of patients received both neoadjuvant and adjuvant trastuzumab ($n = 144$) and 15% adjuvant trastuzumab only ($n = 26$). Sixty-eight patients (39%) achieved pCR after NAC, and this rate was different by ER status [ER negative 53.7% (36/67); ER positive: 29.6% (32/108), $P = 0.002$].

Table 1. Association between baseline, post-NAC TILs and pre- and post-NAC clinicopathological factors (n = 175)

Variables	n	Baseline TILs (%)	P	Post-NAC TILs (%)	P	
Pre-neoadjuvant chemotherapy parameters						
Age	≤45 years	82	27.13	0.7	12.41	0.1
	46–55 years	49	26.47		10.49	
	>55 years	44	24.93		8.61	
Menopausal status	Post	61	27.49	0.5	8.89	0.06
	Pre	113	25.95		11.89	
BMI	19–25	117	24.25	0.054	11.03	0.8
	<19	7	34.29		8.57	
	>25	51	30.24		10.98	
Tumor size	T1	10	32	0.5	6.7	0.2
	T2	113	26.28		10.62	
	T3	52	25.56		12.38	
Nodal status	N0	62	27.81	0.4	10.13	0.4
	N1–N2–N3	113	25.62		11.35	
Histology	Other	5	12.4	0.06	8.4	0.5
	Ductal	169	26.55		10.97	
Grade	I–II	51	24.16	0.2	10.24	0.5
	III	121	27.62		11.33	
Neoadjuvant trastuzumab	No	26	22.01	0.1	13.69	0.1
	Yes	144	27.26		10.47	
Tumor cellularity	<70%	72	27.74	0.3	9.85	0.2
	≥70%	103	25.46		11.67	
Mitotic index	≤10	45	26.93	0.1	9.53	0.5
	11–22	72	23.64		11.18	
	>22	58	29.4		11.67	
ER status (cut-off 1%)	Negative	67	28.99	0.12	11.04	0.9
	Positive	108	24.79		10.84	
PR status (cut-off 1%)	Negative	111	27.51	0.2	9.81	0.1
	Positive	59	23.98		12.49	
Post-NAC parameters						
pCR	Residual disease	107	25.36	0.3	13.94	<0.001
	pCR	68	28.01		6.16	
RCB	0	68	28.01	0.2	6.16	<0.001 (b)
	1	24	29.17		9.79	
	2	69	23.17		13.29	
	3	14	29.64		24.29	
Mitotic index	≤10	153	26.3	0.9	9.43	<0.001 (c)
	11–22	5	25		15.6	
	>22	17	27.65		22.94	
Tumor cellularity	≤5%	91	28.19	0.2	7.27	<0.001 (d)
	6%–15%	33	22.7		10.42	
	>15%	49	25.92		18.16	
Nodal involvement	0	129	26.91	0.78	9.84	0.054
	1–3	38	25.18		13.47	
	≥4 N+	8	23.75		16.25	
Larger nodal metastasis	≤2 mm	24	22.92	0.02 (a)	8.63	0.001 (e)
	3–5 mm	12	18.08		12.92	
	>5 mm	11	36.82		26.36	

Post hoc analyses for significant ANOVA tests (only results with *P*-value 0.05 or below are reported); baseline TIL levels: larger nodal metastasis (a) size >5 mm versus ≤2 mm *P* = 0.05; size >5 mm versus 3–5 mm, *P* = 0.02; post-NAC TIL levels: RCB (b): 2 versus 0, *P* < 0.001; 3 versus 0, *P* < 0.001; 3 versus 1, *P* < 0.001, 3 versus 2, *P* < 0.001; mitotic index (c): >22 versus ≤10, *P* < 0.001; tumor cellularity (d): >15% versus ≤5%, *P* < 0.001, >15% versus 6–15%, *P* < 0.001; larger nodal metastasis (e): >5 mm versus ≤2 mm, *P* < 0.001; >5 mm versus 3–5 mm, *P* = 0.03.

AQ13

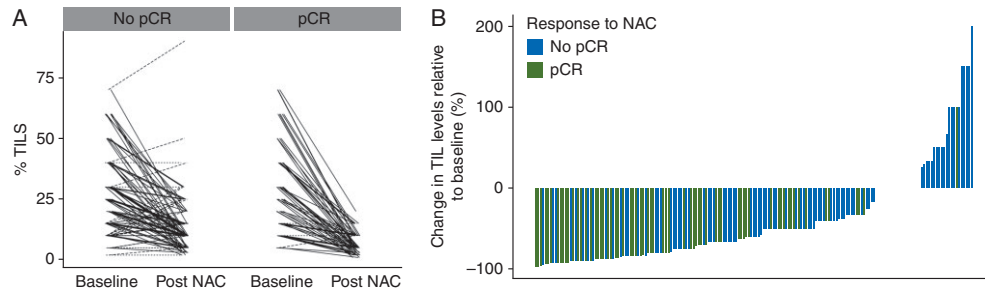
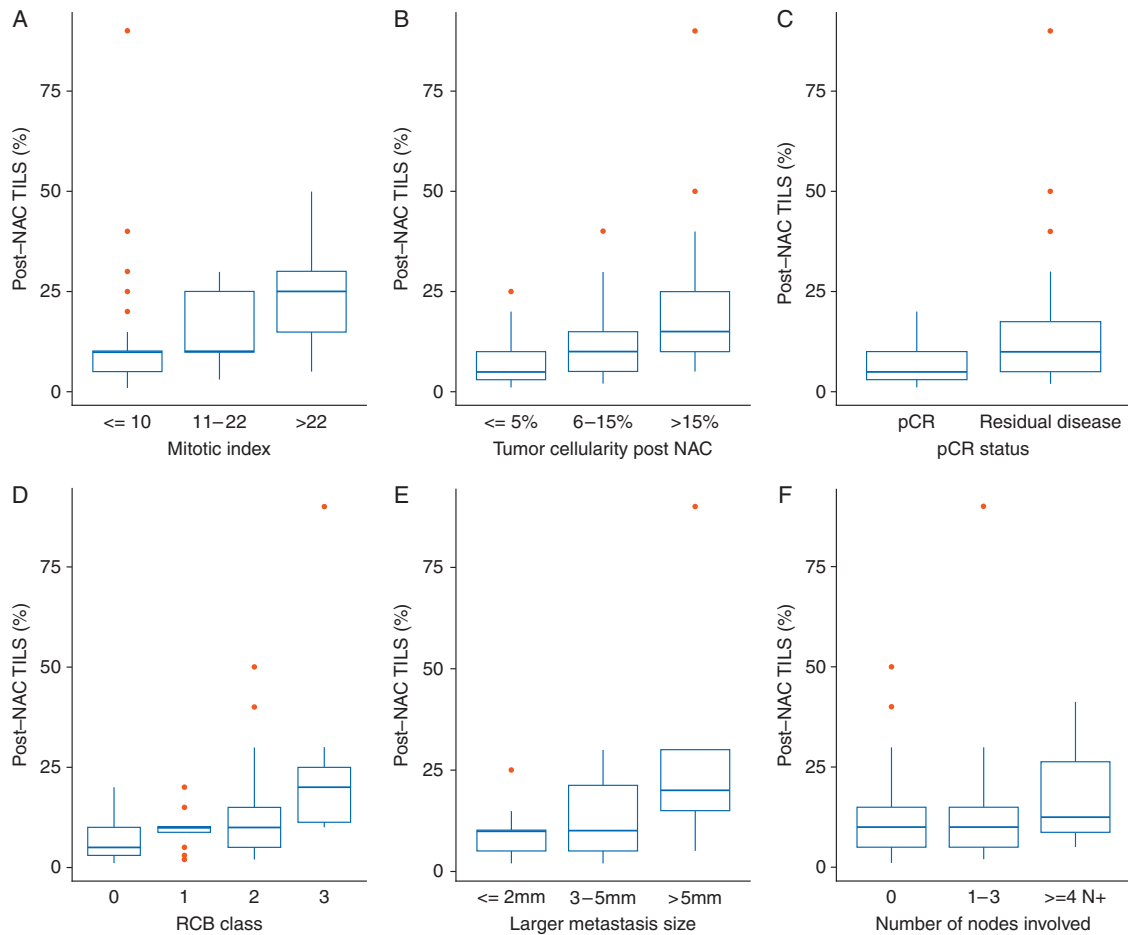


Figure 1. (A) TILs levels before (baseline) and after (post-NAC) chemotherapy. The solid black line represents paired samples displaying a decrease in TILs levels, the dotted lines represent pairs with no change in TILs levels, and the dashed lines represent pairs displaying an increase in TILs levels. (B) Relationship between the change in TILs level (represented as a percentage change relative to baseline levels) and pathological complete response. Plots are sorted by increasing magnitude of change in TILs levels.

AQ12



AQ17

Figure 2. Association between post-NAC TILs percentages and post-NAC parameters: mitotic index (A), tumor cellularity (B), pCR status (C), RCB class (D), larger nodal metastasis size (E) and number of nodes involved (F).

At baseline, the median baseline TIL level was 25% (range: 2%–70%). Baseline TIL levels were higher in patients with BMI >25 ($P=0.05$) and in patients with larger nodal metastasis >5 mm at NAC completion than patients with larger nodal metastasis ≤ 2 mm ($P=0.02$) (Table 1).

After chemotherapy, the median TIL percentage fell to 10% [1–90] [TIL levels decrease ($n=136$, 78%), unchanged ($n=18$, 10%), increase ($n=21$, 12%)]. TIL levels decreased in all but three patients in the pCR group (Figure 1A). The variation of TIL levels was strongly associated with pCR ($P < 10^{-5}$), with the

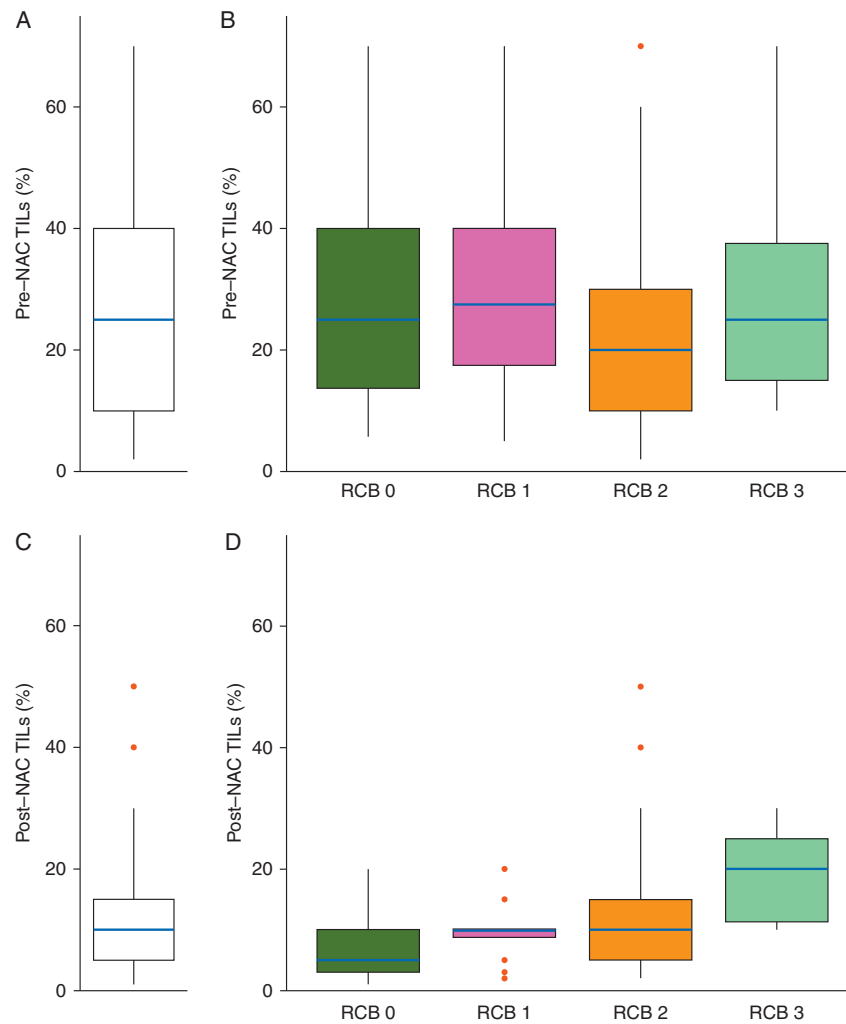


Figure 3. Pre-chemotherapy TILs levels (A) and median levels by RCB class (B). Post-chemotherapy TILs levels (C) and median levels by RCB class (D).

largest decreases of TILs being strongly associated with the achievement of a pCR (Figure 1B). In univariate analysis, baseline TIL levels were not associated with the occurrence of a pCR (supplementary Table S1, available at *Annals of Oncology online*).

AQ8
5

None of the baseline characteristics of the patients or tumors were significantly associated with post-NAC TIL levels (Table 1). Conversely, post-NAC TILs were strongly associated with post-NAC parameters [no pCR, high RCB class, high mitotic index and residual tumor cellularity, $P < 0.001$], as well as a high number of nodes involved ($P = 0.054$), and larger nodal metastases ($P = 0.001$) (Figure 2).

10

Higher RCBs were associated with higher post-NAC TIL levels but not with baseline TIL levels (Figure 3). Overall, these findings indicate that the aggressiveness of the residual tumor is associated with TIL levels after chemotherapy, but not with baseline TIL levels. Analyses carried out after stratifying by ER status did not

15

substantially change the results and are detailed in supplementary Table S2, available at *Annals of Oncology online*.

During a median follow-up of 38.8 months (range 5.5–91.7), 18 patients suffered relapses (one from pCR group). No association was found between DFS and baseline TIL levels (supplementary Table S3, available at *Annals of Oncology online*). In the population of patients without pCR ($n = 107$), levels of TILs higher than 25% were an independent poor prognostic factor together with BMI, tumor grade, post-NAC mitotic index and RCB score (Table 2; supplementary Figure S1, available at *Annals of Oncology online*).

20

25

Discussion

This study of 175 *HER2*-positive pair-matched pre-treatment biopsy and post-treatment surgical specimens provides new insight

30

Table 2. Association of clinicopathological factors and TILs parameters with DFS (population without pCR)

Variable	n	Ev	Univariate analysis				Multivariate analysis			
			HR	95% CI	P	P*	HR	95% CI	P*	
Pre-NAC parameters										
Age	≤45 years	59	9	1						
	46–55 year	31	6	1.14	0.41–3.21	0.8	0.9			
	>55 year	17	2	0.8	0.17–3.71	0.7	0.9			
Menopausal status	post	29	5	1						
	pre	77	12	0.99	0.35–2.83	0.9	0.9			
BMI	19–25	69	6	1				1		
	<19	4	1	2.4	0.29–20.04	0.4		9.57	0.83–110.54	0.07
	>25	34	10	3.76	1.36–10.37	0.01	0.02	5.21	1.8–15.11	0.002
Tumor size	T1	3	0	1						
	T2	70	9	NA	NA		0.2			
	T3	34	8	NA	NA					
Nodal status	N0	37	5	1						
	N1–N2–N3	70	12	1.28	0.45–3.64	0.6	0.6			
Mitotic index	≤22	69	13	1						
	>22	37	4	0.71	0.23–2.17	0.5	0.5			
Grade	I–II	31	10	1				1		
	III	75	7	0.32	0.12–0.85	0.02	0.015	0.11	0.03–0.42	0.001
ER status (cut-off 1%)	Negative	31	7	1						
	Positive	76	10	0.63	[0.24–1.67]	0.4	0.4			
PR status (cut-off 1%)	Negative	54	10	1						
	Positive	48	5	0.58	0.2–1.69	0.3	0.3			
Pre-NAC TILs	<10%	7	1	1						0.924
	10%–60%	91	15	1.28	[0.17–9.7]	0.811				
	>60%	9	1	0.91	[0.06–14.61]	0.947				
Tumor cellularity	<70%	43	8	1						
	≥70%	64	9	0.69	0.27–1.79	0.4	0.4			
Post-NAC parameters										
Nodal involvement	0	61	9	1						
	1–3	38	6	0.89	0.31–2.53	0.829	0.67			
	≥4	8	2	1.82	0.39–8.45	0.445	0.67			
Mitotic index	≤10	85	9	1				1		
	>10	22	8	3.13	1.2–8.12	0.019	0.014	2.98	0.99–8.94	0.051
Tumor cellularity	≤5%	29	1	1						
	>5%	78	16	4.56	0.6–34.51	0.141	0.106			
RCB	1	24	1	1						
	2	69	12	3.35	0.44–25.84	0.245				
	3	14	4	6.1	0.68–54.6	0.106	0.201			
Post-NAC TILs	≤25%	98	13	1				1		
	>25%	9	4	3.23	1.05–9.93	0.041	0.03	7.98	1.68–37.77	0.009

into TILs variation during NAC, and the prognostic significance of residual TIL levels.

First, the association we reported between higher pCR rates and a large decrease in TIL levels after NAC was also found by Ali et al. [13], who first highlighted that an increase in lymphocyte density was associated with a relative chemoresistance. The higher pCR rate may be due to the disappearance of FOXP3+ lymphocyte-T as suggested by Ladoire et al. [14].

Second, the clinical significance of post-NAC TILs seems also complex. Here, we showed that (i) at NAC completion, TILs are present at higher levels in tumors failing to reach pCR than in chemosensitive tumors; (ii) this lymphocyte infiltration does not clear cancer cells effectively. Two hypotheses can be drawn.

On the one hand, TIL presence in patients with residual disease could be active but may not have had sufficient time to completely eradicate the tumor. Furthermore, the immune response may not recognize the tumor cells and post-NAC TILs could be unable to exert their antitumor function, possibly due to a surrounding immunosuppressive milieu.

Interestingly, the opposite pattern has been reported for TNBC by Dieci et al. [15] who showed that high levels of TILs in residual disease were associated with an absence of axillary lymph node metastasis (18 of 27; 66%) and a small tumor size (<2 cm, 22 of 27; 81%) after NAC. Similarly, Miyashita et al. [16] showed that high levels of CD8+ TILs after NAC were associated with a small residual tumor size in a series of 130 TNBCs.

An hypothesis accounting for differences in the predictive and prognostic impacts of TILs between *HER2*-positive BCs and TNBCs is the use of trastuzumab as a neoadjuvant treatment, as interactions between treatment type and the predicted impact of TIL levels have previously been reported [4]. It might also be plausible that TILs significance differs by BC subtype. In TNBC, baseline TIL levels are associated with pCR [2, 4, 17], whereas this effect seems more controversial in *HER2*-positive BC [3, 18–20], and a non-linear association between TILs and pCR had been evidenced in the NeoSPHERE [18] and the NeoALTTO trials [3]. In our study, TIL levels were not associated with pCR (similarly to the NeoSPHERE [18], NeoALTTO [3], and the GeparSepto trials [19]), whereas they were associated with response to chemotherapy in the GeparQuattro and GeparQuinto [20] trials.

Third, to our knowledge, this is the first report of an adverse prognostic impact of high post-NAC TIL levels in such a large cohort of *HER2*-positive BC patients. Our findings are consistent with those of Garcia Martinez et al., who reported an association between high levels of post NAC tumor lymphocyte infiltration and a worse DFS, even though the analysis was carried out on 121 patients of various BC subtypes [21]. To end, Ladoire et al. [22] investigated post-treatment tumor lymphocyte infiltrate in 111 *HER2*-positive BC, and showed that low levels of CD8+ cell infiltration after NAC were associated with poor RFS (HR = 3.85, $P < 0.0001$), and that low levels of FOXP3+ cell infiltration after NAC were associated with better RFS (HR = 0.52, $P = 0.036$). We did not yet perform immunostaining to separate out the immune subpopulations, which will enable us to determine whether there is any enrichment in immunosuppressive signaling. However, there is currently no clear consensus as to which single antibody or antibody combination should be used, and their interpretation is not standardized. One advantage of quantitative TIL assessment is that it could be carried out routinely in any pathology department with no real increase in technical costs; furthermore, our findings suggest that this quantitative information *per se* could be a useful prognostic marker after chemotherapy. Some studies have reported a positive correlation between the numbers of unstained TILs and CD8+ TILs [16], CD3 counts or counts for other immune subpopulations (CD3+, CD20+, CD68+) [21], supporting the notion that quantitative assessments could serve as a relevant surrogate marker. Moreover, Denkert et al. [2] showed that the expression of inflammatory marker genes and proteins was linked to the histopathological infiltrate, even for proteins with reported immunosuppressive functions, such as PD-1, PD-L1, IDO1, and CTLA4.

However, from a research standpoint, extensive characterization of the lymphocyte infiltrate remaining in residual tumors to determine the subsets of TILs present will further improve our understanding of chemoresistance mechanisms and anti-tumor immunology. In addition, immunostaining for PD1, PDL1, CTLA4 may provide theranostic information facilitating selection of the patients most likely to benefit from treatment with drugs restoring sensitivity to anti-tumor treatment.

Conclusion

If TILs variations are confirmed to be predictive of pCR, an early assessment of changes in TIL levels during chemotherapy could

serve as an early surrogate of resistance to treatment and could offer a possibility to a premature switch to second-line treatments. We will investigate whether the residual lymphocyte infiltrate in non-responding tumor is enriched in immunosuppressive cells that could be targeted by immune checkpoint blockade. If our findings are confirmed, we suggest that patients failing to achieve pCR and with high post-NAC TILs should be included in specific second-line drug trials.

Ethics approval

The NEOREP Cohort has been declared to the CNIL under declaration number 1547270. The study was approved by the Breast Cancer Study Group of Institut Curie and was conducted according to institutional and ethical rules concerning research on tissue specimens and patients. Informed consent from the patients was not required.

Funding

We thank Roche France for financial support for the construction of the Institut Curie neoadjuvant database (NEOREP) (no grant numbers apply). The funding source had no role in data analysis and interpretation or in the writing of the manuscript. A-SH-P was supported by an ITMO-INSERM-AVIESAN cancer translational research grant (no grant numbers apply). Funding was also obtained from the Site de Recherche Intégrée en Cancérologie/Institut National du Cancer (Grant No. INCa-DGOS-4654).

Disclosure

The authors have declared no conflicts of interest.

References

- Hamy-Petit A-S, Belin L, Bonsang-Kitzis H et al. Pathological complete response and prognosis after neoadjuvant chemotherapy for *HER2*-positive breast cancers before and after trastuzumab era: results from a real-life cohort. *Br J Cancer* 2015.
- Denkert C, Loibl S, Noske A et al. Tumor-associated lymphocytes as an independent predictor of response to neoadjuvant chemotherapy in breast cancer. *J Clin Oncol* 2010; 28(1): 105–113.
- Salgado R, Denkert C, Campbell C et al. Tumor-infiltrating lymphocytes and associations with pathological complete response and event-free survival in *HER2*-positive early-stage breast cancer treated with lapatinib and trastuzumab: a secondary analysis of the NeoALTTO trial. *JAMA Oncol* 2015; 1(4): 448–454.
- Denkert C, von Minckwitz G, Brase JC et al. Tumor-infiltrating lymphocytes and response to neoadjuvant chemotherapy with or without Carboplatin in human epidermal growth factor receptor 2-positive and triple-negative primary breast cancers. *J Clin Oncol* 2015; 33(9): 983–991.
- Dieci MV, Mathieu MC, Guarneri V et al. Prognostic and predictive value of tumor-infiltrating lymphocytes in two phase III randomized adjuvant breast cancer trials. *Ann Oncol* 2015; 26(8): 1698–1704.
- Loi S, Sirtaine N, Piette F et al. Prognostic and predictive value of tumor-infiltrating lymphocytes in a phase III randomized adjuvant breast cancer trial in node-positive breast cancer comparing the addition of docetaxel

Original article

Annals of Oncology

- to doxorubicin with doxorubicin-based chemotherapy: BIG 02-98. *J Clin Oncol* 2013; 31(7): 860–867.
7. Adams S, Gray RJ, Demaria S et al. Prognostic value of tumor-infiltrating lymphocytes in triple-negative breast cancers from two phase III randomized adjuvant breast cancer trials: ECOG 2197 and ECOG 1199. *J Clin Oncol* 2014; 32(27): 2959–2966.
 8. Loi S, Michiels S, Salgado R et al. Tumor infiltrating lymphocytes are prognostic in triple negative breast cancer and predictive for trastuzumab benefit in early breast cancer: results from the FinHER trial. *Ann Oncol* 2014; 25(8): 1544–1550.
 9. Salgado R, Denkert C, Demaria S et al. The evaluation of tumor-infiltrating lymphocytes (TILs) in breast cancer: recommendations by an International TILs Working Group 2014. *Ann Oncol* 2015; 26(2): 259–271.
 10. [Recommendations for the immunohistochemistry of the hormonal receptors on paraffin sections in breast cancer. Update 1999. Group for Evaluation of Prognostic Factors using Immunohistochemistry in Breast Cancer (GEFPICS-FNCLCC)]. *Ann Pathol* 1999; 19(4): 336–343.
 11. Wolff AC, Hammond MEH, Schwartz JN et al. American Society of Clinical Oncology/College of American Pathologists guideline recommendations for human epidermal growth factor receptor 2 testing in breast cancer. *J Clin Oncol* 2007; 25(1): 118–145.
 12. Symmans WF, Peintinger F, Hatzis C et al. Measurement of residual breast cancer burden to predict survival after neoadjuvant chemotherapy. *J Clin Oncol* 2007; 25(28): 4414–4422.
 13. Ali HR, Dariush A, Provenzano E et al. Computational pathology of pre-treatment biopsies identifies lymphocyte density as a predictor of response to neoadjuvant chemotherapy in breast cancer. *Breast Cancer Res* 2016; 18(1): 21.
 14. Ladoire S, Arnould L, Apetoh L et al. Pathologic complete response to neoadjuvant chemotherapy of breast carcinoma is associated with the disappearance of tumor-infiltrating foxp3+ regulatory T cells. *Clin Cancer Res* 2008; 14(8): 2413–2420.
 15. Dieci MV, Criscitiello C, Goubar A et al. Prognostic value of tumor-infiltrating lymphocytes on residual disease after primary chemotherapy for triple-negative breast cancer: a retrospective multicenter study. *Ann Oncol* 2014; 25(3): 611–618.
 16. Miyashita M, Sasano H, Tamaki K et al. Prognostic significance of tumor-infiltrating CD8+ and FOXP3+ lymphocytes in residual tumors and alterations in these parameters after neoadjuvant chemotherapy in triple-negative breast cancer: a retrospective multicenter study. *Breast Cancer Res* 2015.
 17. West NR, Milne K, Truong PT et al. Tumor-infiltrating lymphocytes predict response to anthracycline-based chemotherapy in estrogen receptor-negative breast cancer. *Breast Cancer Res* 2011; 13(6): R126.
 18. Bianchini G, Pusztai L, Pienkowski T et al. Immune modulation of pathologic complete response after neoadjuvant HER2-directed therapies in the NeoSphere trial. *Ann. Oncol* 2015; mdv395.
 19. Loibl S, Jackisch C, Schneeweiss A et al. Dual HER2-blockade with pertuzumab and trastuzumab in HER2-positive early breast cancer: a subanalysis of data from the randomized phase III GeparSepto trial. *Ann Oncol* 2016.
 20. Ingold Heppner B, Untch M, Denkert C et al. Tumor-infiltrating lymphocytes: a predictive and prognostic biomarker in neoadjuvant treated HER2-positive breast cancer. *Clin Cancer Res* 2016. doi:10.1158/1078-0432.CCR-15-2338.
 21. García-Martínez E, Gil GL, Benito AC et al. Tumor-infiltrating immune cell profiles and their change after neoadjuvant chemotherapy predict response and prognosis of breast cancer. *Breast Cancer Res* 2014; 16(6): 488.
 22. Ladoire S, Mignot G, Dabakuyo S et al. In situ immune response after neoadjuvant chemotherapy for breast cancer predicts survival. *J Pathol* 2011; 224(3): 389–400.

AQ10

Key Message

In HER2-positive BC, TILs decrease is strongly associated with pathological complete response. A high lymphocyte infiltration (>25%) at NAC completion is associated with higher residual tumor burden and with an adverse outcome.

AQ11

Journal: *Annals of Oncology*
Article Doi: 10.1093/annonc/mdx309
Article Title: Stromal lymphocyte infiltration after neoadjuvant chemotherapy is associated with aggressive residual disease and lower disease-free survival in HER2-positive breast cancer
First Author: A.-S. Hamy
Corr. Author: F. Reyat

OXFORD
UNIVERSITY PRESS

INSTRUCTIONS

We encourage you to use Adobe's editing tools (please see the next page for instructions). If this is not possible, (i) print out the proof, mark your corrections clearly in black ink, and fax it to +44 (0)1865 355739 (or scan it and email it to annonc@oup.com), or (ii) send a list of corrections (in an email or Word attachment) listing each change in the following manner: line number, current text, change to be made. Please do not send corrections as track changed Word documents.

Changes should be corrections of typographical errors only. Changes that contradict journal style will not be made.

These proofs are for checking purposes only. They should not be considered as final publication format. The proof must not be used for any other purpose. In particular we request that you do not post them on your personal/institutional web site, and do not print and distribute multiple copies (please use the online offprint order form). Neither excerpts nor all of the article should be included in other publications written or edited by yourself until the final version has been published and the full citation details are available. You will be sent these when the article is published.

- 1. Author groups:** Please check that all names have been spelled correctly and appear in the correct order. Please also check that all initials are present. Please check that the author surnames (family name) have been correctly identified by pink background. If this is incorrect, please identify the full surname of the relevant authors. Occasionally, the distinction between surnames and forenames can be ambiguous, and this is to ensure that the authors' full surnames and forenames are tagged correctly, for accurate indexing online. Please also check all author affiliations.
 - 2. Figures:** If applicable figures have been placed as close as possible to their first citation. Please check that they are complete and that the correct figure legend is present. Figures in the proof are low resolution versions that will be replaced with high resolution versions when the journal is printed.
 - 3. Missing elements:** Please check that the text is complete and that all figures, tables, and their legends are included.
 - 4. URLs:** Please check that all web addresses cited in the text, footnotes and reference list are up-to-date, and please provide a 'last accessed' date for each URL. Please specify format for last accessed date as: Month Day, Year.
 - 5. Funding:** Please provide a Funding statement, detailing any funding received. Remember that any funding used while completing this work should be highlighted in a separate Funding section. Please ensure that you use the full official name of the funding body, and if your paper has received funding from any institution, such as NIH, please inform us of the grant number to go into the funding section. We use the institution names to tag NIH-funded articles so they are deposited at PMC. If we already have this information, we will have tagged it and it will appear as coloured text in the funding paragraph. Please check the information is correct.
-

Author Query Form

Journal: *Annals of Oncology*
Article Doi: 10.1093/annonc/mdx309
Article Title: Stromal lymphocyte infiltration after neoadjuvant chemotherapy is associated with aggressive residual disease and lower disease-free survival in HER2-positive breast cancer
First Author: A.-S. Hamy
Corr. Author: F. Reyal

AUTHOR QUERIES – TO BE ANSWERED BY THE CORRESPONDING AUTHOR

The following queries have arisen during the typesetting of your manuscript. Please click on each query number and respond by indicating the change required within the text of the article. If no change is needed please add a note saying “No change.”

- AQ1:** Please check that all names have been spelled correctly and appear in the correct order. Please also check that all initials are present. Please check that the author surnames (family name) have been correctly identified by a pink background. If this is incorrect, please identify the full surname of the relevant authors. Occasionally, the distinction between surnames and forenames can be ambiguous, and this is to ensure that the authors’ full surnames and forenames are tagged correctly, for accurate indexing online. Please also check all author affiliations.
- AQ2:** Author name ‘A.-S. Hamy’ is mismatched with funding statement ‘A-S Hamy-Petit’. Please check and correct if necessary.
- AQ3:** As per journal style, affiliations are renumbered in sequential order. Please check and correct if necessary.
- AQ4:** Please provide division/department for author affiliations wherever applicable.
- AQ5:** Please check that the tel/fax numbers are identified correctly.
- AQ6:** Please indicate with an underline (in the proofs) any characters not already italicized that represent genes (in text and in reference list).
- AQ7:** Please check that the text is complete and that all figures, tables and their legends are included.
- AQ8:** If applicable figures have been placed as close as possible to their first citation. Please check that they are complete and that the correct figure legend is present. Figures in the proof are low resolution versions that will be replaced with high resolution versions when the journal is printed.
- AQ9:** Remember that any funding used while completing this work should be highlighted in a separate Funding section. Please ensure that you use the full official name of the funding body.
- AQ10:** Please update references [16, 18–20].
- AQ11:** We have included the ‘key message’ text for online-only publication at the end of this proof. Please note that the character limit is 400 characters or fewer, including spaces. Please reduce the ‘key message’ to fit this.
- AQ12:** The figures in your proof have been recolored in the standard journal-specific style palette. Please check and confirm that there are no errors and make any necessary edits to the figure captions.
- AQ13:** Please provide significance for bold values in Tables 1 and 2.
- AQ14:** Please provide significance for “*” in Table 2.
- AQ15:** Please check that the ethical statement section is OK.
- AQ16:** Please check and confirm the author initials “A-SH-P” as it mismatches with the author name “A.-S. Hamy.”
- AQ17:** Please note there is a charge of £350/€525/\$600 per figure for colour reproduction in print. Please confirm you accept this charge. Alternatively, figures can be published online in colour and printed in black and white free of charge.

Supplementary file

1. Supplementary methods

1.1. Patients

We analyzed a cohort of 175 T1-3NxM0 patients with *HER2*-positive invasive BC treated by neoadjuvant chemotherapy (NAC) at Institut Curie, between 2002 and 2012. Trastuzumab use changed over time and was introduced in an adjuvant setting in 2004. Its use was generalized both in the neoadjuvant and the adjuvant setting in 2006. Since the results of the NOAH study¹, the standard of care in *HER2*-positive BC patients receiving neoadjuvant chemotherapy became the use of both neoadjuvant trastuzumab in addition to NAC and adjuvant trastuzumab after NAC completion. Overall, 144 patients received both neoadjuvant and adjuvant and the analyses on this subpopulation are provided in the supplementary results, section 2.2.

1.2. Tumor samples and pathology review

1.2.1. Evaluation of tumor-infiltrating lymphocytes and pathology review

Pathological specimens (pretreatment core needle biopsy and post-NAC surgical specimens) were reviewed by one expert breast pathologist (ML) for the purpose of the study following the recommendations of the international TILs Working Group². They were evaluated independently for the presence of a mononuclear cells infiltrate (including lymphocytes and plasma cells, excluding polymorphonuclear leukocytes), on hematoxylin and eosin-stained sections without additional staining as recommended by Salgado *et al*³.

Both IT and stromal TILs were assessed, but exhaustive results are reported for stromal TILs only. Intratumoral TILs (IT TILs) were defined as intraepithelial mononuclear cells within tumor nests or in direct contact with tumor cells and stromal TILs (Str TILs) were defined as mononuclear inflammatory cells within intratumoral stromal area and were reported as percentage of stromal area. Pearson's correlation coefficients were calculated for the relationships between IT and stromal TIL levels.

To study their association with pathological complete response (pCR), stromal TILs levels were categorized into the following classes: <10%, 10 to 60%, >60%, according to Denkert *et al* (SABCS 2016, oral presentation)

1.2.2. Immunostaining

To further validate that the unstained mononuclear cells infiltrate we considered as TILs in the analyses indeed corresponded to tumor-infiltrating lymphocytes, we performed a CD3+ immunostaining on a subset of 20 surgical specimens selected within the cases without pathological complete response. For immunohistochemistry, 4 µm sections were cut from the tissue specimen, deparaffinated, rehydrated and processed with standard methods using an automatized stainer (Autostainer Link 48, DAKO, Carpinteria, CA, USA) and the following antibodies was used CD3 (A0452, Dako). CD3 positivity was assessed blind to the levels of unstained TILs. We assessed correlations between unstained TILs and CD3+ cells by calculating the Pearson's correlation coefficient.

1.2.3. Tumor samples

In the main manuscript, cases were considered estrogen receptor (ER) or progesterone receptor (PR) positive if at least 1% of the tumor cells expressed estrogen and/or progesterone receptors (ER/PR), according to the ASCO/CAP guideline recommendations³. However, French guidelines⁴ recommend to use a 10% positivity threshold for ER and PR to consider a tumor as ER or PR-positive respectively. The results of the supplementary results section 2.3 are provided with the 10% threshold for ER and PR positivity.

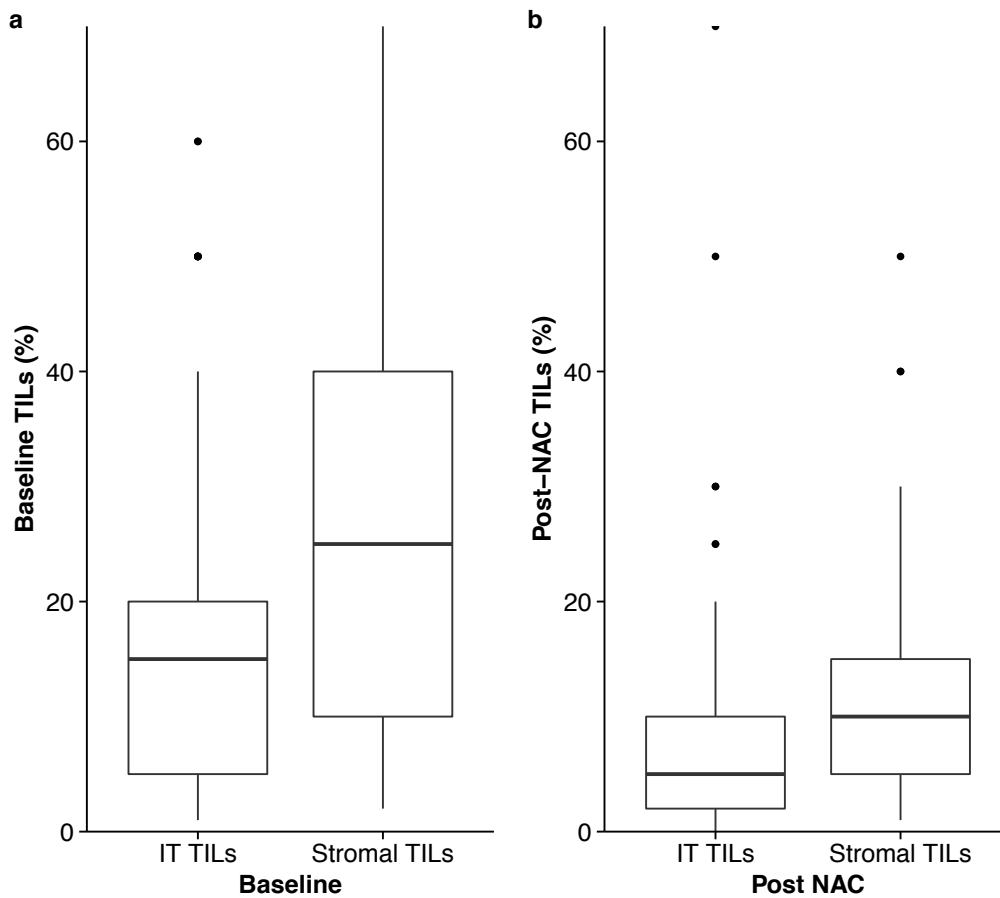
2. Supplementary results

2.1. Supplementary pathological results

2.1.1. Correlation between IT and stromal TILs

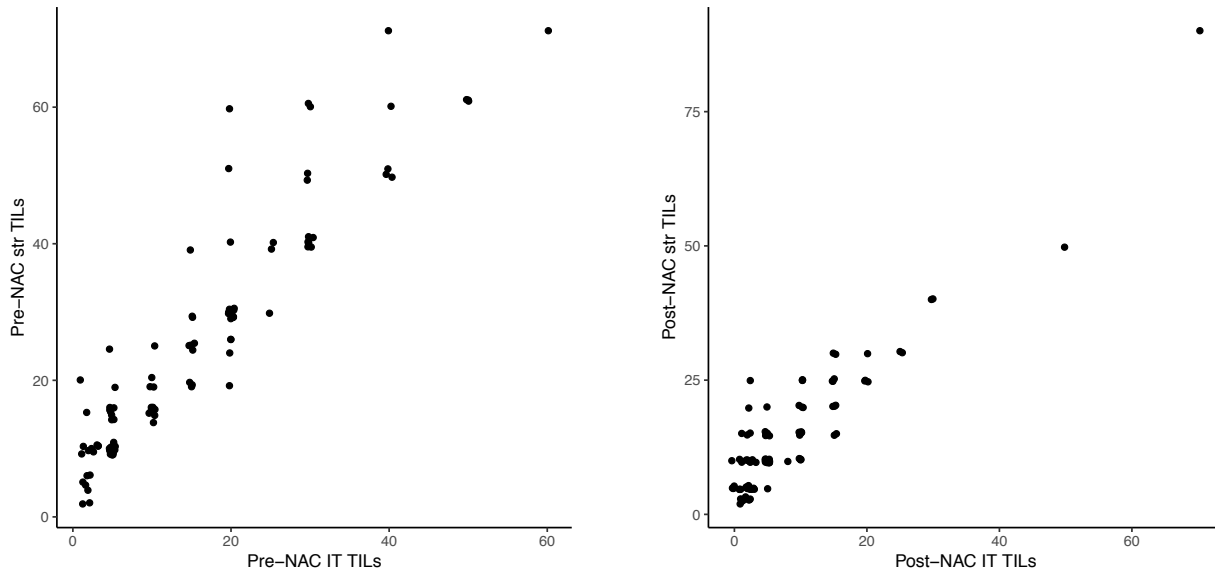
Stromal TILs were assessed on 175 pretreatment and post-treatment samples, and IT TILS were assessed in 175 pretreatment samples and 107 surgical specimens with residual disease.

The median baseline IT TIL level was 15% (range: 1-60%), and the median baseline Str TIL level was 25% (range: 2-70%) (Supplementary Figure 2).



Supplementary Figure 2: IT and Str TIL levels before (1a) and after (1b) neoadjuvant chemotherapy. The bar represents the median value, and the box plot represents the interquartile ranges.

IT and Str TIL levels were strongly correlated both before (Pearson correlation coefficient, $r=0.93$ [0.91-0.95], $p<10^{-16}$, supplementary Figure 3a) and after NAC (Pearson correlation coefficient, $r=0.92$ [0.89-0.95], $p<10^{-16}$, supplementary Figure 3b).



Supplementary Figure 3: Correlation between pre-NAC intra tumoral (IT) TIL levels and pre-NAC stromal TIL levels. (3a) : Correlation between post-NAC intra tumoral (IT) TIL levels and post-NAC stromal TIL levels (3b)

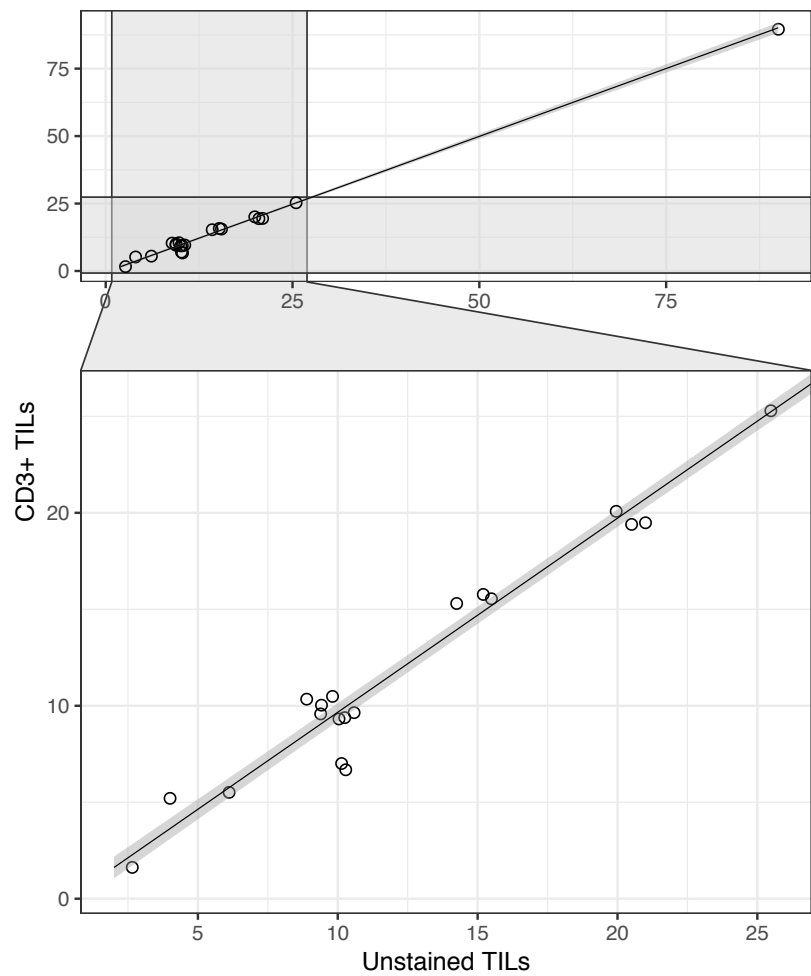
2.1.2. Correlation between unstained TILs and CD3+ TILs

A subset of 20 surgical specimen with residual disease were stained with a CD3 antibody.

Correspondence between unstained TILs and CD3+ TILs are detailed in supplementary table

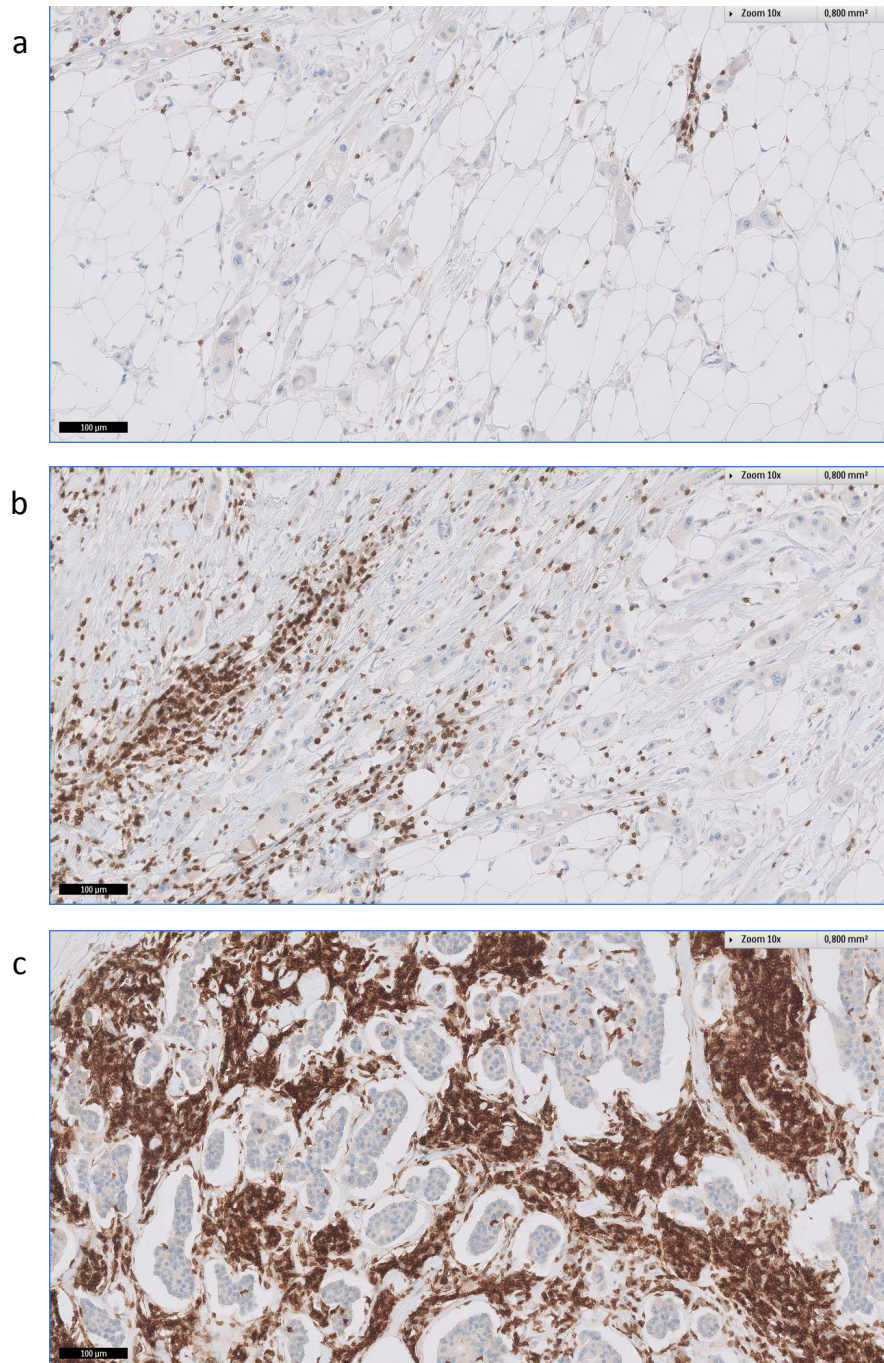
3 and represented on supplementary Figure 4.

Case #	Unstained TILs	CD3+ TILs
1	20	20
2	10	10
3	5	5
4	5	5
5	10	7
6	15	15
7	10	10
8	10	10
9	2	2
10	15	15
11	10	10
12	10	10
13	20	20
14	90	90
15	20	20
16	25	25
17	10	10
18	10	10
19	10	7
20	15	15



Supplementary Table 3 and supplementary figure 4: Relationships between unstained TILs and CD3+ TILs

CD3 staining ranged from 2 to 90%. Supplementary Figure 5 shows pictures of a 5% (5a) – 25% (5b) and 90% (5c) CD3+ immunostaining

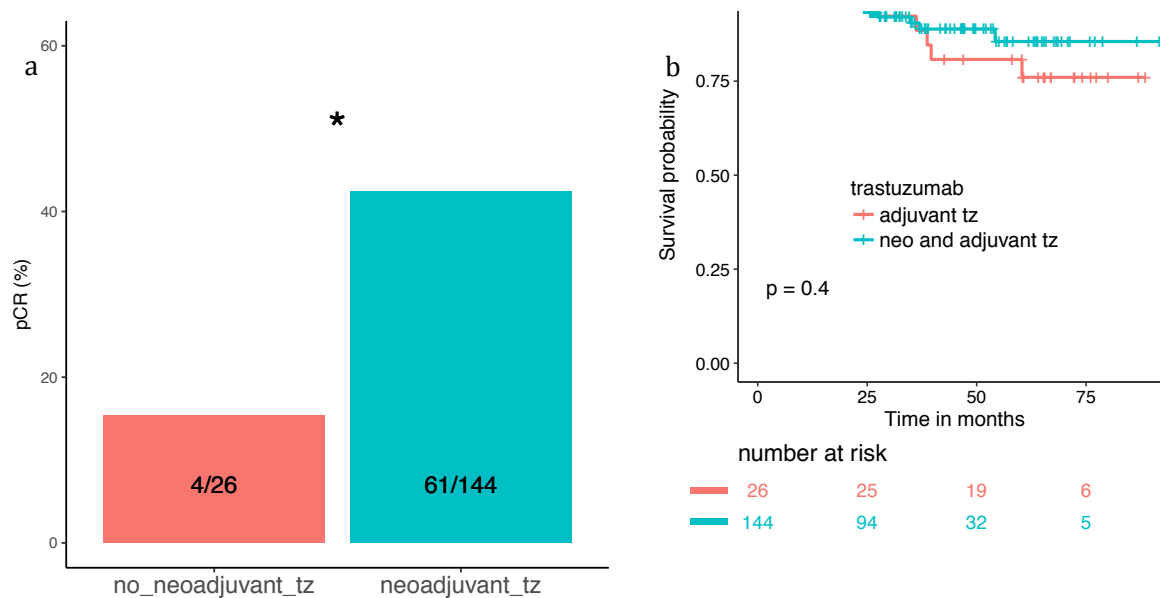


Supplementary Figure 5: Evaluation of stromal TILs percentages with a CD3 immunostaining. **5a:** stromal TILs: 5%; **5b:** stromal TILs: 25%; **5c:** stromal TILs : 90%

Overall, CD3+ and unstained TILs showed an excellent correlation ($r=0.99$, $p<0.00001$).

2.2. Results within the population treated with both neoadjuvant and adjuvant trastuzumab only (n=144)

In the whole cohort (n=175), trastuzumab combined with neoadjuvant chemotherapy was associated with higher pCR rates than chemotherapy alone (15.4% versus 42.4%, $p=0.01$) (Supplementary Figure 6a). DFS was not different by use of neoadjuvant trastuzumab (yes versus no, HR=0.65 [0.24 - 1.77], $p=0.4$) (Supplementary Figure 6b).



Supplementary Figure 6: **6a:** Pathological complete response rates according to the use of neoadjuvant trastuzumab (no neoadjuvant trastuzumab, red plot (no_neoadjuvant_tz); neoadjuvant trastuzumab, blue plot (neoadjuvant_tz)). **6b:** Kaplan Meier Disease Free survival curve by use of neoadjuvant trastuzumab.

All the following results are presented in the population of patients treated with both neoadjuvant and adjuvant trastuzumab (n=144). The median age was 48 years (range: 27-78 years), 64.6% (n=93) were premenopausal, and 29.2% (n=42) were overweight or obese (BMI>25). Clinically, most patients were classified as having stage T2 (n=96, 66.7%), node-positive breast cancer (61.8%, n=89). Most of the tumors were grade 3 tumors (71.3%, n=102), and 36.8% were ER-negative (n=53). All patients (but one) received anthracycline-taxane based regimen as neoadjuvant treatment.

At baseline, the median baseline TIL level was 25% (range: 5-70%). PR negativity was associated with higher baseline TIL levels ($p<0.001$), and baseline TIL levels were higher in patients with larger nodal metastasis $> 5\text{mm}$ at NAC completion than patients with larger nodal metastasis $\leq 2\text{ mm}$ ($p=0.03$) (Supplementary Table 4).

Variables		<i>n=144</i>				
		<i>n</i>	baseline TILs (%)	<i>p</i>	post-NAC TILs (%)	<i>p</i>
Pre-neoadjuvant chemotherapy parameters						
Age	$\leq 45\text{ y}$	67	26.87	0.75	12	0.23
	46-55 y	38	29.08		9.45	
	$>55\text{ y}$	39	26.15		8.85	
Menopausal status	post	51	29.22	0.5	8.63	0.1
	pre	93	26.18		11.48	
Body mass index (BMI)	19 to 25	98	25.56	0.24	10.91	0.36
	<19	4	28.75		3.75	
	>25	42	31.07		10.1	
Tumor size	T1	8	33.75	0.57	8	0.43
	T2	96	26.77		10.02	
	T3	40	27.12		12.05	
Nodal status	N0	55	28.36	0.56	10.42	0.96
	N1-N2-N3	89	26.57		10.51	
Grade	I-II	41	24.27	0.19	9.98	0.69
	III	102	28.58		10.73	
Tumor cellularity	$< 70\%$	61	28.52	0.46	10.25	0.82
	$\geq 70\%$	83	26.33		10.64	
Mitotic index	≤ 10	36	26.94	0.33	9.94	0.65
	11-22	54	24.81		9.81	
	>22	54	29.91		11.48	
ER status (cut-off 1%)	negative	53	30.57	0.09	9.85	0.57
	positive	91	25.33		10.84	
PR status	negative	91	29.01	<0.001	9.19	0.04
	positive	51	24.51		12.78	

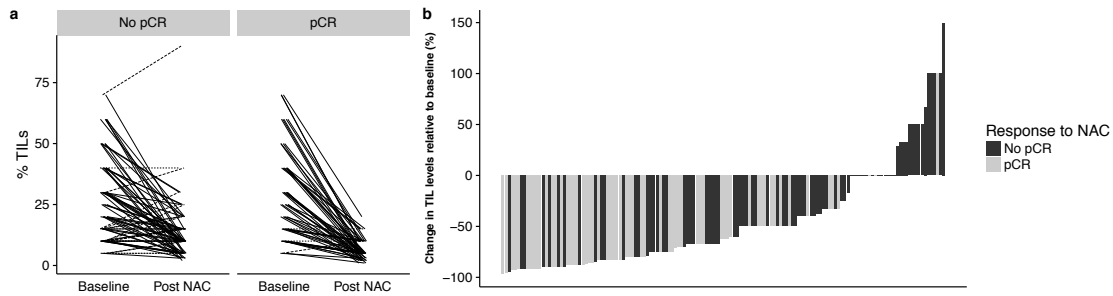
Variables		n=144				
		n	baseline TILs (%)	p	post- NAC TILs (%)	p
Post-NAC parameters						
pCR	Residual disease	83	25.9	0.29	13.55	<0.001
	pCR	61	29.1		6.28	
Residual cancer burden (RCB)	0	61	29.1	0.44	6.28	<0.001 [⊙]
	1	22	28.18		10	
	2	53	24.15		12.83	
	3	8	31.25		28.12	
Mitotic index	≤ 10	130	27.19	0.91	9.46	<0.001 [⊙]
	11-22	3	31.67		19.33	
	>22	11	26.82		20	
Tumor cellularity	≤ 5%	82	29.27	0.34	7.55	<0.001 [⊙]
	6-15%	26	24.23		11.31	
	>15%	34	25.29		17.06	
Nodal involvement	0	113	27.43	0.91	9.6	0.11
	1 - 3 N+	25	27.2		13.12	
	≥ 4 N+	6	24.17		15.83	
Larger nodal metastasis	≤ 2 mm	19	22.89	0.03 [⊙]	7.89	0.001 [⊙]
	3-5mm	4	16.25		9.5	
	>5mm	8	40.63		29.38	

Supplementary Table 4: Association between baseline TIL levels, post-NAC TILs levels and clinical and pathological factors before and after chemotherapy in the population treated with neoadjuvant and adjuvant trastuzumab only (n=144).

Abbreviations: Body mass index (BMI); Estrogen Receptor (ER); Progesterone Receptor (PR); neoadjuvant chemotherapy (NAC); pathological complete response (pCR); Residual cancer burden (RCB), events (Ev).

Post hoc analyses for significant ANOVA tests (only results with p-value 0.05 or below are reported): (a) Larger nodal metastasis size: >5mm versus ≤ 2 mm, p=0.04; (b) RCB class: 2 versus 0: p<0.001, 3 versus 0, p<0.001, 3 versus 1 p<0.001, 3 versus 2 p<0.001; (c) Mitotic index ≤ 10 versus >22 p<0.001; (d) Post NAC tumor cellularity: >15% versus ≤ 5% p<0.001, >15% versus 6-15% p=0.05; (e) Larger nodal metastasis size: >5mm versus ≤ 2 mm p<0.001.

After chemotherapy, the median TIL percentage fell to 10% [1-90] (TIL levels decrease (n=113, 78.5%), unchanged (n=15, 10.4%), increase (n=16, 11.1%)). TIL levels decreased in all but three patients in the pCR group (Supplementary Figure 7a). The variation of TIL levels was strongly associated with pCR (p<10⁻⁵), with the largest decreases of TILs being strongly associated with the achievement of a pCR (Supplementary Figure 7b).



Supplementary Figure 7: 7a: Str TIL levels before (Baseline) and after (post NAC) chemotherapy (population of patients treated with both neoadjuvant and adjuvant trastuzumab (n=144)). The solid black line represents paired samples displaying a decrease in TIL levels, the dotted lines represent pairs with no change in TIL levels, and the dashed lines represent pairs displaying an increase in TIL levels. **7b:** Relationship between the change in Str TIL level (represented as a percentage change relative to baseline levels) and pathological complete response. Plots are sorted by increasing magnitude of change in Str TIL levels.

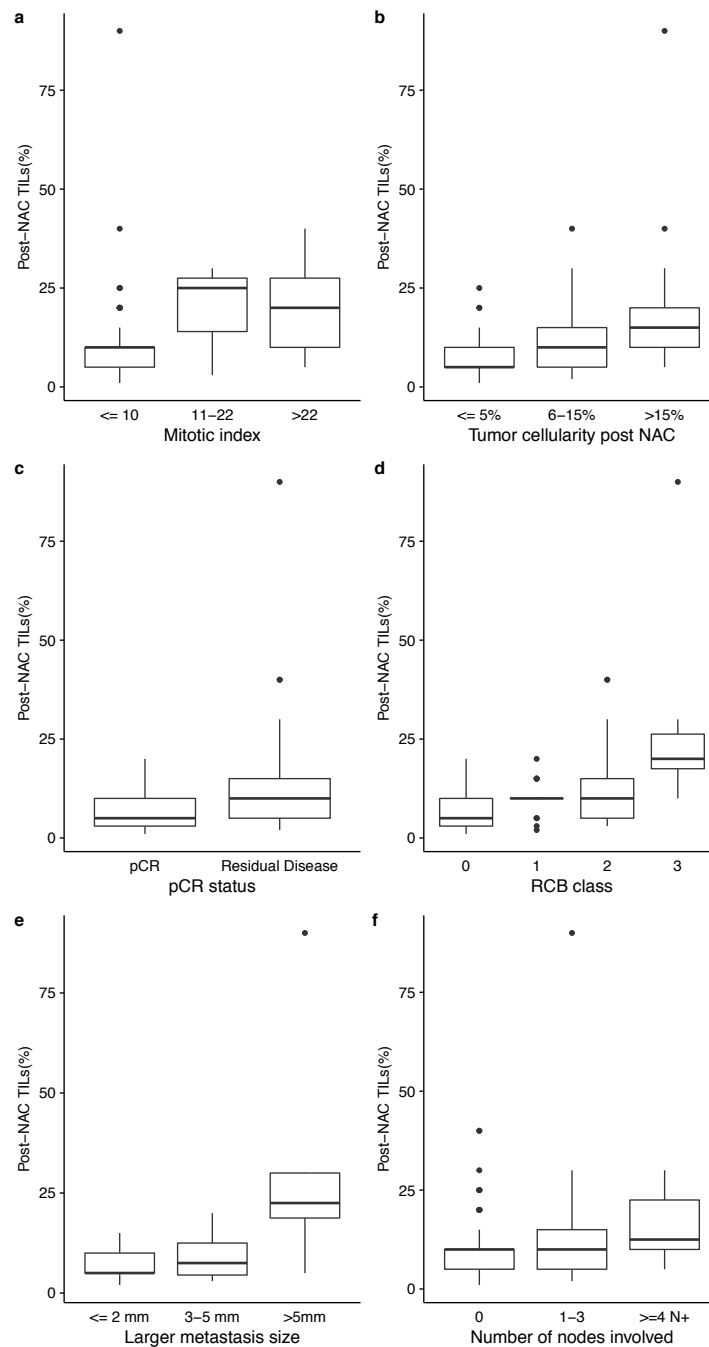
Overall, pCR was reached in 61 patients (42.4%), and this rate was significantly different by ER status (ER negative, 32/53 (60.4%), ER positive 29/91 (31.9%), $p < 0.001$). In univariate analysis, age, menopausal status, ER and PR status were associated with the occurrence of a pCR, but baseline TIL levels were not (Supplementary Table 5). After multivariate analysis, only PR status remained significantly associated with pCR.

Variables	<i>n</i>	pCR (n)	pCR (%)	Univariate			Multivariate		
				OR	95% IC	<i>p</i>	OR	95% IC	<i>p</i>
Age	≤45 y	67	21	31.3 %	1				
	46-55 y	38	16	42.1 %	1.59	[0.7 - 3.65]	0.269		
	>55 y	39	24	61.5 %	3.5	[1.55 - 8.17]	0.003		
Menopausal status	post	51	28	54.9 %	1				
	pre	93	33	35.5 %	0.45	[0.22 - 0.9]	0.025		
Body mass index (BMI)	19 to 25	98	43	43.9 %	1				
	<19	4	3	75%	3.84	[0.47 - 79.07]	0.251		
	>25	42	15	35.7 %	0.71	[0.33 - 1.49]	0.37		
Tumor size	T1	8	5	62.5 %	1				
	T2	96	39	40.6 %	0.41	[0.08 - 1.77]	0.241		
	T3	40	17	42.5 %	0.44	[0.08 - 2.06]	0.308		
Nodal status	N0	55	25	45.5 %	1				
	N1-N2-N3	89	36	40.4 %	0.82	[0.41 - 1.61]	0.555		
Mitotic index	≤ 10	36	15	41.7 %	1				
	nov-22	54	22	40.7 %	0.96	[0.41 - 2.28]	0.93		
	>22	54	24	44.4 %	1.12	[0.48 - 2.65]	0.794		
Grade	I-II	41	19	46.3 %	1				
	III	102	42	41.2 %	0.81	[0.39 - 1.69]	0.573		
ER status (cut-off 1%)	negative	53	32	60.4 %	1				
	positive	91	29	31.9 %	0.31	[0.15 - 0.62]	0.001		
PR status (cut-off 1%)	negative	91	51	56%	1			1	
	positive	51	10	19.6 %	0.19	[0.08 - 0.42]	<0.001	0.19	[0.08 - 0.42] <0.001
Tumor cellularity	<70%	61	27	44.3 %	1				
	≥70%	83	34	41 %	0.87	[0.45 - 1.71]	0.692		
TILS (3 classes)	< 10%	8	3	37.5 %	1				
	10-60%	121	50	41.3 %	1.17	[0.28 - 5.93]	0.832		
	≥ 60%	15	8	53.3 %	1.9	[0.34 - 12.24]	0.472		

Supplementary Table 5: Association of baseline clinicopathological factors and pCR in the population treated with neoadjuvant and adjuvant trastuzumab only (n=144).

None of the baseline characteristics of the patients or tumors were significantly associated with post-NAC TIL levels (Supplementary Table 4), except for PR positivity, that was significantly associated with higher TILs than PR negativity ($p=0.04$). Conversely, post-NAC TILs were strongly associated with post-NAC parameters, and were higher in tumours with aggressive characteristics (no pCR, high RCB class, high mitotic index and residual tumor

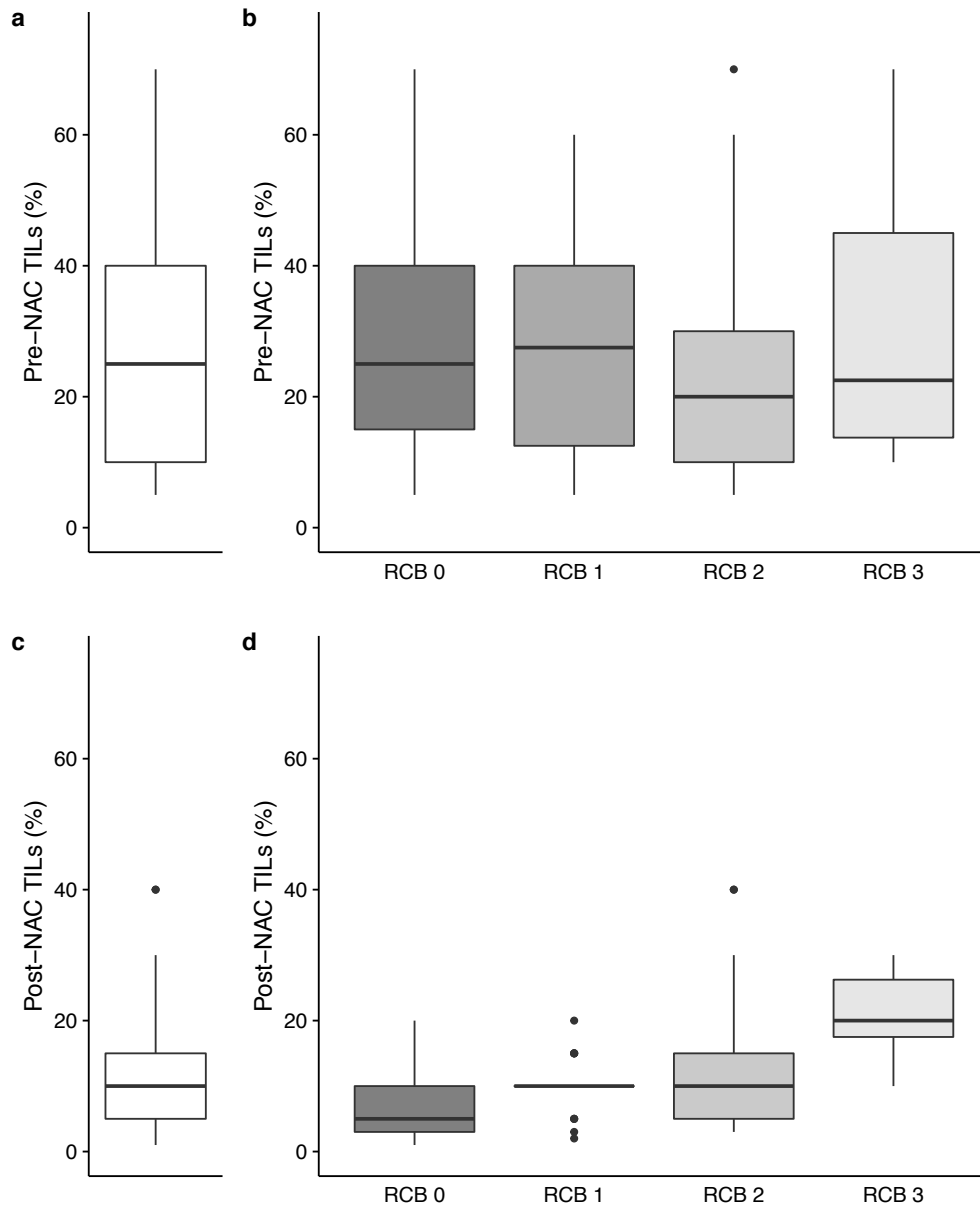
cellularity, $p < 0.001$), and in tumors with larger nodal metastases ($p = 0.001$) (Supplementary Figure 8).



Supplementary Figure 8: Association between post-NAC Str TIL percentages and post-NAC parameters (population of patients treated with both neoadjuvant and adjuvant trastuzumab ($n = 144$)): mitotic index (a), tumor cellularity (b), pCR status (c), RCB class (d), larger nodal metastasis size (e) and number of nodes involved (f).

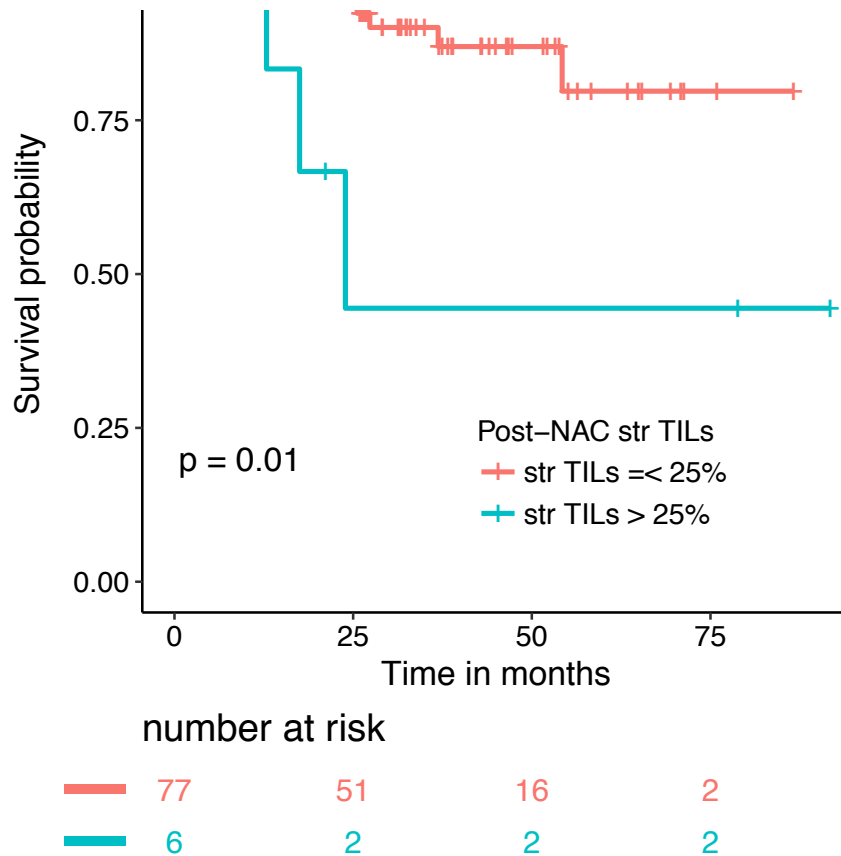
Higher RCBs were associated with higher post-NAC TIL levels but not with baseline TIL

levels (Supplementary Figure 9).



Supplementary Figure 9: Pre-chemotherapy median Str TIL level in the whole population (a) and by RCB class (b). Post-chemotherapy median Str TIL level in the whole population (c) and by RCB class (d).

During a median follow-up of 31.3 months (range: 5.5-91.7), 12 patients suffered relapses (one from pCR group). No association was found between DFS and baseline TIL levels (Supplementary Table 5). In the population of patients without pCR (n=83), post-NAC TIL levels higher than 25% were an independent poor prognostic factor (Supplementary Table 6, Supplementary Figure 10).



Supplementary Figure 10: Kaplan Meier DFS survival curve by post-NAC TIL levels (cut-off: 25%) in the population of patients treated with both neoadjuvant and adjuvant trastuzumab and with residual disease at NAC completion (n=83)

				Univariate analysis			Multivariate analysis			
				Neoadjuvant and Immunity						
Variables		n	events (n)	HR	95% IC	p	p (global)	HR	95% IC	p
Pre-neoadjuvant chemotherapy parameters										
Age	≤45 y	46	6	1						
	46-55 y	22	4	1.27	[0.36 - 4.53]	0.71	0.708			
	>55 y	15	1	0.51	[0.06 - 4.3]	0.54				
Menopausal status	post	23	2	1						
	pre	60	9	2	[0.43 - 9.27]	0.378	0.368			
Body mass index (BMI)	19 to 25	55	4	1						
	<19	1	0	0	[0 - Inf]	0.998	0.072			
	>25	27	7	3.76	[1.1 - 12.85]	0.035				
Tumor size	T1-T2	60	7	1						
	T3	23	4	1.78	[0.52 - 6.13]	0.363	0.363			
Nodal status	N0	30	4	1						
	N1-N2-N3	53	7	1.1	[0.32 - 3.76]	0.88	0.88			
Mitotic index	≤ 10	21	5	1						
	11-22	32	4	0.47	[0.13 - 1.75]	0.261	0.279			
	>22	30	2	0.31	[0.06 - 1.63]	0.168				
Grade	I-II	22	6	1				1		
	III	60	5	0.34	[0.1 - 1.12]	0.077	0.064	0.27	[0.08 - 0.92]	0.036
ER status (cut-off 1%)	negative	21	5	1						
	positive	62	6	0.4	[0.12 - 1.33]	0.135	0.135			
PR status (cut-off 1%)	negative	40	7	1						
	positive	41	4	0.54	[0.16 - 1.83]	0.32	0.32			
Tumor cellularity	< 70%	34	5	1						
	≥ 70%	49	6	0.8	[0.25 - 2.64]	0.718	0.718			
TILs (3 classes)	< 10%	5	1	1						
	10-60%	71	9	0.67	[0.08 - 5.28]	0.702	0.1			
	≥ 60%	7	1	0.86	[0.05 - 13.96]	0.918				
Post-NAC parameters										
Nodal involment	0	52	6	1						
	1 to 3 N+	25	4	1.41	[0.4 - 5.02]	0.592	0.831			
	≥ 4 N+	6	1	1.54	[0.18 - 12.84]	0.69				
Mitotic index	≤ 10	69	7	1						
	11-22	3	1	4.17	[0.5 - 34.56]	0.186	0.221			
	>22	11	3	2.31	[0.59 - 8.96]	0.228				
Tumor cellularity	< 70%	27	1	1						
	≥ 70%	56	10	4.14	[0.53 - 32.42]	0.176	0.141			
Residual Cancer Burden (RCB)	1	22	1	1						
	2	53	9	3.3	[0.42 - 26.04]	0.258	0.487			
	3	8	1	2.64	[0.16 - 42.22]	0.493				
TIL levels (cut-off: 25%)	≤25%	77	8	1				1		
	> 25%	6	3	4.91	[1.28 - 18.81]	0.02	0.01	6.61	[1.65 - 26.46]	0.008

Supplementary Table 6: Association of clinicopathological factors and TILs parameters with DFS (population treated with both neoadjuvant and adjuvant trastuzumab and without pCR (n=83))

2.3. Results in the whole population (n=175) with a 10%-threshold for ER and PR positivity

Results in the main manuscript are provided with a 1% positivity threshold for ER and PR positivity according to American guidelines. In France, cases are considered estrogen receptor (ER) or progesterone receptor (PR) positive if at least 10% of the tumor cells expressed estrogen and/or progesterone receptors (ER/PR). Results below are provided using a 10% positivity threshold in accordance with guidelines used in France.

With the 10% threshold value, the distribution of the population by ER status was as follows: ER negative (n=77, 44%), ER positive (n=98, 56%). Concerning PR status, the distribution of the population was similar using a 1% or a 10% threshold. ER negativity was associated with higher baseline TIL levels 30.61 versus 23.08, $p=0.003$, but not with the level of post-NAC TILs (Supplementary Table 7).

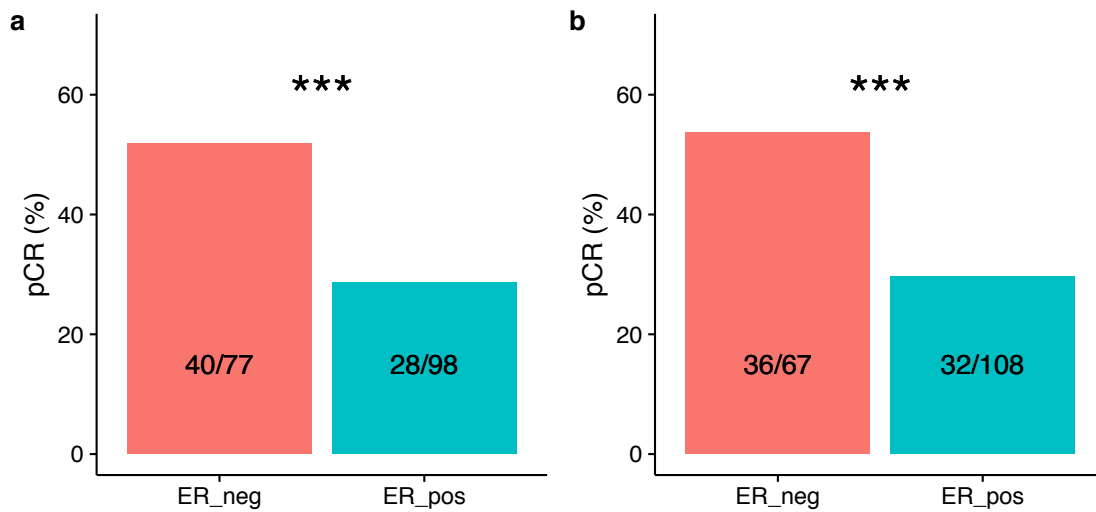
Variables		n	baseline TILs (%)	p	post-NAC TILs (%)	p
ER status (cut-off 10%)	negative	77	30.61	0.003	11.26	0.6
	positive	98	23.08		10.65	
ER status (cut-off 1%)	negative	67	28.99	0.12	11.04	0.9
	positive	108	24.79		10.84	

Supplementary Table 7: Association between baseline, post-NAC TILs and ER status with the two thresholds for ER positivity (cut-off 10% according to French guidelines and cut-off 1% according to American guidelines respectively).

Irrespective of the 10% or the 1% threshold, ER negativity was strongly associated with higher pCR rates (OR : 0.37 and 0.36 respectively, p-value=0.002, Supplementary Table 8 and Supplementary Figure 11).

					Univariate analysis		
		n	pCR	%	OR	95% CI (OR)	p
ER status (cut-off 10%)	negative	77	40	51.9	1		
	positive	98	28	28.6	0.37	0.2-0.69	0.002
ER status (cut-off 1%)	negative	67	36	53.7	1		
	positive	108	32	29.6	0.36	[0.19 - 0.68]	0.002

Supplementary Table 8: Association between pCR and ER status with the two thresholds for ER positivity (cut-off 10% according to French guidelines and cut-off 1% according to American guidelines respectively).



Supplementary Figure 11: pCR rates by ER status and by ER-positivity threshold. **a** : cut-off 10% according to French guidelines. **b** : cut-off 1% according to American guidelines.

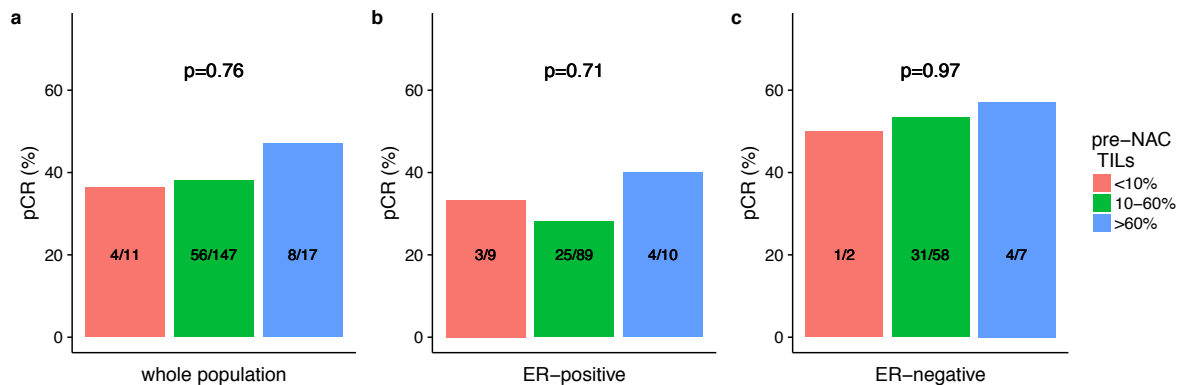
In the population without pCR, ER status was not associated with DFS, neither with the 1 nor the 10% threshold (HR=0.63 [0.24-1.67], $p=0.4$ and HR=0.85 [0.32-2.23], $p=0.7$ respectively, Supplementary Table 9).

			Univariate analysis				
Variable		n	Ev	HR	95% CI	p	p*
ER status (cut-off 10%)	negative	37	7	1			
	positive	70	10	0.85	[0.32-2.23]	0.7	0.7
ER status (cut-off 1%)	negative	31	7	1			
	positive	76	10	0.63	[0.24-1.67]	0.4	0.4

Supplementary Table 9: Association between DFS and ER status with the two thresholds for ER positivity (cut-off 10% according to French guidelines and cut-off 1% according to American guidelines respectively; population without pCR, $n=107$).

2.4. pCR rates by TILs in classes according to Denkert and by ER status

We investigated in the whole population (n=175) if pre-NAC TIL levels were associated with pCR. Pre-NAC TILs were not associated with pCR (Supplementary Figure 12), neither in the whole population (a), nor in the ER-positive (b) or the ER-negative (c) subgroup.



Supplementary Figure 12: pCR rates by pre-NAC TIL levels by classes, according to Denkert et al (<10%, 10 to 60%, >60%), in the whole population (a), in the ER-positive population (b), and in the ER-negative population (c) (cut-off for ER positivity: 1%).

3. References

1. Gianni, L. *et al.* Neoadjuvant and adjuvant trastuzumab in patients with HER2-positive locally advanced breast cancer (NOAH): follow-up of a randomised controlled superiority trial with a parallel HER2-negative cohort. *Lancet Oncol.* **15**, 640–647 (2014).
2. Salgado, R. *et al.* The evaluation of tumor-infiltrating lymphocytes (TILs) in breast cancer: recommendations by an International TILs Working Group 2014. *Ann. Oncol. Off. J. Eur. Soc. Med. Oncol. ESMO* **26**, 259–271 (2015).
3. Hammond, M. E. H. *et al.* American Society of Clinical Oncology/College Of American Pathologists guideline recommendations for immunohistochemical testing of estrogen and progesterone receptors in breast cancer. *J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol.* **28**, 2784–2795 (2010).
4. [Recommendations for the immunohistochemistry of the hormonal receptors on paraffin sections in breast cancer. Update 1999. Group for Evaluation of Prognostic Factors using Immunohistochemistry in Breast Cancer (GEFPICS-FNCLCC)]. *Ann. Pathol.* **19**, 336–343 (1999).

Anne-Sophie Hamy^{1,2*}, H el ene Bonsang-Kitzis^{1,3*}, Diane De Croze⁴, Enora Laas³, Lauren Darrigues³, Lucian Topciu⁵, Emmanuelle Menet⁴, Anne Vincent-Salomon⁵, Florence Lerebours⁶, Jean-Yves Pierga^{2,7}, Etienne Brain⁶, Jean-Guillaume Feron³, Gabriel Benchimol³, Giang-Thanh Lam^{3,8}, Marick La  ^{5*}, Fabien Reyat^{1,3,7*}

* Anne-Sophie Hamy and H el ene Bonsang-Kitzis contributed equally to this work. Fabien Reyat and Marick Lae contributed equally to this work.

1. Residual Tumor & Response to Treatment Laboratory, RT2Lab, PSL Research University, INSERM, U932 Immunity and Cancer, Institut Curie, Paris, F-75248, France.
2. Department of Medical Oncology, PSL Research University, Institut Curie, Paris, F-75248, France.
3. Department of Surgery, PSL Research University, Institut Curie, Paris, F-75248, France.
4. Department of Tumor Biology, H opital Ren e Huguenin, 35, rue Dailly, Saint-Cloud, F-92210, France
5. Department of Tumor Biology, PSL Research University, Institut Curie, Paris, F-75248, France.
6. Department of Medical Oncology, H opital Ren e Huguenin, 35, rue Dailly, Saint-Cloud, F-92210, France
7. Universit e Paris Descartes, Paris, France

Corresponding author:

2.5 Article n°14 : Hamy, A.-S. et al., CCR Apr 2019, in press

425

Pr Fabien REYAL, Institut Curie, Department of Surgery, 26 rue d'Ulm, 75005 Paris

00 33 144324087, 00 33 615271980, fabien.reyal@curie.fr

Keywords: Tumor-infiltrating lymphocytes – *HER2*-positive - TNBC– prognosis interaction
– neoadjuvant chemotherapy

Word count: 3847

The authors declare no potential conflicts of interest.

In breast cancer (BC), the evaluation of tumor infiltration lymphocytes (TILs) is encouraged in routine practice. However, little is known on their variations between before and after neoadjuvant chemotherapy (NAC), and few data are available on their value after treatment. We investigated TIL levels before and after neoadjuvant chemotherapy (NAC) in 716 paired biopsy and surgical specimens.

Pre-NAC TILs levels were associated to pathological complete response (pCR) in a non-linear manner in triple negative BC and were not associated with pCR in *HER2*-positive BC. TIL levels decrease after chemotherapy completion and this decrease was strongly associated with pCR. High post-NAC TIL levels were associated with impaired survival in *HER2*-positive BC but not in the other subtypes. TILs subsetting would be critical (i) to further identify the different immune subpopulations in residual specimen (ii) and understand if their localization, their quantity or their state of activation is associated with the non-linear predictive impact and/or their different prognostic value before and after NAC among BC subtypes.

Purpose: High levels of tumor-infiltrating lymphocytes (TILs) before neoadjuvant chemotherapy (NAC) are associated with higher pathological complete response (pCR) rates, and better survival in TNBC and *HER2*-positive breast cancers (BCs). We investigated the value of TIL levels by evaluating lymphocyte infiltration before and after NAC.

Patients and methods: We assessed stromal TIL levels in 716 pre- and post-treatment matched paired specimens, according to the guidelines of the international TIL working group.

Results: Pre-NAC TIL levels were higher in tumors for which pCR was achieved than in cases with residual disease (33.9% versus 20.3%, $p=0.001$). This was observed in luminal tumors and TNBCs, but not in *HER2*-positive BCs, ($p_{\text{Interaction}}=0.001$). The association between pre-NAC TIL levels and pCR was non-linear in TNBCs ($p=0.005$). Mean TIL levels decreased after chemotherapy completion (pre-NAC TILs: 24.1% versus post-NAC TILs: 13.0%, $p<0.001$). This decrease was strongly associated with high pCR rates, and the variation of TIL levels was strongly inversely correlated with pre-NAC TIL levels ($r=-0.80$, $p<0.001$). Pre-NAC TILs and disease-free survival (DFS) were associated in a non-linear manner ($p<0.001$). High post-NAC TIL levels were associated with aggressive tumor characteristics and with impaired DFS in *HER2*-positive BCs (HR=1.04, CI [1.02-1.06], $p=0.001$), but not in luminal tumors or TNBCs ($p_{\text{Interaction}}=0.04$).

Conclusion: The associations of pre and post-NAC TIL levels with response to treatment and DFS differ between BC subtypes. The characterization of immune subpopulations may improve our understanding of the complex interactions between pre- or post-NAC setting, BC subtype, response to treatment and prognosis.

Background

Breast cancer (BC) is the most frequently diagnosed cancer, and the leading cause of cancer-related death in women. Neoadjuvant chemotherapy (NAC) is increasingly prescribed for patients with locally advanced BC and provides opportunities for studying and monitoring the treatment sensitivity of tumors “*in vivo*”. A pathological complete response (pCR) after NAC is a surrogate marker of good prognosis in triple-negative BC (TNBC) and *HER2*-positive BC, and is now used in FDA trials as a means of accelerating the approval of new drugs.

The role of **tumor-infiltrating lymphocytes (TILs)** in BC has been studied over the last decade. Many studies have reported associations between high TIL levels at diagnosis and a better response to NAC (1–3), and a better prognosis in both neoadjuvant and adjuvant chemotherapy settings (4–7), particularly for TNBC and *HER2*-positive BC. In 2015, an international consortium provided guidelines for the standardized evaluation of TILs in clinical practice(8), and their assessment is encouraged in routine practice, although the results of such evaluations currently have no impact on therapeutic strategy in clinical practice.

The analysis of residual tumor burden after systemic neoadjuvant treatment is an underexplored area that may help us to understand the mechanisms of resistance to specific treatments in BC. However, only a few studies have investigated the variation of TIL levels in response to NAC. Furthermore, studies of the prognostic significance of post-chemotherapy TILs have focused almost exclusively on TNBCs(9,10).

The aim of this study was to report and compare the predictive and prognostic values of TIL levels (before and after NAC) as a function of BC subtype, in a real-life cohort of 718 BC patients treated with NAC.

Patients and treatments

We analyzed a cohort of 718 patients with non-metastatic BC treated with NAC with or without trastuzumab, followed by surgery, at the Institut Curie (Paris and Saint Cloud, France). The cohort and treatments have been described in detail elsewhere and are summarized in the Supplementary material. This study was approved by the institutional review board of Institut Curie and was conducted in accordance with the ethical standards laid down in the 1964 declaration of Helsinki. By the law, no informed consent from the patient was required in this observational study.

Tumor samples

BC tumors were classified into subtypes (TNBC, *HER2*-positive, and luminal *HER2*-negative [referred to hereafter as “luminal”]) on the basis of immunohistochemistry and fluorescence *in situ* hybridization (see the Supplementary material). In accordance with the guidelines used in France(11), cases were considered estrogen receptor (ER)-positive or progesterone receptor (PR)-positive if at least 10% of the tumor cells expressed estrogen and/or progesterone receptors (ER/PR) and endocrine therapy was prescribed when this threshold was exceeded.

Pathological review

Pretreatment core needle biopsy specimens and the corresponding post-NAC surgical specimens were reviewed independently by two experts in breast diseases (ML and DdC).

Formalin-fixed paraffin-embedded (FFPE) tumor tissue samples were studied. Tumor-infiltrating lymphocytes (TILs), residual cancer burden (RCB) indices and pre- and post-NAC cellularity were reviewed simultaneously, specifically for the purposes of this study, between January 2015 and March 2017. In accordance with the recommendations of the international TILs Working Group (12), we checked for presence of a mononuclear cell infiltrate in the stroma on hematoxylin and eosin-stained sections without additional staining, after excluding areas around ductal carcinomas *in situ* (DCIS), and tumor zones with necrosis and artifacts.

occupied by mononuclear cells. After NAC, we assessed TIL levels within the borders of the residual tumor bed, as defined by the RCB index(13). Nothing is known about the clinical, biological and prognostic significance of TILs in the area of regression in cases of pathological response, but the TILs international working group recently called for their evaluation for research purposes. In cases of pCR, the scar area was measured on macroscopic examination. The scar appeared as a white area in the breast parenchyma corresponding to the tumor bed modified by NAC. It was characterized by the presence of histiocytes, lymphocytes, macrophages, fibrosis and elastosis. The whole fibro-inflammatory scar was evaluated on HE sections (size in mm and stromal TIL level evaluation) (Supplementary Fig. S1). We determined the RCB index, as described by Symmans(13), with the web-based calculator freely available via the Internet (www.mdanderson.org/breastcancer_RCB). Invasive tumor cellularity before and after NAC was determined as the percentage of the tumor area occupied by invasive cancer.

Study endpoints

We defined pathological complete response (pCR) as the absence of invasive residual tumor from both the breast and axillary nodes (ypT0/is N0). Disease-free survival (DFS) was defined as the time from surgery to death, locoregional recurrence or distant recurrence, and overall survival (OS) was defined as the time from surgery to death. For patients for whom none of these events were recorded, we censored data at the time of last known contact.

Quantitative data handling and statistical analysis

Pre- and post-NAC TIL levels were analyzed as continuous variables, after performing linearity tests (see the complementary statistical methods section of the Supplementary material). RCB index was assessed as a continuous variable in both univariate and multivariate analysis. All analyses were performed on the whole population and after

ANOVA, with post-hoc Tukey analysis when required, or in Mann Whitney *U* or Kruskal-Wallis tests, where indicated. Absolute and relative changes in TIL levels were calculated as the difference between pre- and post-NAC TIL levels, and as these levels divided by pre-NAC TIL levels, respectively. Changes in mean values were investigated in paired *t*-tests. The classical statistical methods used to analyze univariate and multivariate associations with pCR (logistic regression models) and survival (Cox proportional hazard models) are described in the complementary statistical methods section of the Supplementary material.

Associations between pre-NAC TILs, clinicopathological patterns, response to treatment and survival

Patient and tumor characteristics before NAC

In total, 718 patients were included in the cohort (luminal ($n=223$), TNBC ($n=320$), *HER2*-positive ($n=175$), Supplementary Table S1). Mean pre-NAC TIL level was 24.2% (luminal: 16.2%; TNBC: 28.5%; *HER2*-positive: 26.5%, $p<0.001$), and the distribution of TILs differed between BC subtypes (Fig. 1A-B).

Pre-NAC TILs and response to treatment

Pre-NAC TIL levels were significantly higher in tumors for which pCR was achieved than for tumors for which residual disease (RD) was detected, except in *HER2*-positive BCs (Supplementary Table S2, $p_{\text{Interaction}}=0.001$). Pre-NAC TILs were significantly associated with pCR (all: OR=1.03, CI [1.02 - 1.04], $p<0.001$) (Table 1). However, after stratification by BC subtype, this association was found to be significant only in TNBCs (luminal, OR=1.03 CI [1 - 1.06], $p=0.058$; TNBC, OR=1.03 CI [1.02 - 1.04], $p<0.001$; *HER2*-positive, OR=1.01, CI [0.99 - 1.03], $p=0.341$) (Supplementary Table S3). The association between TILs and pCR (Fig. 1C) was linear for all groups (Figs. 1D-E-G) except TNBCs, for which it was best fitted by a cubic spline ($p=0.006$) (Fig. 1F). In univariate and multivariate analysis, pre-NAC TIL levels were significantly associated with pCR in the whole population and in the TNBC subtype.

Prognostic impact of pre-NAC TILs

Pre-NAC TIL levels were significantly associated with DFS in the whole population (HR=0.988, CI [0.979-0.998], $p=0.017$) (Table 2) and in the TNBC subgroup (HR=0.982 CI [0.971-0.993], $p=0.002$), but not in the other subgroups (luminal, HR=0.994, CI [0.971-1.018], $p=0.641$; *HER2*-positive HR=1.007, CI [0.981-1.032], $p=0.611$) (Supplementary Table S4). Statistical tests revealed significant deviations from the assumption of linearity in

prognostic effect of TILs. No such deviation from linearity was observed in the *HER2*-positive population (Figs. 1 H-K). In addition, the interaction test between pre-NAC TILs and chemotherapy regimen on DFS was significant ($P_{\text{interaction}}=0.05$), suggesting that the positive impact of TILs on DFS was different according to the NAC used (Anthra-taxanes, HR=0.993, 95%CI [0.983-1.003], $p=0.18$, Others, HR=0.968, 95%CI [0.944-0.994], $p=0.014$).

TIL variations before and after NAC

After chemotherapy, TIL levels decreased in 61.6% of tumors ($n=441$), did not change in 17.7% ($n=127$) and increased in 20.7% ($n=148$). Mean TIL levels were higher before than after NAC (all: 24.1 *versus* 13.0%, $p<0.001$; luminal: 16.0 *versus* 11.2 %; TNBC: 28.5 *versus* 15.4 %; *HER2*-positive: 26.5 *versus* 10.9 %, $p<0.001$) (Fig. 2A). These results were similar according to NAC regimen (Supplementary Fig. S2).

Mean TIL variation differed according to pCR status (pCR: -25.2 *versus* no pCR: -5.6, $p<0.001$). TIL levels were more likely to increase or remain stable after NAC if pre-NAC TIL levels were low than if they were high (Fig. 2B-D). PCR status was strongly associated with the magnitude of TIL level decrease (Fig. 2E); however, the variation of TIL level was strongly inversely correlated with pre-NAC TIL levels ($r=-0.80$, $p<0.001$) regardless of pCR status (Fig. 2F). Overall, these findings suggest a strong inverse correlation between pre-NAC TIL levels and the variation of TIL levels, both of which are also strongly associated with pCR (Supplementary Fig. S3). This was true irrespective of BC subtypes and NAC regimen (Supplementary Fig. S4 and S5).

Association between post-NAC TILs, clinicopathological patterns, and survival

Association between post-NAC TILs and tumor characteristics

(TNBC: 15.4%; luminal: 11.3%, *HER2*-positive: 10.9%, $p < 0.001$, Fig. 3A-B).

Post-NAC TIL levels differed significantly between tumors with and without pCR (no pCR/
pCR: 14.7% *versus* 8.8 %, $p < 0.001$) (Fig. 3C, Supplementary Table S5) except in luminal
BCs (TNBC: 18.6% *versus* 10.3%, $p < 0.001$; *HER2*-positive: 14.0% *versus* 6.2 %, $p < 0.001$;
luminal: 11.4% *versus* 7.8 % $p = 0.27$). Post-NAC TIL levels were associated with aggressive
tumor characteristics in the *HER2*-positive population, but not in luminal tumors and TNBCs
(Fig. 3D-E). Significant interactions were observed for the association between post-NAC
TILs, BC subtype, and post-NAC mitotic index ($p_{\text{Interaction}}: 0.037$), invasive tumor cellularity
($p_{\text{Interaction}} < 0.001$), and RCB class ($P_{\text{interaction}} = 0.05$, Figure 3F).

Survival as a function of post-NAC TIL levels

Post-NAC TIL levels were not associated with DFS in the whole population (HR=1.01,
95%CI [0.099-1.02], $p = 0.325$, Table 2), but a significant interaction with BC subtype was
observed ($p_{\text{Interaction}} = 0.04$). Post-NAC TILs had no impact on prognosis in the luminal
subgroup (HR=0.996, CI [0.964-1.029], $p = 0.79$ or TNBC subtypes (HR=0.998, CI [0.983-
1.013], $p = 0.786$), but had a significant adverse impact in *HER2*-positive BCs (HR= 1.04,
95%CI [1.016-1.064], $p = 0.001$, Supplementary Table S4). No significant deviation from the
assumption of linearity was observed. In the population with residual disease, an adverse
impact of post-NAC TILs was observed for patients with *HER2*-positive disease (HR=1.029,
CI [1.002-1.057], $p = 0.034$), whereas a trend towards a protective effect of high post-NAC
TIL levels was observed for TNBC (HR=0.984, CI [0.966-1.003], $p = 0.095$).

Multivariate survival analyses

After multivariate analysis, pre-NAC TIL levels, BC subtype, RCB index, and post-NAC
mitotic index were significantly associated with DFS (Table 2). In TNBCs, pre-NAC TIL
levels were an independent predictor of better DFS (Supplementary Table S4), whereas post-

436 NAC TIL levels were an independent predictor of impaired DFS in the *HER2*-positive Neoadjuvant and Immunity subgroup. Neither pre-NAC nor post-NAC TIL levels were associated with DFS in the luminal subgroup.

TIL analyses by RCB class and trastuzumab use

Relationship between TIL levels and survival, by RCB class

Detailed analyses performed after stratification by RCB class are provided in the Supplementary Tables S6-7. The association between post-NAC TILs and DFS was not significant in the RCB-0-I or RCB-II classes, whereas post-NAC TILs were associated with poor outcome in the RCB-III class (HR=1.02, CI [1.001-1.037], $p=0.036$).

Survival analysis in the HER2-positive population, according to neoadjuvant trastuzumab use and ER status

We investigated survival as a function of neoadjuvant trastuzumab use ($n=144$, 82.3%) or non-use ($n=31$, 17.7%) in the *HER2*-positive population (Supplementary Table S8).. Pre-NAC TIL levels were not associated with DFS in either of the two groups, and post-NAC TIL levels were significantly associated with impaired DFS only in the population treated with neoadjuvant trastuzumab (HR=1.038, CI [1.011-1.065], $p=0.005$)

We analyzed the *HER2*-positive population according to ER status (ER positive, $n=98$, ER negative, $n=77$) (Supplementary Table S9). Tumors from the ER-/*HER2*+ subgroup were of higher grade, and TILs levels were higher before chemotherapy than in the ER+/*HER2*+ subgroup (30.6% versus 23.2%, $p<0.01$). After chemotherapy, there was no difference in the TIL levels.

Pre-NAC TILs levels were neither associated with pCR nor DFS in any of the ER positive or ER negative subgroups. Post-NAC TILs were associated with impaired DFS in ER positive population (HR= 1.04, 95%CI [1.02-1.07], $p<0.01$) but not in the ER negative population

(HR= 1.04, 95%CI [0.99-1.09], p=0.13). This difference might be explained by a lack of
2.5 Article n°14 : Hamy, A.-S. et al., CCR Apr 2019, in press **437**

statistical power ($P_{interaction\ with\ ER\ status} = NS$).

We report here detailed analyses of associations between baseline and post-treatment immune infiltration levels in a large cohort of paired pre- and post-NAC BC samples. Our findings extend existing knowledge in this field in several ways.

First, our results confirm the widely reported association between pre-NAC TILs and pCR(1–3,14–18), but we nevertheless observed (i) a non-linear effect in TNBCs; (ii) a significant interaction with BC subtype.

Non-linear effects have been reported for the association of pCR and TIL levels in *HER2*-positive tumors(2,19,20); but linearity has never been investigated in detail for TNBCs in the neoadjuvant setting (no linearity test reported(1,3,9,10,16–18,21–28)). Additionally, our data also revealed a non-linear prognostic impact of TILs, differing by BC and by NAC regimens.

Second, significant interactions with BC subtype have been described only in the GeparSixto trial(3) so far. It is unclear why pre-NAC TILs were associated with pCR only in TNBC.

While the relationship we found here is almost constant in TNBC studies (1,3,18,24), this effect seems less clear in *HER2*-positive BC(2,15,29,30). Several studies on *HER2*-positive BC (NeoSPHERE (29), NeoALTTO (2), GeparSepto (30)) - including ours - showed no association between pre-NAC TILs and pCR, whereas other did (GeparQuattro and GeparQuinto (15)). Several hypotheses could explain such differences: (i) Differences in tumor biology; (ii) quantitative and qualitative differences in the immune infiltration and corresponding threshold values for defining high-TILs tumors (2,19); (iii) the use, the type, and the interaction of TILs with anti-*HER2* targeted therapies (2,7,31) (iv) the type and the sequences of NAC regimen, as interactions have been previously described between TILs, subtype and chemotherapy regimen(4,5)(3)); (v) and the difference in the percentages of ER-positive disease in the different *HER2*-positive BC cohorts. Regarding luminal BC, the number of patients whose tumor reached pCR was very low, and a lack of statistical power may partially explain why the association between pre-NAC TILs and pCR failed to reach statistical significance ($p=0.058$).

Group(14) analyzing the relationships between TIL levels in baseline samples and oncologic outcomes in a large cohort of 3771 patients receiving NAC. Denkert and colleagues(1) found that higher TIL levels were associated with a DFS benefit in *HER2*-positive and TNBC tumors; but with a poor OS in luminal *HER2*-negative BCs. The authors concluded that the biological features of the immunological infiltrate are probably important in luminal *HER2*-negative BCs and that the use of genomic parameters, such as mutational signatures or copy number variations, should be considered for stratification purposes.

Second, we demonstrated a decrease in mean TIL levels when comparing levels before and after chemotherapy. Only a few cohorts (9,16,22,25–28,32–35) have reported pathological TIL evaluations on paired matched samples, and all these previous cohorts were small (Supplementary Table S10). Two studies assessing lymphocyte density by computational pathology on large cohorts of patients from neoadjuvant trials (Neo-tAnGo (26), ARTEMIS(28)) found that both pre-NAC immune infiltration and a decrease in immune infiltration were associated with pCR, and another study found that larger decreases in CD3 levels after treatment were associated with better DFS and OS(16). Due to the strong association between pre-NAC TILs, TILs changes, pCR status, post-NAC TILs and DFS, their respective part regarding the association with prognosis remains unknown. Notably, TILs changes might be an interesting parameter, as it was both strongly associated with pCR and DFS. As these data on TILs variation are unprecedented on a large cohort of BC patients in the literature, it calls for further validation of this endpoint on independent cohorts.

Third, regarding the immune infiltration after treatment, there was almost no post-NAC LPBCs (TILs \geq 60%), highlighting the need for a revision of TIL level cutoff points after NAC. Post-NAC TIL levels were higher in tumors with RD than in areas of scarring in tumors displaying pCR. TIL levels have never been reported from pCR specimens, but recent guidelines(36) have suggested that these levels could be evaluated for research purposes. We

might suggest that, once the immune cells have eradicated the tumor, they would move into the periphery similar to responses to infection or other anomalies eliciting an immune response. Research on post-NAC TILs in pCR specimen could be of interest notably to analyze their association with the rare subgroup of patients experiencing relapse after their tumor reached pCR.

In cases of RD, TILs were associated with aggressive post-NAC patterns only in the *HER2*-positive subgroup. It remains unclear whether this difference reflects inherent differences between the three BC subtypes, the use of neoadjuvant trastuzumab, or differences in the immune infiltration in RD. Two hypotheses can be drawn. On the one hand, TILs in specimen with residual disease could be active but may not have had sufficient time to completely eradicate the tumor. Our data do not support this hypothesis, because we found no correlation at all between time from biopsy to surgery and post-NAC TIL levels (Supplementary Figure S6). On the other hand, the immune response may not recognize the tumor cells and post-NAC TILs could be unable to exert their antitumor function, possibly due to a surrounding immunosuppressive milieu.

Finally, our results suggest that pre- and post-NAC TIL levels may have different impacts on outcome. In TNBC, high pre-NAC TIL levels were an independent predictor of good prognosis, whereas, in *HER2*-positive BC, high post-NAC TIL levels were an independent predictor of poor outcome. We are currently characterizing the immune subpopulations, immune checkpoint and immune checkpoint ligand expression in residual tumor specimens, in the hope that this will shed further light on the mechanisms underlying the observed differences in the prognostic impact of post NAC-TILs in the 3 BC subtypes. Analyses of spatial and temporal dynamics, particularly to determine whether TIL location (intratumoral *versus* stromal) has a differential effect on outcome, will also be of interest.

matched pre- and post-NAC samples for 716 patients. In addition, the patients and samples were derived from an institutional cancer center cohort, and therefore reflect real-life conditions more faithfully than analyses of results for randomized trials including only highly selected patients. The limitations of this study include the lack of data on TILs during NAC (*i.e.* on-treatment biopsies), which would have (i) provided insights into the mechanisms underlying immune response to chemotherapy (ii) confirmed if pre-NAC TIL levels go straightforward to the levels observed after NAC, or if it is preceded by an initial increase. On-treatment data from the I-SPY trial suggest that the immune genes expression decreases as soon as 1 to 4 days after NAC beginning(37). Additionally, the study was performed at a single center, making external validation necessary. Large integrative and collaborative analyses may make it possible to decipher the role of immune infiltration in BC in more detail, particularly in cases of residual disease after NAC. We therefore provide our original data as an open-access resource for the medical and scientific community, for pooling with existing datasets (Supplementary Table S11).

Our results have several implications. First, they suggest that future studies should include interaction and linearity tests, to help determining and validating TILs thresholds values relevant to each BC subtype, both in the pre and in the post-NAC setting. Second, due to the multiplicity of interactions (breast cancer subtypes, NAC regimen, benefit from targeted therapy, RCB score), efforts should be paid in routinely score TILs both in the pre and the post-NAC setting, and share data within collaborative projects, as such complex associations may only be deciphered with a very large amount of patients and samples. Finally, the adverse outcome associated with high TIL levels after the completion of NAC in some subgroups (*HER2*-positive patients; RCB-III tumors) highlights the urgent need for second-line trials in the post-NAC setting. Immunotherapies may theoretically be of interest for the treatment of tumors with an immune infiltrate associated with a poor prognosis.

We thank Roche France for financial support for the construction of the Institut Curie neoadjuvant database (NEOREP). Funding was also obtained from the *Site de Recherche Intégrée en Cancérologie/Institut National du Cancer* (Grant No. INCa-DGOS-4654). A-S Hamy-Petit was supported by an ITMO-INSERM-AVIESAN translational cancer research grant. We thank Dr Bernard Asselain for his helpful advice and for validating the statistical methodology used in this study.

Table 1: Association between clinical and pathological factors with pathological complete response (Univariate and multivariate analysis, whole population).

Odds ratio for pCR and corresponding confidence interval are calculated with a univariate logistic regression model. Pre-NAC TILs are considered as a continuous variable in the analyses. Due to the difficulty to translate a continuous variable into a pCR rate, we also reported pre-NAC TILs binned by 10% increment to enable comparison with further studies using other TILs threshold values.

Table 2: Association with clinical and pathological pre and post-NAC parameters with disease-free survival (whole population, univariate and multivariate analysis).

Due to a significant deviation to the linearity assumption, pre-NAC TILs are considered as a continuous variable but are modeled with a fractional polynomial. Post-NAC TILs are considered as a continuous, linear variable.

Figure legends:

Figure 1: Associations between pre-NAC TIL levels, clinical and pathological factors and response to treatment

A: Distribution of pre-NAC TIL levels, by BC subtype (kernel density plot); B: Barplot of the repartition of the percentage of tumors according to pre-NAC TIL levels binned by 10% increment by BC subtype). The proportion of tumors with TILs $\geq 60\%$ is 11% ($n=80$) [luminal: 2.3%, $n=5$; *HER2*-positive: 9.7%, $n=17$; TNBC: 18.2%, $n=58$). C: Percentage of pathological complete response rate (pCR) by pre-NAC TIL levels, in the global population and by BC subtype (TILs were binned by increments of 10%, as previously described(6)). The shape of the TNBCs bars enables a visual representation of the deviation to the linearity assumption. D to G: Graphical representation of the best statistical model retained for analyzing the association between pre-NAC TIL levels and pCR. X-axis represents the increasing value of pre-NAC TILs, and y-axis represents the increasing odds ratio for pCR: D: whole population, linear model; E: luminal, linear model; F: TNBC: restricted cubic spline,

data for the association between pre-NAC TILs and DFS. X-axis represents the increasing value of pre-NAC TILs, and y-axis represents the increasing hazard ratio for DFS: H: whole population, second-order fractional polynomial; I: luminal, restricted cubic spline; J:TNBC, second-order fractional polynomial; K: *HER 2*-positive, linear model.

Figure 2: TIL levels variation before and after NACA : Bar plots of TIL levels before and after NAC in the whole population and in the various BC subtypes Lower and upper bars of the boxplots represent the first and third quartile respectively, the medium bar is the median, and whiskers extend to 1.5 times the inter-quartile range; B : Repartition (as percentages) of TIL variation classes, according to the pre-NAC TIL levels, binned by increments of 10%. TIL level variation is classified into three categories (TIL level decrease: yellow/ no change: blue/ increase: red); C: Variation of TIL levels according to the pre-NAC TIL levels binned by increments of 10%. Lines represent pre and post-NAC paired TIL levels values of a given patient, and are colored according to TIL variation category; (TIL level decrease: yellow/ no change: blue/ increase: red). D: Waterfall plot representing the variation of TILs according to the pre-NAC TILs levels, binned by increments of 10%. Each bar represents the absolute TIL variation, i.e. the difference between TIL levels after and before NAC and is colored according to the pre-NAC TIL levels; Within each pre-NAC TIL levels category, the change in TIL levels is ranked by increasing TIL level variation.

E : Association between pre-NAC TIL levels by 10% increment and absolute difference in TIL levels before and after NAC, by pCR status(No pCR tumor, left panel, pCR tumor, right panel)); each boxplot represents the median value and associated interquartile range.

F. Waterfall plot representing the variation of TIL levels according to pCR status ; each bar represents one sample, and samples are ranked by increasing order of TIL level change. Paired samples for which no change was observed have been removed from the graph.

A: Distribution of post-NAC TIL levels, by BC subtype (kernel density plot); B: Barplot of the repartition of the percentage of tumors according to post-NAC TIL levels binned by 10% increment by BC subtype); The proportion of tumors with TILs $\geq 60\%$ is 2% ($n=16$) (luminal: 1%, $n=3$; *HER2*-positive: 1%, $n=1$; TNBC: 4%, $n=12$). C: Post-NAC TIL levels by pCR status and by BC subtype; D: Associations between post-NAC TIL levels and post-NAC mitotic index, E: Associations between post-NAC TIL levels and post-NAC cellularity. F: Associations between post-NAC TIL levels and RCB in the whole population, and after stratification by BC subtype. Lower and upper bars of the boxplots represent the first and third quartile respectively, the medium bar is the median, and whiskers extend to 1.5 times the inter-quartile range. The results are considered statistically significant at a p-value <0.05 (*), <0.01 (**) or <0.001 (***)).

References

Automatic citation updates are disabled. To see the bibliography, click Refresh in the Zotero toolbar.

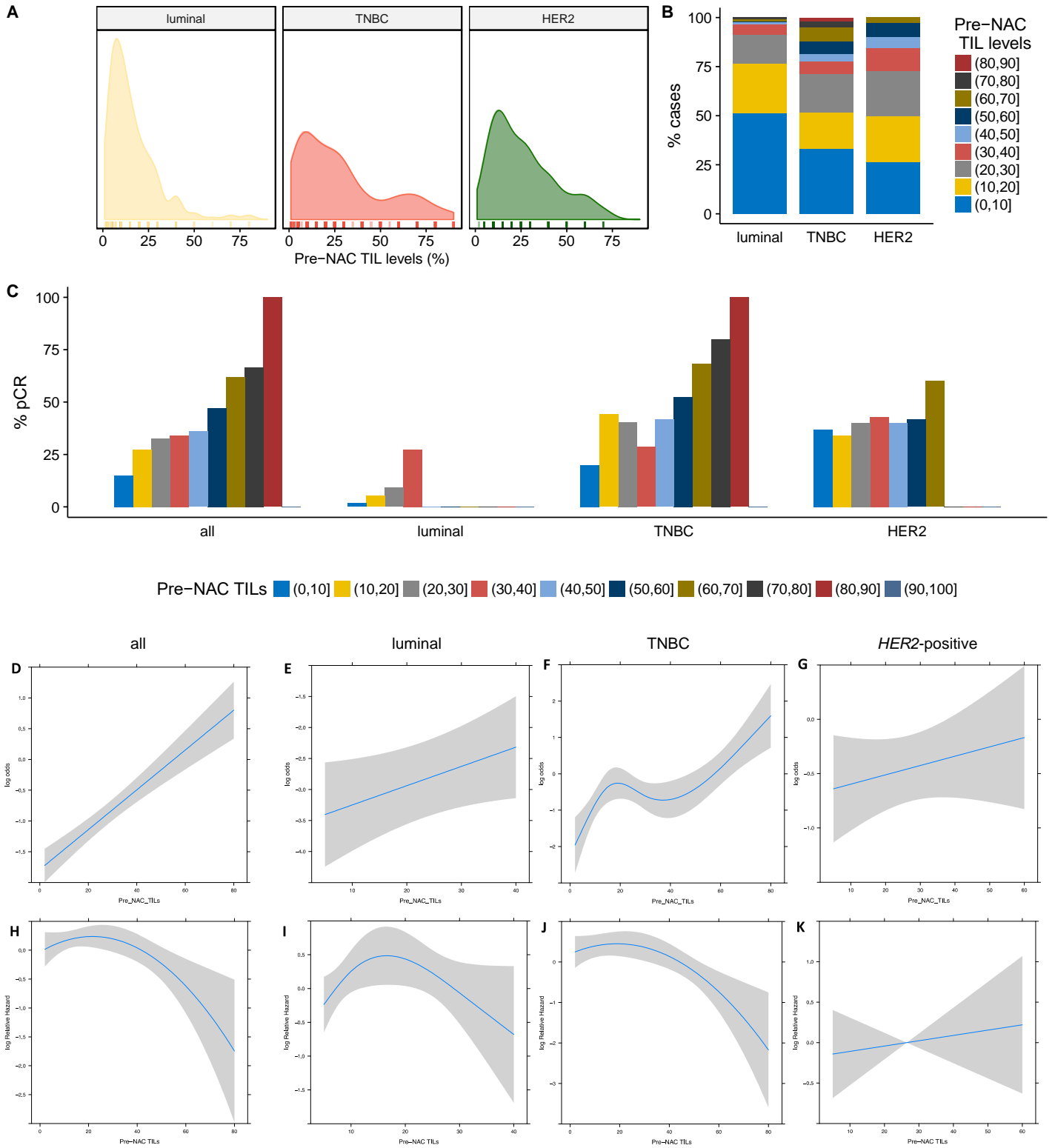


Figure 2

Neoadjuvant and Immunity

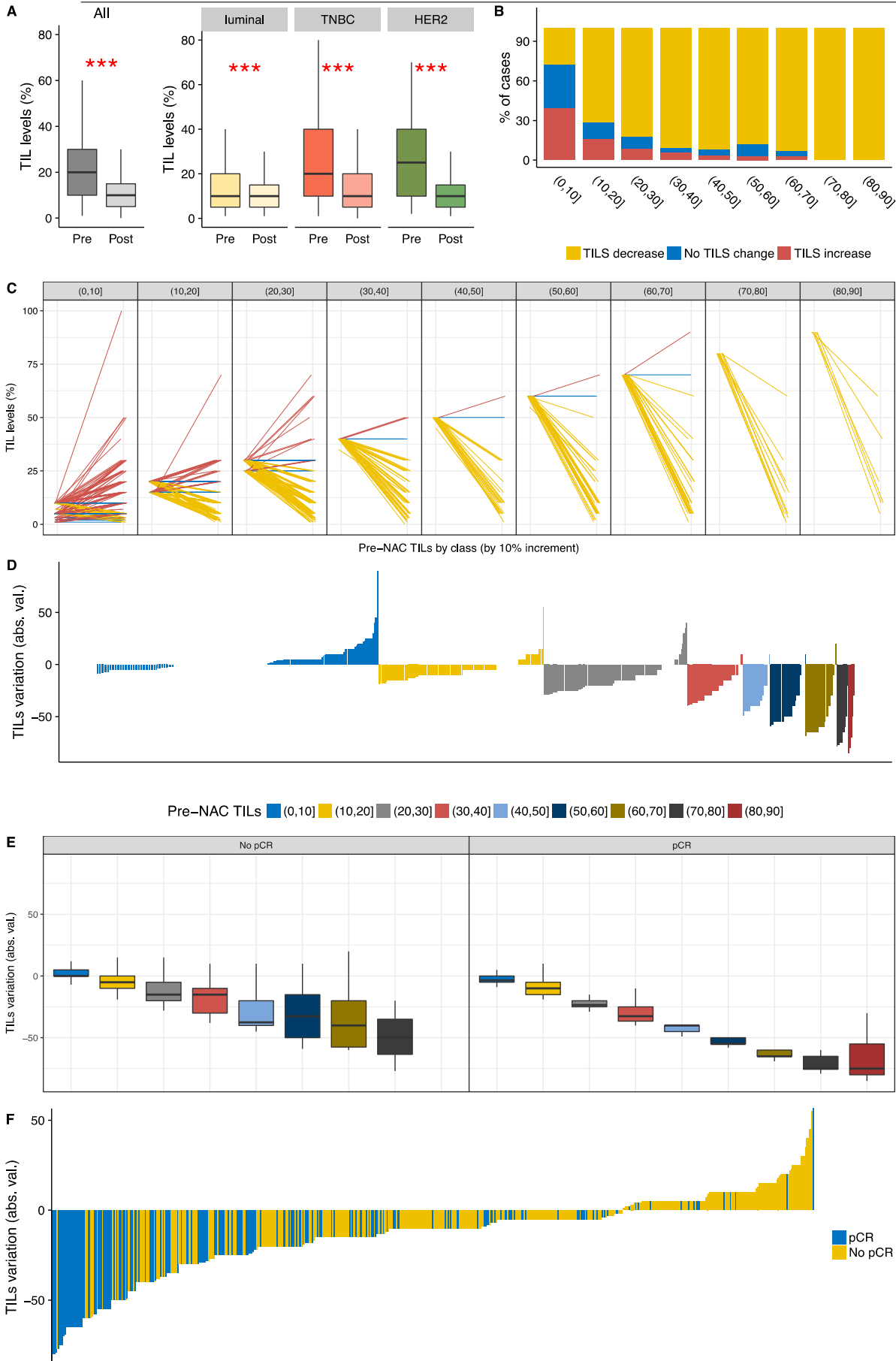


Figure 3

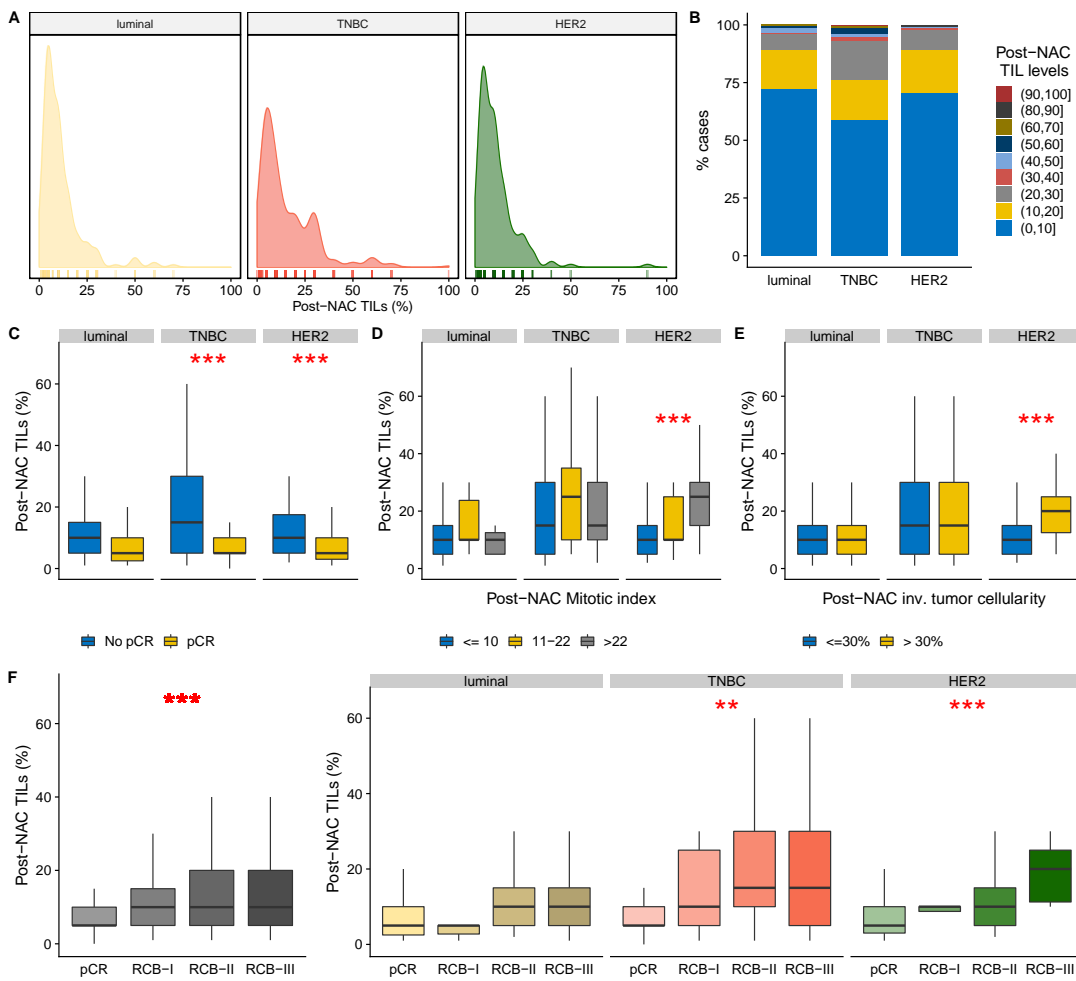


Table1

Table 1: Association between clinical and pathological factors with pathological complete response (Univariate and multivariate analysis, whole population).

Odds ratio for pCR and corresponding confidence interval are calculated with a univariate logistic regression model. Pre-NAC TILs are considered as a continuous variable in the analyses. Due to the difficulty to translate a continuous variable into a pCR rate, we also reported pre-NAC TILs binned by 10% increment to enable comparison with further studies using other TILs threshold values.

Characteristics	Class	Total population			Univariate			Multivariate		
		n	pCR	%	OR	[95% CI]	pval	OR	[95% CI]	p
Pre-NAC Parameters										
Age (years)	< 45	286	76	26.6 %	1					
	45-55	254	66	26 %	0.97	[0.66 - 1.42]	0.877			
	> 55	178	60	33.7 %	1.4	[0.93 - 2.11]	0.101			
Menopausal status	Post	259	80	30.9 %	1					
	Pre	452	119	26.3 %	0.8	[0.57 - 1.12]	0.193			
BMI class	[19-25]	414	125	30.2 %	1					
	< 19	41	8	19.5 %	0.56	[0.24 - 1.19]	0.156			
	[25-30]	166	41	24.7 %	0.76	[0.5 - 1.14]	0.186			
	> 30	96	27	28.1 %	0.9	[0.55 - 1.47]	0.69			
Tumor size	T1-T2	529	155	29.3 %	1					
	T3	189	47	24.9 %	0.8	[0.54 - 1.16]	0.245			
Clinical nodal status	N0	282	83	29.4 %	1					
	N1-N2-N3	435	119	27.4 %	0.9	[0.65 - 1.26]	0.546			
ER status	Negative	397	163	41.1 %	1					
	Positive	321	39	12.1 %	0.2	[0.13 - 0.29]	<0.001			
PR status	Negative	474	183	38.6 %	1					
	Positive	221	17	7.7 %	0.13	[0.08 - 0.22]	<0.001			
HER2 status	Negative	543	134	24.7 %	1					
	Positive	175	68	38.9 %	1.94	[1.35 - 2.78]	<0.001			
BC subtype	Luminal	223	11	4.9 %	1			1		
	TNBC	320	123	38.4 %	12.03	[6.58 - 24.27]	<0.001	10.96	[5.64 - 24]	<0.001
	HER2	175	68	38.9 %	12.25	[6.46 - 25.36]	<0.001	11.08	[5.52 - 24.8]	<0.001
Histology	NST	661	188	28.4 %	1					
	Other	53	13	24.5 %	0.82	[0.41 - 1.52]	0.543			
Grade	I-II	211	33	15.6 %	1					
	III	491	164	33.4 %	2.71	[1.8 - 4.16]	<0.001			
Ki67	< 20	33	6	18.2 %	1					
	≥ 20	146	53	36.3 %	2.56	[1.05 - 7.23]	0.051			
NAC regimen	Anthra-tax	610	169	27.7 %	1					
	Anthra	62	17	27.4 %	0.99	[0.54 - 1.74]	0.962			
	Taxane-based	23	7	30.4 %	1.14	[0.43 - 2.73]	0.774			
	Others	23	9	39.1 %	1.68	[0.69 - 3.9]	0.236			
Mitotic index	< 11	176	36	20.5 %	1					
	[11-22]	202	53	26.2 %	1.38	[0.86 - 2.25]	0.187			
	> 22	319	110	34.5 %	2.05	[1.34 - 3.19]	0.001			
Tumor (inv) cellularity	≤ 60%	372	107	28.8 %	1					
	> 60%	344	95	27.6 %	0.94	[0.68 - 1.31]	0.733			
DCIS component	Yes	605	174	28.8 %	1					
	No	112	28	25 %	0.83	[0.51 - 1.3]	0.417			
Pre-NAC TIL levels (10% increment)	[0.10]	266	40	15.0%	1					
	[10.20]	157	43	27.4%	2.13	[1.65 - 2.62]	0.002			
	[20.30]	135	44	32.6%	2.73	[2.24 - 3.22]	<0.001			
	[30.40]	53	18	34.0%	2.91	[2.25 - 3.57]	0.002			
	[40.50]	25	9	36.0%	3.18	[2.29 - 4.06]	0.01			
	[50.60]	34	16	47.1%	5.02	[4.27 - 5.77]	<0.001			
	[60.70]	29	18	62.1%	9.25	[8.42 - 10.07]	<0.001			
	[70.80]	12	8	66.7%	11.3	[10.05 - 12.55]	<0.001			
	[80.90]	6	6	100.0%						
	[90.100]	0	0							
Pre-NAC TILs	(linear)				1.03	[1.02 - 1.04]	<0.001	1.03	[1.02 - 1.03]	<0.001

Table 1: Association between clinical and pathological factors with pathological complete response (Univariate and multivariate analysis, whole population). Odds ratio 1
Abbreviations: BC: breast cancer, BMI: body mass index (kg/m2), DCIS: ductal carcinoma in situ, ER: estrogen receptor, NAC: neoadjuvant chemotherapy, NST: no specific t

Table2

Table 2: Association with clinical and pathological pre and post-NAC parameters with disease-free survival (whole population, univariate and multivariate analysis).

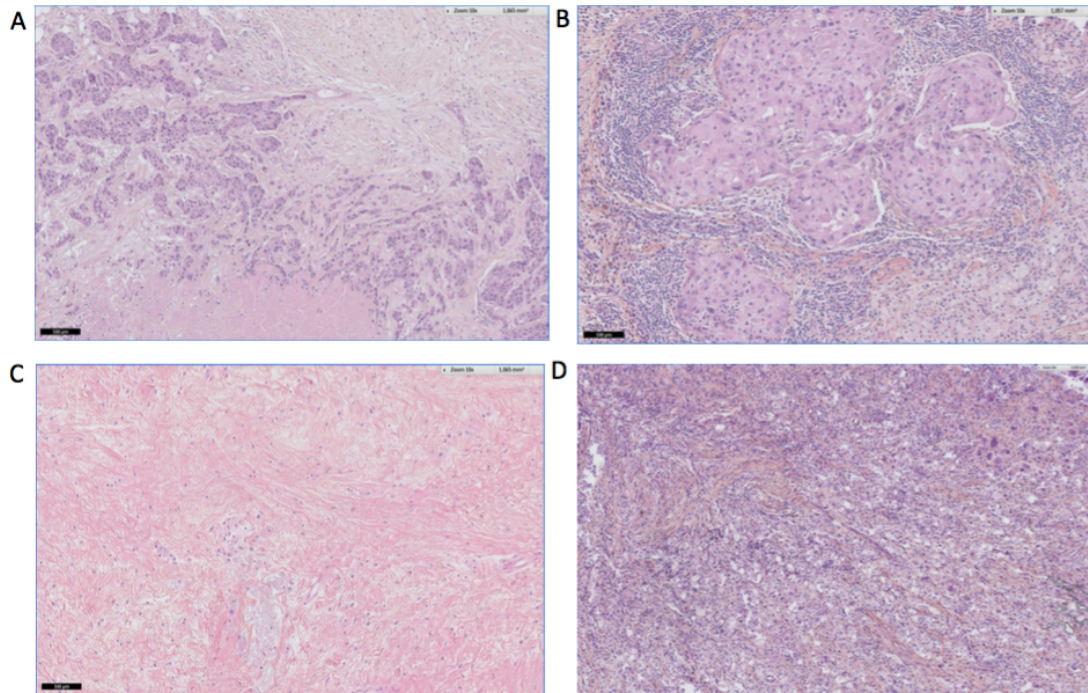
Due to a significant deviation to the linearity assumption, pre-NAC TILs are considered as a continuous variable but are modeled with a fractional polynomial. Post-NAC TILs are considered as a continuous, linear variable.

				All						
				Univariate			Multivariate			
Characteristics	Class	n	ev	HR	[95% CI]	p	<i>p wald</i>	HR	[95% CI]	p
Pre-NAC parameters										
Age (years)	< 45	286	60	1			0.666			
	[45-55]	254	54	0.98	[0.68 - 1.41]					
	> 55	178	31	0.83	[0.53 - 1.27]					
Menopausal status	Post	259	53	1			0.864			
	Pre	452	89	0.97	[0.69 - 1.36]					
BMI class	< 25	455	84	1			0.143			
	≥ 25	262	61	1.28	[0.92 - 1.78]					
Tumor size	T1-T2	529	96	1			0.004			
	T3	189	49	1.66	[1.18 - 2.34]					
Clinical node status	N0	282	54	1			0.449			
	N1-N2-N3	435	91	1.14	[0.81 - 1.6]					
ER status	Negative	397	91	1			0.009			
	Positive	321	54	0.64	[0.46 - 0.9]					
PR status	Negative	473	106	1			0.006			
	Positive	222	32	0.57	[0.38 - 0.84]					
HER2 status	Negative	543	127	1			0.002			
	Positive	175	18	0.45	[0.28 - 0.74]					
BC subtype	Luminal	223	44	1			<0.001	1	-	-
	TNBC	320	83	1.64	[1.14 - 2.37]			2.45	[1.55 - 3.87]	<0.001
	HER2	175	18	0.61	[0.35 - 1.05]			1.05	[0.53 - 1.7]	0.95
Histology	NST	661	130	1			0.206			
	Other	53	14	1.43	[0.82 - 2.48]					
Grade	I-II	211	41	1			0.344			
	III	491	101	1.19	[0.83 - 1.71]					
Ki 67	< 20%	33	7	1			0.292			
	≥ 20%	146	41	1.54	[0.69 - 3.43]					
Tumor cellularity (inv)	≤ 60%	372	82	1			0.345			
	> 60%	344	63	0.85	[0.61 - 1.19]					
Mitotic index	< 11	176	27	1			0.061			
	[11-22]	202	43	1.47	[0.91 - 2.37]	0.119				
	> 22	319	73	1.7	[1.1 - 2.65]	0.018				
Pre-NAC TILs (as FP*)							<0.001			0.01
Post-NAC parameters										
pCR	No pCR	516	131	1			<0.001			
	pCR	202	14	0.26	[0.15 - 0.46]					
RCB index	continuous			1.63	[1.42 - 1.86]		<0.001	1.66	[1.4 - 1.95]	<0.001
Post-NAC TILs (linear)				1.01	[0.99-1.02]		0.325			
Mitotic index	< 11	524	64	1			<0.001	1	-	-
	[11-22]	34	8	1.94	[0.93 - 4.04]	0.078		0.95	[0.43 - 2.1]	0.89
	> 22	120	61	5.54	[3.9 - 7.88]	<0.001		2.92	[1.95 - 4.35]	<0.001
Tumor (inv) cellularity	≤ 30%	456	59	1			<0.001			
	> 30%	237	79	2.6	[1.85 - 3.64]	<0.001				
Size of nodal metastasis (mm)	≤ 2	135	45	1			0.046			
	[3-5]	73	14	0.51	[0.28 - 0.93]	0.028				
	> 5	59	21	1.15	[0.68 - 1.93]	0.604				

Abbreviations: BC: breast cancer, BMI: body mass index (kg/m²), DCIS: ductal carcinoma in situ^[1], ER: estrogen receptor, NAC: neoadjuvant chemotherapy, NS

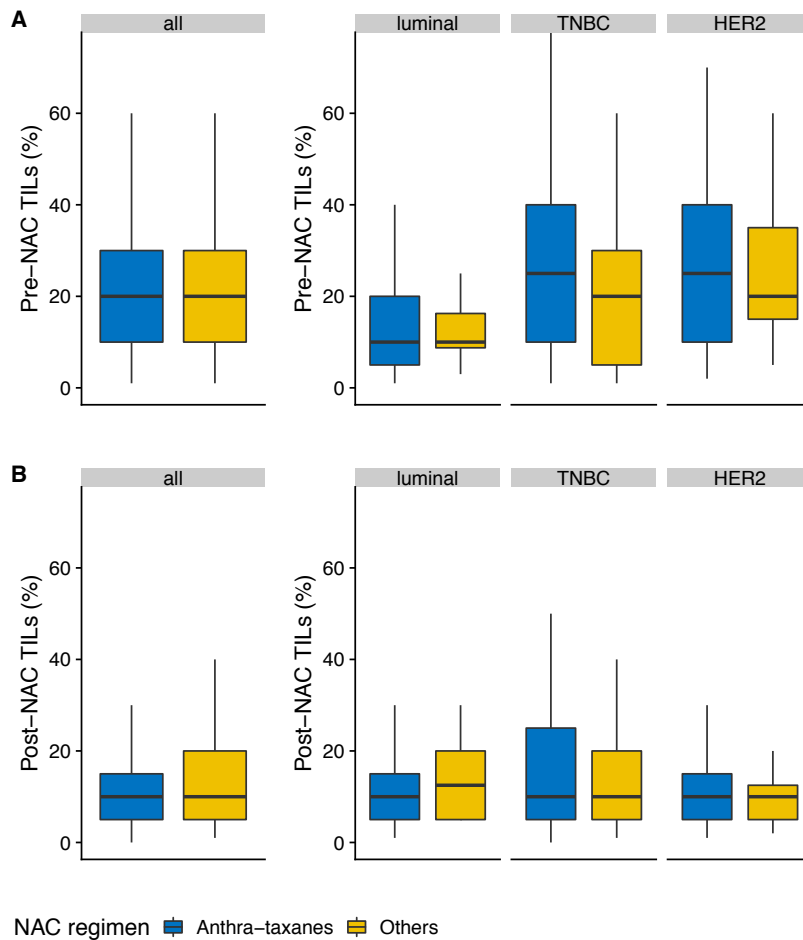
Supplementary Figures

Supplementary Figure S1



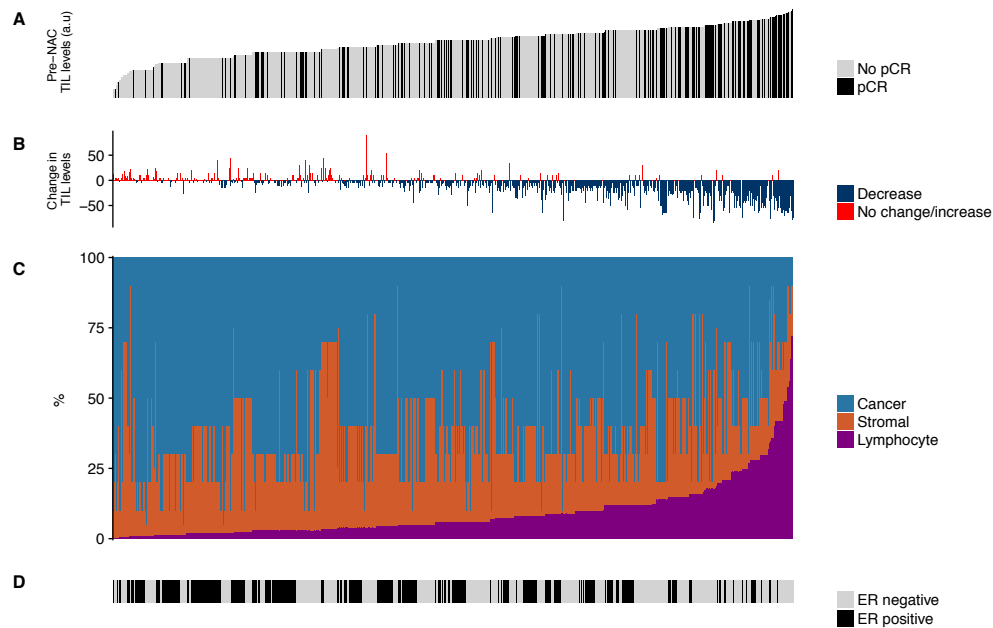
Supplementary Figure S1: TILs in surgical specimens obtained after NAC (H&E (hematoxylin and eosin) staining). A: RD with low TIL levels (5%); B: RD with high TIL levels (60%); C: pCR with low TIL levels (5%); D: pCR with high TIL levels (60%). Abbreviations: RD: residual disease, pCR: pathological complete response.

Supplementary Figure S2

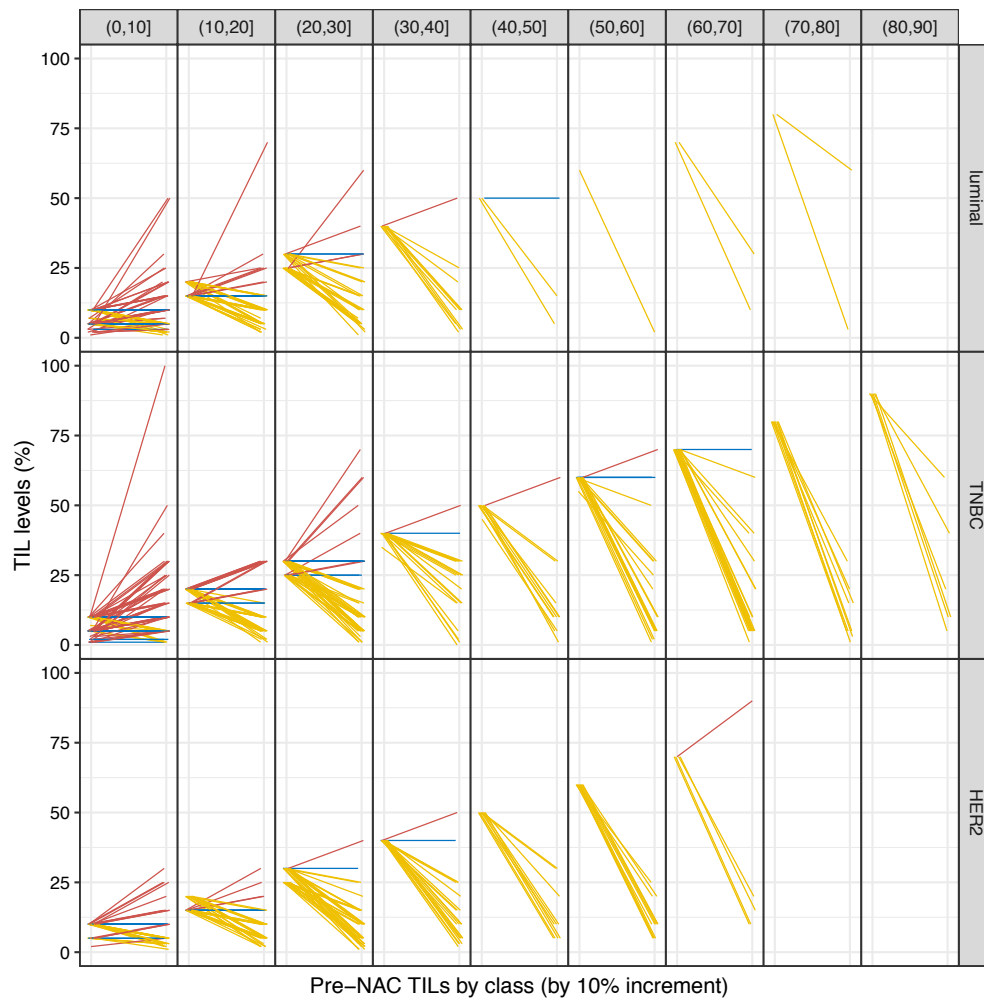


Supplementary Figure S2 : Association between pre-NAC TIL levels (A), post-NAC TIL levels (B) according to chemotherapy regimen in the whole population and after stratification by BC subtype. Lower and upper bars of the boxplots represent the first and third quartile respectively, the medium bar is the median, and whiskers extend to 1.5 times the inter-quartile range. The results are considered statistically significant at a p-value <0.05 (*), <0.01 (**) or <0.001 (***) .

Supplementary Figure S3



Supplementary Figure S3: Association between pre-NAC TIL levels and chemotherapy response (A), change in TIL levels (B), cellularity (C), and ER status (D). Pre-NAC TIL levels have been rescaled to values between zero and one for illustration. Abbreviations: a.u.: arbitrary units, ER: estrogen receptor, pCR: pathological complete response. We ranked 716 paired samples in increasing order of pre-NAC TIL levels (figure adapted from computational pathology analyses performed in the Neo-tAnGo (10) and ARTemis(11) trials).

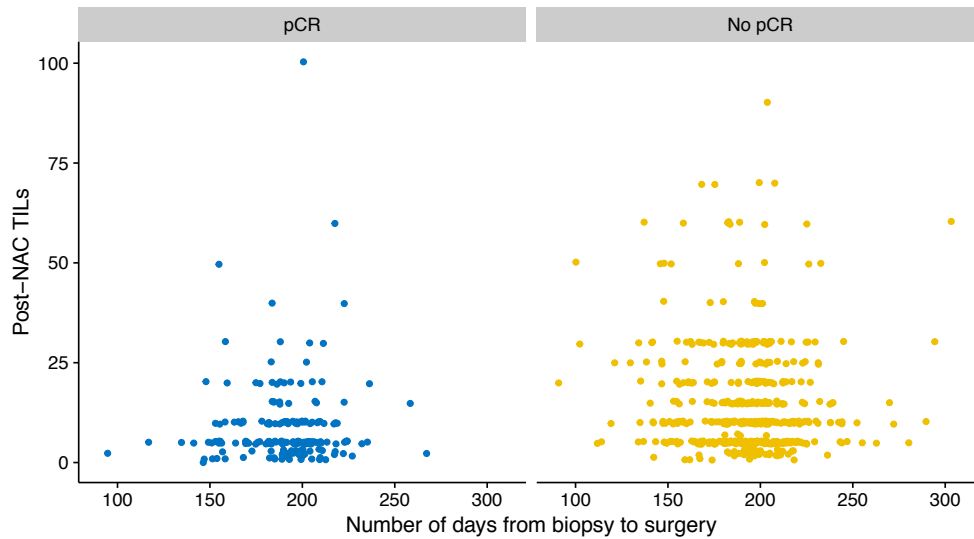
Supplementary Figure S4:

Supplementary Figure S4: C: Variation of TIL levels according to the pre-NAC TIL levels binned by increments of 10%. Lines represent pre and post-NAC paired TIL levels values of a given patient, and are colored according to TIL variation category; (TIL level decrease: yellow/ no change: blue/ increase: red). Upper panel represents luminal BC, middle panel represents TNBCs, and lower panel represents *HER2*-positive BC.

Supplementary Figure S5:

Association between TIL level variation and pCR according to the BC subtype; each bar represents one sample, and samples are ranked by increasing order of TIL level change. Paired samples with no change observed have been removed from the graph.

Supplementary Figure S6:



Supplementary Figure S6: Post-NAC TIL levels as a function of time from biopsy to surgery (number of days).

Supplementary material

1. Patients and treatments

1.1. Patients

In total, 718 patients with T1-3NxM0 invasive breast cancer (BC) (NEOREP Cohort, CNIL declaration number 1547270) treated at Institut Curie (Paris and Saint Cloud) between 2002 and 2012 were included in this study. We included unilateral, non-recurrent, non-inflammatory, non-metastatic tumors, excluding T4 tumors. NAC regimens changed over time (anthracycline-based regimen or sequential anthracycline-taxane regimen) with trastuzumab used in an adjuvant and/or neoadjuvant setting since 2005 for *HER2*-positive tumors. All but 10 patients underwent radiotherapy. Endocrine therapy (tamoxifen or aromatase inhibitor) was prescribed when indicated. This study was approved by the Breast Cancer Study Group of Institut Curie.

1.2. Treatments

The wide majority of patients (610/718, 85%) received an anthracyclines and taxanes based regimen, while the other patients received anthracyclines-based regimen without taxanes (62/718, 9%), taxanes-based regimen without anthracyclines (23/718, 3%), or other various regimen (23/718, 3%). Due to the very low number of patients who did not receive anthracyclines and taxanes-based regimen, we grouped the three other regimens into a single category (anthracyclines based regimen without taxanes / taxanes based regimen without anthracyclines, or others, total n=108).

2. Tumor samples and pathological review

2.1. ER, PR, *HER2* status and BC subtype

Cases were considered to be estrogen receptor (ER)-positive or progesterone receptor (PR)-positive if at least 10% of the tumor cells expressed estrogen and/or progesterone receptors (ER/PR). *HER2* expression was determined by immunohistochemistry, with scoring according to the American Society of Clinical Oncology (ASCO)/College of American Pathologists (CAP) guidelines (1). Scores of 3+ were reported as positive, and scores of 1+/0 as negative. Tumors with scores of 2+ were further tested by fluorescence *in situ*

hybridization (FISH). For *HER2* gene amplification, we evaluated a mean of 40 tumor cells per sample and calculated the mean *HER2* signal per nucleus. A *HER2/CEN17* ratio ≥ 2 was considered positive, and a ratio < 2 was considered negative (1). BC subtype was defined as follows (luminal: ER+ or PR+/*HER2*; TNBC: ER-/PR-/*HER2*; *HER2*-positive BC: *HER2*).

2.2. Other pathological parameters

Histological grade was determined as described by Elston Ellis. Mitotic cells were counted on 10 high-power fields (HPF) (x40 objective; field diameter = 0.62 mm) and cutoffs of <11 , 12–22 and >22 mitoses were used to define low, intermediate and high mitotic indices, respectively, according to the international recommendations(2). Due to significant differences in distribution before and after NAC, invasive tumor cellularity was binned according to the median value (pre-NAC: 60%; post-NAC: 30%).

2.3. Immunostaining

We checked that the infiltrating mononuclear cells considered to be TILs in the analyses were indeed lymphocytes, by immunostaining for CD3 on a subset of 20 surgical specimens. CD3 immunostaining was strongly correlated with the levels of unstained TILs(3) ($r=0.99$, $p<0.00001$).

3. Statistical analysis

3.1. Handling of missing data

For tumors for which a pCR (pathological complete response) was achieved, we considered mitotic index and tumor cellularity to be “Non Available” for descriptive and univariate pCR and disease-free survival (DFS) analysis. As multivariate analysis must be performed after the exclusion of missing data, pCR cases were imputed to the “ < 11 ” mitotic index category, and the “ $\leq 30\%$ ” category for tumor cellularity, to avoid the need to exclude these patients from the multivariate analysis.

3.2. Complementary statistical methods

We investigated the linearity of the association between TILs and pCR/DFS, by comparing the model in which TIL levels were considered to vary linearly with models based on restricted cubic spline fits and fractional polynomials, as previously described(16). If the TIL variable was found to be linear, TILs were analyzed as a continuous variable; if significant deviation from the assumption of linearity was observed, the variable was modeled with the model yielding the best fit to the data.

Factors predictive of pCR were introduced into a univariate logistic regression model. For variables that were significantly correlated, collinearity was avoided by retaining only one variable, based on its clinical relevance or likelihood ratio. A multivariate logistic model with a forward stepwise selection procedure was then applied, the covariates included having a likelihood ratio test p -value ≤ 0.05 . Survival probabilities were estimated by the Kaplan–Meier method, and survival curves were compared in log-rank tests. Hazard ratios and their 95% confidence intervals were calculated with the Cox proportional hazards model. Variables with a p -value for the likelihood ratio test ≤ 0.05 in univariate analysis were included in the multivariate model. Forward selection was used to establish the final multivariate model. The significance threshold was 5%.

3.3. Software and libraries

Analyses were performed with R software(8), version 3.1.2, with the libraries ggplot2, performanceanalytics, cowplot, survival, survminer, rms, grid, dplyr, reshape2, tableone, ggpubr, and ggsci.

References

1. Wolff AC, Hammond MEH, Schwartz JN, Hagerty KL, Allred DC, Cote RJ, et al. American Society of Clinical Oncology/College of American Pathologists guideline recommendations for human epidermal growth factor receptor 2 testing in breast cancer. *J Clin Oncol*. 2007;25:118–45.
2. WHO Classification of Tumours of the Breast. Fourth Edition - WHO - OMS - [Internet]. [cited 2018 Feb 9]. Available from: <http://apps.who.int/bookorders/WHP/detart1.jsp?sesslan=1&codlan=1&codcol=70&codcch=4004>
3. Hamy A-S, Pierga J-Y, Sabaila A, Laas E, Bonsang-Kitzis H, Laurent C, et al. Stromal lymphocyte infiltration after neoadjuvant chemotherapy is associated with aggressive residual disease and lower disease-free survival in HER2-positive breast cancer. *Ann Oncol Off J Eur Soc Med Oncol*. 2017;28:2233–40.
4. de Cremoux P, Valet F, Gentien D, Lehmann-Che J, Scott V, Tran-Perennou C, et al. Importance of pre-analytical steps for transcriptome and RT-qPCR analyses in the context of the phase II randomised multicentre trial REMAGUS02 of neoadjuvant chemotherapy in breast cancer patients. *BMC Cancer*. 2011;11:215.
5. Valet F, de Cremoux P, Spyrtos F, Servant N, Dujaric ME, Gentien D, et al. Challenging single- and multi-probesets gene expression signatures of pathological complete response to neoadjuvant chemotherapy in breast cancer: experience of the REMAGUS 02 phase II trial. *Breast Edinb Scotl*. 2013;22:1052–9.
6. Hamy AS, Bieche I, Lehmann-Che J, Scott V, Bertheau P, Guinebretière JM, et al. BIRC5 (survivin): a pejorative prognostic marker in stage II/III breast cancer with no response to neoadjuvant chemotherapy. *Breast Cancer Res Treat*. 2016;
7. Pierga J. Whole expression genome array can be used in daily practice for selecting neoadjuvant treatment. *Ann Oncol Abstr ESMO*. 2012;Volume 23,.
8. <http://cran.r-project.org/web/packages/citbcmst/index.html>.

Chapter 3 Comedication, neoadjuvant and Immunity

ANTICANCER RESEARCH 38: 1485-1490 (2018)
doi:10.21873/anticancerres.12375

COX2/PTGS2 Expression Is Predictive of Response to Neoadjuvant Celecoxib in HER2-negative Breast Cancer Patients

PATRICIA DE CREMOUX^{1,2}, ANNE-SOPHIE HAMY³, JACQUELINE LEHMANN-CHE^{1,2}, VÉRONIQUE SCOTT⁴, BRIGITTE SIGAL⁵, MARIE-CHRISTINE MATHIEU⁶, PHILIPPE BERTHEAU^{2,3,4,5,6,7}, JEAN MARC GUINEBRETIERE⁸, JEAN YVES PIERGA^{9,10}, SYLVIE GIACCHETTI¹¹, ETIENNE BRAIN¹², MICHEL MARTY¹³, BERNARD ASSELAIN¹⁴, FRÉDÉRIQUE SPYRATOS^{15*} and IVAN BIÈCHE^{15*}

¹Molecular Oncology Unit, ⁷Pathology Department, ¹¹Breast Disease Unit, Assistance Publique–Hôpitaux de Paris, University Saint-Louis Hospital, Paris, France;
²University Paris-Diderot, Sorbonne Paris Cité, INSERM/CNRS UMR944/7212, Paris, France;
³PSL Research University, Translational Research Department INSERM U932, Immunity and Cancer, Residual Tumor & Response to Treatment Laboratory (RT2 Lab), ⁵Pathology Department, ⁹Medical Oncology Department, and ¹⁵Pharmacogenomics Unit, Curie Institute, Paris, France;
⁴Translational Research Laboratory, and ⁶Pathology Department, Gustave Roussy Cancer Center, Villejuif, France;
⁸Tumor Biology Department, ¹²Medical Oncology Department, Curie Institute, Saint-Cloud, France;
¹⁰University Paris-Descartes, Sorbonne Paris Cité, Paris, France;
¹³Centre for Therapeutic Innovations in Oncology and Hematology, Assistance Publique–Hôpitaux de Paris, Saint-Louis Hospital, Paris, France;
¹⁴UMR 8081 “IM4R”, Université Paris Sud, Paris, France

Abstract. *Background:* The prognostic and predictive role of cyclo-oxygenase-2 (COX2) in breast cancer is still debated, and in particular, its role as a target of COX2 inhibitor (celecoxib) in neoadjuvant setting. *Materials and Methods:* We analyzed a series of 156 breast cancer samples from patients of the COX2 inhibitor-treated arm included in the REMAGUS-02 randomized phase II trial. COX2 gene expression was assessed by reverse transcription and quantitative polymerase chain reaction using ribonucleic acid from frozen biopsies. Pathological complete response (pCR) was the surrogate endpoint. *Results:* Significantly higher rates of grade 3, and estrogen and progesterone receptor negativity were observed in tumors with the highest expression of COX2. pCR rates were significantly higher in COX2-overexpressing tumors in

patients receiving celecoxib. The test for interaction between COX2 gene expression and the celecoxib effect was statistically significant ($p < 0.01$), but was not retained in the multivariate analysis. *Conclusion:* COX2 overexpression is predictive of pCR in patients with celecoxib-treated tumors. The efficacy of celecoxib in breast cancer might be improved by quantification of COX2 gene expression.

Many human cancers exhibit elevated prostaglandin levels due to up-regulation of cyclo-oxygenase-2 (COX2), a key enzyme in eicosanoid biosynthesis. COX2 overexpression has been observed in different malignant tumors and especially in breast cancer (1). Experimental studies showed that COX2 overexpression and a related production of prostaglandins stimulates angiogenesis and proliferation, promotes cell invasion and development of metastases (2). Consequently, selective COX2 inhibitors such as celecoxib have been explored as therapeutic or chemopreventive agents in different settings (3-5).

The level of COX2 has been associated with poor outcomes in many tumor models and clinical studies (6-8). However, there is no consensus on the prognostic or predictive value of COX2 expression in invasive breast carcinoma (9-11). Very few studies addressed the neoadjuvant context and response to celecoxib associated to chemotherapy in primary or metastatic breast cancer (12).

This article is freely accessible online.

*These Authors contributed equally to this study.

Correspondence to: Dr. Patricia de Cremoux, Molecular Oncology Unit, Saint-Louis Hospital, 1 avenue Claude Vellefaux, 75010 Paris, France. Tel +33 142499388. e-mail: patricia.de-cremoux@aphp.fr

Key Words: Breast cancer, neoadjuvant, COX2 inhibitors, chemotherapy, predictive, COX2.

ANTICANCER RESEARCH 38: 1485-1490 (2018)

Table I. Patient and tumor characteristics.

Variable	n (%)
Age (years)	
<40	34 (22.3%)
40 to 49	66 (43.4%)
≥50	52 (34.2%)
Clinical tumor stage	
T2	88 (57.9%)
T3 and T4	64 (42.1%)
Clinical lymph node status*	
N0	57 (37.8%)
N1, N2, N3	94 (62.2%)
Histological subtype	
Ductal	127 (83.6%)
Lobular	15 (9.8%)
Other	10 (6.6%)
Elston-Ellis grade*	
I	13 (8.8%)
II	60 (40.8%)
III	74 (50.3%)
Lymphovascular invasion*	
No	128 (84.8%)
Yes	23 (15.2%)
ER	
Negative	54 (35.5%)
Positive	98 (64.5%)
PR*	
Negative	83 (55.7%)
Positive	66 (44.3%)
Triple-negative status	
Yes	53 (35.3%)
No	99(64.7%)
Celecoxib treatment (per protocol)	
No	89 (58.6%)
Yes	63 (41.4%)
pCR	
No	132 (86.8%)
Yes	20 (13.2%)

ER: Estrogen receptor; PR: progesterone receptor; pCR: pathological complete response. *Missing data: lymph node status, n=1; grade, n=5; lymphovascular invasion, n=1; and PR, n=3.

One could expect to obtain better results with a better selection of patients receiving celecoxib. We present here data obtained in the human epidermal growth factor receptor 2 (*HER2*)-negative arm of the prospective neoadjuvant randomized phase II trial, REMAGUS-02 (R02), suggesting that the quantification of *COX2* gene transcripts predicts the pathological response to neoadjuvant celecoxib associated with chemotherapy including anthracyclines and taxanes.

Patients and Methods

Patients. The present study concerns a series of 220 breast cancer samples from patients included in the *HER2*-negative arm of the prospective randomized phase II trial REMAGUS-02 (R02) for

Table II. Tumor characteristics in the subgroups of tumors with low and high levels of cyclo-oxygenase-2 (*COX2*) mRNA expression.

Variable	COX2 mRNA expression		p-Value
	Low n (%)	High n (%)	
Age (years)			
<40	23 (22.5)	11 (22.0)	0.99
40 to 49	44 (43.1)	22 (44.0)	
≥50	35 (34.3)	17 (34.0)	
Clinical tumor stage			
T2	61 (59.8)	27 (54.0)	0.61
T3 and T4	41 (40.2)	23 (46.0)	
Clinical lymph node status*			
N0	36 (35.3)	21 (42.9)	0.47
N1, N2, N3	66 (64.7)	28 (57.1)	
Histological subtype			
Ductal	86 (84.3)	41 (82.0)	0.11
Lobular	12 (11.8)	3 (6.0)	
Other	4 (3.9)	6 (12.0)	
Elston-Ellis grade*			
1	11 (10.9)	2 (4.3)	0.01
2	48 (47.5)	12 (26.1)	
3	42 (41.6)	32 (69.6)	
Lymphovascular invasion*			
No	87 (86.1)	41 (82.0)	0.67
Yes	14 (13.9)	9 (18.0)	
ER			
Negative	23 (22.5)	31 (62.0)	<0.01
Positive	79 (77.5)	19 (38.0)	
PR*			
Negative	45 (45.0)	38 (77.6)	<0.01
Positive	55 (55.0)	11 (22.4)	
Triple-negative status			
Yes	22 (21.6)	31 (62.0)	<0.01
No	80 (78.4)	19 (38.0)	

ER: Estrogen receptor; PR: progesterone receptor; pCR: pathological complete response. *Missing data: lymph node status, n=1; grade, n=5; lymphovascular invasion, n=1; and PR, n=3. Significant data are indicated in bold.

patients with T2/T3/T4 advanced breast cancer. The patients were treated by sequential epirubicin/cyclophosphamide followed by docetaxel with or without the randomized addition of celecoxib (400 mg twice daily, orally) (arms A and B, respectively). Surgery was performed for 218 patients after eight cycle of chemotherapy, as previously described (13, 14). The primary objective of the trial was pathological complete response (pCR) evaluated according to Chevallier criteria (15). Secondary objectives were to define genomic profiles of success (pCR) or failure of each type of treatment and were published elsewhere together with quality control criteria (16-18).

The trial and ancillary studies were reviewed by the Ethics Committee of Bicêtre (CPP IDF VII), no. 03-55, 14th October 2004, in compliance with the Helsinki Declaration. All patients were informed and prospectively gave their signed consent to participate in the trial and ancillary studies,

Due to safety concerns, the use of celecoxib was suspended by the French Health Products Safety Agency (AFSSAPS) from

De Cremoux *et al.*: *COX2/PTGS2* Expression in HER2-negative BC PatientsTable III. Pathological response in patients as a function of tumor cyclo-oxygenase-2 (*COX2*) expression and celecoxib treatment.

Population	Celecoxib	<i>COX2</i> expression*	n	pCR, n (%)	OR 95% CI	p-Value
Whole population (n=152)	No	-	89	10 (11.2%)	1	0.407
	Yes	-	63	10 (15.9%)	1.49 (0.57-3.87)	
Whole population (n=152)	-	Low level	102	7 (6.9%)	1	0.002
	-	High level	50	13 (26%)	4.77 (1.81-13.58)	
No celecoxib (n=89)	-	Low level	58	6 (10.3%)	1	0.716
	-	High level	31	4 (12.9%)	1.28 (0.31-4.89)	
Celecoxib (n=63)	-	Low level	44	1 (2.3%)	1	0.001
	-	High level	19	9 (47.4%)	38.7 (6.27-757)	

pCR: Pathological complete response; OR: odds ratio; CI: confidence interval. *Low level=tertiles 1 and 2; high level=tertile 3. Significant data are indicated in bold.

December 2004 to September 2005 and thereafter authorized with revision of the informed consent form (13, 14). Thirteen patients randomized to receive celecoxib did not receive it. Consequently, the analyses were performed in the per protocol population.

Of the cases, 152 samples out of the 218 patients were available for reverse transcription and quantitative polymerase chain reaction (RT-qPCR) analysis on the basis of RNA of high quality from frozen pretreatment biopsies with more than 30% invasive epithelial tumor cells. There was no difference between the 152 patients with RT-qPCR data and the remaining 66 patients of the *HER2*-negative arm regarding age, menopausal status, clinical tumor size or nodal involvement, and hormone receptor status (data not shown).

Tissue samples and real-time RT-qPCR analysis. Total RNA extraction from frozen pretreatment biopsies, reverse transcription and qPCR analysis conditions and quality controls were previously described in detail (16-18). Primer and probe sequences for *COX2* mRNA expression are available on request. Large ribosomal protein (*RPLPO*), TATA box-binding protein (*TBP*), transferrin receptor (*TFR*), beta-actin (*ACTB*), beta-glucuronidase (*GUS*), and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) were used as endogenous reference genes. *COX2* mRNA levels were normalized to the median of the six reference genes.

Statistical analysis. As no consensual threshold was defined for RT-qPCR analyses and to ensure the robustness of the results, *COX2* gene expression was arbitrarily split according to tertiles (low, intermediate and high). As the magnitude of odds ratios for pCR of the two lower tertiles (tertiles 1 and 2) was similar, we chose to group these two tertiles and analyze them (low and intermediate expression) versus the third one (tumors with the highest *COX2* mRNA levels).

To analyze the association between clinical, pathological, *COX2* mRNA and pCR data, we performed a univariate analysis using the chi-square test and a univariate logistic regression model to estimate odds ratios (OR) and their 95% confidence intervals.

Association between *COX2* expression (tertile 1&2 versus tertile 3) and pCR were performed.

Analyses were performed with R software, version 3.1.2 (R Development Core Team, 2011).

Results

Patient population. The characteristics of the population of 152 patients are described in Table I. Patients and tumor characteristics were similar in patients treated with and without celecoxib. However, the tumor characteristics were significantly different in subgroups with low (n=102) and high (n=50) *COX2* gene expression level (Table II). We observed higher rates of grade 3 (69.6% versus 41.6%, $p=0.01$), ER-negative (62.0% versus 22.5%, $p<0.01$) and PR-negative (77.6% versus 45.0%, $p<0.01$) tumors in the population with the highest expression of *COX2* when compared with the lower tertiles (Table II).

Pathological response. A pCR was observed in 20/152 patients (13.2%). Considering the whole population, no effect of celecoxib was observed on tumor pCR (15.9% in celecoxib-treated patients vs. 11.2% in those without celecoxib; $p=0.41$) (Table III). However, the pCR rate was higher in the group of patients with highest tertile of *COX2* in the overall population and in patients receiving celecoxib ($p=0.002$) (Table III).

Taking into account the level of *COX2* expression in patients who received celecoxib, the pCR rate was significantly higher in those with tumors with the highest tertile of *COX2* (47.4%) than in those with low expression of *COX2* (2.3%) (Table III). The magnitude of the OR for pCR in the group of patients who received celecoxib suggested an interaction between *COX2* gene expression and the effect of celecoxib. The test for interaction was statistically significant ($p<0.01$), meaning that the effect of celecoxib on pCR was significantly different according to *COX2* gene expression. On the contrary, in the arm without celecoxib, no difference in pCR rate was observed according to *COX2* expression (Table III). However, after multivariate analysis, the interaction between celecoxib and *COX2*

expression failed to reach statistical significance ($p=0.12$), and only initial tumor size (T3 and T4 *versus* T2: OR=0.14, 95% CI=0.03-0.5, $p=0.006$) and ER status (OR=0.03, 95% CI=0-0.12, $p<0.001$) were significantly associated with pCR.

Discussion

In this biologically-driven analysis of the *HER2*-negative arm of the breast cancer neoadjuvant REMAGUS 02 trial, we found that *COX2* expression analyzed by RT-qPCR could be a target for celecoxib treatment. The effect of celecoxib in addition to neoadjuvant chemotherapy was different according to *COX2* expression level in patients with *HER2*-negative breast cancer in terms of pCR. To our knowledge, this phase II neoadjuvant trial is the first to investigate in a prospective randomized trial the efficacy of the selective *COX2* inhibitor celecoxib in addition to chemotherapy according to *COX2* gene expression in breast cancer.

The main finding of the current study was that patients with high *COX2* gene expression who received celecoxib had a significantly higher pCR rate compared with patients with low *COX2* gene expression. It was previously shown in 42 patients with metastatic breast cancer patients treated with anthracyclines with and without taxanes, that the combination of celecoxib with capecitabine was more effective in patients overexpressing *COX2* (19). Our results are also supported by interesting data published on lung cancer. Edelman *et al.* re-analyzed the negative results of the CALGB 30203 trial in advanced non-small cell lung cancer (20). Their analysis of the *COX2* expression data indicate that the benefit of 400 mg celecoxib twice per day was greatest in those with tumors with a higher level of *COX2* expression (20, 21).

Regarding the relationship between *COX2* and other tumor characteristics, we observed a positive correlation of *COX2* overexpression with higher tumor grade, as observed in the recent retrospective Chilkman's study. Their study was designed for a subgroup of 303 high-grade breast cancers and they analyzed *COX2* expression by immunohistochemistry (9). In contrast, they did not find any correlation with ER expression and only eight out of 18 studies cited in their article found an inverse correlation between ER and *COX2* expression as observed in our study (9). Furthermore, in a recent study performed on a retrospective cohort of 446 breast carcinomas treated in the adjuvant setting where *COX2* expression was analyzed by RT-qPCR, the authors found an inverse correlation between *COX2* expression and ER and PR expression (22).

A recent meta-analysis including 21 studies and 6739 patients with breast cancer showed that the presence of high levels of *COX2* predicts a greater tumor size and lymph node metastasis (11). The occurrence of *COX2*-overexpressing tumors in each study ranged from 27.9% to 81.4%. As illustrated by this meta-analysis (11), most published studies on prognostic or predictive value of *COX2* were performed

using immunohistochemistry. But the methods used for immunohistochemical analysis of *COX2* were diverse, with various antibodies, lack of standardization of staining, and analysis of tumor *COX2* expression. Classifications of *COX2* positivity and negativity differed significantly between published studies, which make comparisons between studies difficult. Recent studies pointed out several problems related to this method and explained discrepancies between studies. Urban *et al.* pointed out the importance of taking into account the stromal component of the tumor and not only epithelial cells, as is usually done (23). Only a few studies used RT-qPCR to assess *COX2* expression (22, 24, 25). The main advantage of RT-qPCR is its truly quantitative approach but there is still no standardized method. However, this could be adapted, since to date, new standardized tests using quantification of target genes by RT-qPCR are available for routine use of a molecular signature for luminal breast cancer (26, 27). However, a recent publication showed a good correlation between *COX2* expression analyzed by immunohistochemistry and by RT-qPCR (22).

We conclude that *COX2* expression in breast cancer is associated with histological type and grade, and inversely correlated with ER and PR expression. We also showed that higher *COX2* expression is associated with an increase in pCR rate in patients treated with celecoxib. Taken together, our findings support the fact that drug trials using celecoxib should include pre-stratification by *COX2* status. The efficacy of *COX2* inhibitors in combination with chemotherapy might benefit from the quantitative evaluation of the target as a predictive biomarker (companion diagnosis). These results need to be confirmed in independent prospective study.

In this article, we included the essential elements of the Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK) (28).

Ethics Approval and Consent to Participation

Remagus 02 trial: "A randomized phase II study of cytotoxic chemotherapy or cytotoxic chemotherapy combined with celecoxib or trastuzumab as primary chemotherapy for patients with high risk localized breast cancer not amenable to breast conserving therapy." was reviewed by the Ethic Committee of Bicêtre (CPP IDF VII), no. 03-55 14th October 2004.

Competing Interests

None of the Authors have any competing interest in regard to this study.

Acknowledgements

This work was supported by Academic Grants from the French Ministry of Health (French Programme Hospitalier de Recherche Clinique ISRCTN10059974, PHRC: AOM/2002/0211) and

Industrial Grants from Pfizer Inc., Roche, Sanofi-Aventis ISRCTN100599. A.S. Hamy was supported by an ITMO-INSERM-AVIESAN cancer translational research grant.

The Authors thank K. Tran-Perennou, C. Barbaroux and S. Vacher for their helpful technical contribution, and Dr. O. Tembo for clinical study monitoring. The Authors thank all the participants of the REMAGUS 02 trial.

References

- Singh-Ranger G, Salhab M and Mokbel K: The role of cyclo-oxygenase-2 in breast cancer: review. *Breast Cancer Res Treat* 109: 189-198, 2008.
- Howe LR: Inflammation and breast cancer Cyclo-oxygenase/prostaglandin signaling and breast cancer. *Breast Cancer Res* 9: 210, 2007.
- Evans JF and Kargman SL: Cancer and cyclo-oxygenase-2 (COX2) inhibition. *Curr Pharm Des* 10: 627-634, 2004.
- Menter DG, Schilsky RL and DuBois RN: Cyclo-oxygenase-2 and cancer treatment: understanding the risk should be worth the reward. *Clin Cancer Res* 16: 1384-1390, 2010.
- Regulski M, Regulska K, Prukała W, Piotrowska H, Stanisław B and Murias M: COX2 inhibitors: a novel strategy in the management of breast cancer. *Drug Discov Today* 21: 598-615, 2016.
- Ristimäki A, Sivula A, Lundin J, Lundin M, Salminen T, Haglund C, Joensuu H and Isola J: Prognostic significance of elevated cyclo-oxygenase-2 expression in breast cancer. *Cancer Res* 62: 632-635, 2002.
- Denkert C, Winzer KJ, Müller BM, Weichert W, Pest S, Köbel M, Kristiansen G, Reles A, Siegert A, Guski H and Hauptmann S: Elevated expression of cyclo-oxygenase-2 is a negative prognostic factor for disease free survival and overall survival in patients with breast carcinoma. *Cancer* 97: 2978-2987, 2003.
- Kim HS, Moon HG, Han W, Yom CK, Kim WH, Kim JH and Noh DY: COX2 overexpression is a prognostic marker for Stage III breast cancer. *Breast Cancer Res Treat* 132: 51-59, 2012.
- Chikman B, Vasyanovich S, Lavy R, Habler L, Tolstov G, Kapiev A, Halevy A and Sandbank J: COX2 expression in high-grade breast cancer: evidence for prognostic significance in the subset of triple-negative breast cancer patients. *Med Oncol* 31: 989, 2014.
- Simonsson M, Björner S, Markkula A, Nodin B, Jirstrom K, Rose C, Borgquist S, Ingvar C and Jernstrom H: The prognostic impact of COX2 expression in breast cancer depends on oral contraceptive history, preoperative NSAID use, and tumor size. *Int J Cancer* 140: 163-175, 2017.
- Xu F, Li M, Zhang C, Cui J, Liu J, Li J and Jiang H: Clinicopathological and prognostic significance of COX2 immunohistochemical expression in breast cancer: a meta-analysis. *Oncotarget* 8: 6003-6012, 2017.
- Chuah BY, Putti T, Salto-Tellez M, Charlton A, Iau P, Buhari SA, Wong CI, Tan SH, Wong AL, Chan CW, Goh BC and Lee SC: Serial changes in the expression of breast cancer-related proteins in response to neoadjuvant chemotherapy. *Ann Oncol* 22: 1748-1754, 2011.
- Pierga JY, Delaloge S, Espié M, Brain E, Sigal-Zafrani B, Mathieu MC, Bertheau P, Guinebretière JM, Spielmann M, Savignoni A and Marty M: A multicenter randomized phase II study of sequential epirubicin/cyclophosphamide followed by docetaxel with or without celecoxib or trastuzumab according to HER2 status, as primary chemotherapy for localized invasive breast cancer patients. *Breast Cancer Res Treat* 122: 429-437, 2010.
- Giacchetti S, Hamy AS, Delaloge S, Brain E, Berger F, Sigal-Zafrani B, Mathieu MC, Bertheau P, Guinebretière JM, Saghatchian M, Lerebours F, Mazouni C, Tembo O, Espié M, Reyat F, Marty M, Asselain B and Pierga JY: Long-term outcome of the REMAGUS 02 trial, a multicenter randomised phase II trial in locally advanced breast cancer patients treated with neoadjuvant chemotherapy with or without celecoxib or trastuzumab according to HER2 status. *Eur J Cancer* 75: 323-332, 2017.
- Chevallier B, Roche H, Olivier JP, Chollet P and Hurloup P: Inflammatory breast cancer Pilot study of intensive induction chemotherapy (FEC-HD) results in a high histologic response rate. *Am J Clin Oncol* 16: 223-228, 1993.
- de Cremoux P, Valet F, Gentien D, Lehmann-Che J, Scott V, Tran-Perennou C, Barbaroux C, Servant N, Vacher S, Sigal-Zafrani B, Mathieu MC, Bertheau P, Guinebretière JM, Asselain B, Marty M and Spyratos F: Importance of pre-analytical steps for transcriptome and RT-qPCR analyses in the context of the phase II randomised multicentre trial REMAGUS02 of neoadjuvant chemotherapy in breast cancer patients. *BMC Cancer* 11: 215, 2011.
- Valet F, de Cremoux P, Spyratos F, Servant N, Dujaric ME, Gentien D, Lehmann-Che J, Scott V, Sigal-Zafrani B, Mathieu MC, Bertheau P, Guinebretière JM, Pierga JY, Delaloge S, Giacchetti S, Brain E, Tembo O, Marty M and Asselain B: Challenging single- and multi-probesets gene expression signatures of pathological complete response to neoadjuvant chemotherapy in breast cancer: experience of the REMAGUS 02 phase II trial. *Breast* 22: 1052-1059, 2013.
- Hamy AS, Bieche I, Lehmann-Che J, Scott V, Bertheau P, Guinebretière JM, Matthieu MC, Sigal-Zafrani B, Tembo O, Marty M, Asselain B, Spyratos F and de Cremoux P: BIRC5 (survivin): a pejorative prognostic marker in stage II/III breast cancer with no response to neoadjuvant chemotherapy. *Breast Cancer Res Treat* 159: 499-511, 2016.
- Fabi A, Metro G, Papaldo P, Mottolese M, Melucci E, Carlini P, Sperduti I, Russillo M, Gelibter A, Ferretti G, Tomao S, Milella M and Cognetti F: Impact of celecoxib on capecitabine tolerability and activity in pretreated metastatic breast cancer: results of a phase II study with biomarker evaluation. *Cancer Chemother Pharmacol* 62: 717-725, 2008.
- Edelman MJ, Watson D, Wang X, Morrison C, Kratzke RA, Jewell S, Hodgson L, Mauer AM, Gajra A, Masters GA, Bedor M, Vokes EE and Green MJ: Eicosanoid modulation in advanced lung cancer: cyclo-oxygenase-2 expression is a positive predictive factor for celecoxib + chemotherapy—Cancer and Leukemia Group B Trial 30203. *J Clin Oncol* 26: 848-55, 2008.
- Edelman MJ, Wang X, Hodgson L, Watson D, Cheney RT, Baggstrom MQ, Sachdev T, Gajra A, Bertino EM, Reckamp KL, Molina J, Schiller J, Mitchell-Edwards K, Friedman P, Ritter J, Milne G, Stinchcombe TE, Hahn O and Vokes EE: Phase III randomized, placebo-controlled, double blind trial of celecoxib in addition to standard chemotherapy for advanced non-small-cell lung cancer with cyclo-oxygenase-2 overexpression: CALGB 30801 *J Clin Oncol* 35: 2184-2192, 2017.

- 22 Tury S, Becette V, Assayag F, Vacher S, Benoist C, Kamal M, Marangoni E, Bièche I, Lerebours F and Callens C: Combination of COX2 expression and PIK3CA mutation as prognostic and predictive markers for celecoxib treatment in breast cancer. *Oncotarget* 7: 85124-85141, 2016.
- 23 Urban J, Kuźbicki Ł, Szatkowski G, Stanek-Widera A, Lange D, and Chwirot BW: Stromal, rather than epithelial cyclo-oxygenase-2 (COX2) expression is associated with overall survival of breast cancer patients. *BMC Cancer* 14: 732, 2014.
- 24 Boneberg EM, Legler DF, Senn HJ and Fürstenberger G: Reduced expression of cyclo-oxygenase-2 in primary breast cancer. *J Natl Cancer Inst* 100: 1042-1043, 2008.
- 25 Fahlén M, Zhang H, Löfgren L, Masironi B, von Schoultz E, von Schoultz B and Sahlin L: Expression of cyclo-oxygenase-1 and cyclo-oxygenase-2, syndecan-1 and connective tissue growth factor in benign and malignant breast tissue from premenopausal women. *Gynecol Endocrinol* 33: 353-358, 2017.
- 26 Filipits M, Rudas M, Jakesz R, Dubsy P, Fitzal F, Singer CF, Dietze O, Greil R, Jelen A, Sevela P, Freibauer C, Müller V, Jänicke F, Schmidt M, Kölbl H, Rody A, Kaufmann M, Schroth W, Brauch H, Schwab M, Fritz P, Weber KE, Feder IS, Hennig G, Kronenwett R, Gehrman M, Gnant M and EP Investigators: A new molecular predictor of distant recurrence in ER-positive, HER2-negative breast cancer adds independent information to conventional clinical risk factors. *Clin Cancer Res* 17: 6012-20, 2011.
- 27 Nielsen T, Wallden B, Schaper C, Ferree S, Liu S, Gao D, Barry G, Dowidar N, Maysuria M and Storhoff J: Analytical validation of the PAM50-based Prosigna Breast Cancer Prognostic Gene Signature Assay and nCounter Analysis System using formalin-fixed paraffin-embedded breast tumor specimens. *BMC Cancer* 14: 177, 2014.
- 28 McShane LM, Altman DG, Sauerbrei W, Taube SE, Gion M, and Clark GM: REporting recommendations for tumor MARKer prognostic studies (REMARK). *Nat Clin Pract Uro* 2: 416-422, 2005.

Received December 5, 2017

Revised January 10, 2018

Accepted January 16, 2018

original report

Celecoxib With Neoadjuvant Chemotherapy for Breast Cancer Might Worsen Outcomes Differentially by COX-2 Expression and ER Status: Exploratory Analysis of the REMAGUS02 Trial

Anne-Sophie Hamy, MD¹; Sandrine Tury, PhD¹; Xiaofei Wang, PhD²; Junheng Gao, MS²; Jean-Yves Pierga, MD, PhD, Pr¹; Sylvie Giacchetti, MD³; Etienne Brain, MD, PhD¹; Barbara Pistilli, MD⁴; Michel Marty, MD, PhD, Pr³; Marc Espié, MD³; Gabriel Benchimol, MD¹; Enora Laas, MD¹; Marick Laë, MD¹; Bernard Asselain, MD, PhD¹; Brice Aouchiche, MPharm¹; Martin Edelman, MD⁵; and Fabien Reyat, PhD, Pr¹

abstract

PURPOSE The overexpression of cyclooxygenase 2 (COX-2) gene, also known as prostaglandin-endoperoxide synthase 2 (*PTGS2*), occurs in breast cancer, but whether it affects response to anticox drugs remains unclear. We investigated the relationships between *PTGS2* expression, celecoxib use during neoadjuvant chemotherapy (NAC), and both event-free survival (EFS) and overall survival (OS).

MATERIALS AND METHODS We analyzed a cohort of 156 patients with human epidermal growth factor receptor 2–negative breast cancer from the REMAGUS02 (ISRCTN Registry No. 10059974) trial with pretreatment *PTGS2* expression data. Patients were treated by sequential NAC (epirubicin plus cyclophosphamide followed by docetaxel with or without celecoxib). Experimental validation was performed on breast cancer cell lines. The Cancer and Leukemia Group B (CALGB) 30801 (ClinicalTrials.gov identifier: NCT01041781) trial that tested chemotherapy with or without celecoxib in patients with lung cancer served as an independent validation cohort.

RESULTS After 94.5 months of follow-up, EFS was significantly lower in the celecoxib group (hazard ratio [HR], 1.7; 95% CI, 1 to 2.88; $P = .046$). A significant interaction between *PTGS2* expression and celecoxib use was detected ($P_{\text{interaction}} = .01$). In the *PTGS2*-low group ($n = 100$), EFS was lower in the celecoxib arm (HR, 3.01; 95% CI, 1.45 to 6.24; $P = .002$) than in the standard treatment arm. Celecoxib use was an independent predictor of poor EFS, distant relapse-free survival, and OS.

Celecoxib in addition to docetaxel enhanced cell viability in *PTGS2*-low cell lines but not in *PTGS2*-high cell lines. In CALGB 30801, a trend toward poorer progression-free survival was observed in the patients with low urinary metabolite of prostaglandin E2 who received celecoxib (HR = 1.57; 95% CI, 0.87 to 2.84; $P = .13$).

CONCLUSION Celecoxib use during chemotherapy adversely affected survival in patients with breast cancer, and the effect was more marked in *PTGS2*-low and/or estrogen receptor–negative tumors. COX-2 inhibitors should preferably be avoided during docetaxel use in patients with breast cancer who are undergoing NAC.

J Clin Oncol 37. © 2019 by American Society of Clinical Oncology

INTRODUCTION

Cyclooxygenase-2 (COX-2; also known as *PTGS2* [prostaglandin-endoperoxide synthase 2]) is an isoform of the key enzyme in eicosanoid biosynthesis *PTGS*, which catalyzes the rate-limiting step in prostaglandin synthesis. COX-2 overexpression has been observed in various malignant tumors, including lung,¹ colon,² and breast^{3,4} cancers. Preclinical studies have shown that COX-2 overexpression and the resulting production of prostaglandins stimulated angiogenesis and proliferation, which promoted cell invasion and metastasis development.^{5,6} High COX-2 levels are associated with poor outcome in many tumor models and clinical studies.⁷⁻⁹ However, there is no consensus

about the prognostic or predictive value of COX-2 expression in invasive breast carcinoma.¹⁰⁻¹²

The selective COX-2 inhibitor celecoxib was released onto the market in 2000 for the symptomatic treatment of arthritis. Celecoxib binds reversibly to a hydrophilic pocket near the active site of COX-2 and thus inhibits the conversion of arachidonic acid to prostaglandin H₂. This results in anti-inflammatory and pain-relieving effects. Selective COX-2 inhibitors have also been explored as therapeutic or preventive agents in various oncologic settings.^{13,14} Several studies have evaluated celecoxib in the neoadjuvant setting for breast cancer as a monotherapy^{15,16} or combined with endocrine therapy.^{17,18} In addition to toxicity and safety

ASSOCIATED CONTENT

Appendix

Data Supplement

Author affiliations and support information (if applicable) appear at the end of this article.

Accepted on December 3, 2018 and published at [jco.org](https://doi.org/10.1200/JCO.18.00636) on January 31, 2019; DOI <https://doi.org/10.1200/JCO.18.00636>

A.-S.H. was a part of the ANR-10-IDEX-0001-02 PSL, the ANR-11-LABX-0043, and the INCa-DGOS-Inserm 12554 units.

ASCO

Journal of Clinical Oncology®

1

concerns, the benefits of such strategies to patients with breast cancer were not sufficiently high for these agents to be incorporated into standard care, and the development of COX-2 inhibitors in oncology thus fell short of initial expectations.^{19,20}

The REMAGUSO2 (ISRCTN Registry No. 10059974) study was a multicenter, randomized, phase II trial that included 340 patients with locally advanced breast cancer. Patients were randomly assigned to receive neoadjuvant sequential chemotherapy (NAC; either epirubicin plus cyclophosphamide, followed by docetaxel alone or docetaxel plus celecoxib [400 mg twice per day orally] for human epidermal growth factor receptor 2 (*HER2*)-negative tumors [n = 220]; or docetaxel alone or docetaxel plus trastuzumab for *HER2*-positive tumors [n = 120]). The trial found no benefit of celecoxib in terms of pathologic complete response²¹ (primary objective) or disease-free survival²² (DFS; secondary objective).

Predictive biomarkers are biologic indicators of the likely response of a patient to a particular drug. Estrogen receptor (ER), progesterone receptor, and *HER2* status, which are used to determine the potential benefits of endocrine and trastuzumab treatments, are currently the only predictive markers used in clinical settings in breast cancer. However, many patients still do not respond to these therapies, and the identification of additional biomarkers to provide personalized treatment to population subgroups remains an important task in breast oncology.

In this study, we investigated the dependence of the effects of celecoxib on COX-2 expression by performing a post hoc exploratory analysis of the REMAGUSO2 trial to evaluate survival as a function of *PTGS2* expression, as assessed by reverse transcription quantitative polymerase chain reaction (RT-qPCR). We validated our findings experimentally on breast cancer cell lines, and we performed analyses in an independent cohort of patients with non-small-cell lung cancer (NSCLC) from the Cancer and Leukemia Group B (CALGB) 30801 (ClinicalTrials.gov identifier: NCT01041781).

MATERIALS AND METHODS

Patients

In total, 220 patients with locally advanced breast cancer were included in the *HER2*-negative stratum of the REMAGUSO2 phase II randomized trial. The patients received sequential chemotherapy with, first, epirubicin plus cyclophosphamide alone followed by docetaxel with or without celecoxib 400 mg administered twice per day orally with random assignment to arm 1 (without celecoxib) or arm 2 (with celecoxib), as previously described.^{21,22} The full protocol (REMAGUSO2 protocol; Appendix, online only), CONSORT diagram (Appendix Fig A1, online only), and results of the clinical trial (REMAGUSO2 trial; Appendix) are provided. The use of celecoxib was suspended by the French Health Products Safety Agency from December 2004 to September 2005 because of safety concerns.

Thereafter, the use of this agent was authorized but with a revision of the informed consent form. As a result, 13 patients randomly assigned to the celecoxib group did not receive this drug. Analyses of the results of this study were performed on an intention-to-treat basis and per-protocol analyses are provided in the Appendix. For the 220 patients who were randomly assigned, 156 (71%) had frozen pretreatment biopsy specimens that contained more than 30% invasive epithelial tumor cells and that were available for RT-qPCR analysis (raw data in Data Supplement). Among them, 139 patients had Affymetrix U133A chips (Thermo Fisher Scientific, Waltham, MA) with baseline gene expression data available (standard treatment, n = 72; celecoxib, n = 67).

PTGS2 (COX-2) Expression

Total RNA extraction from frozen pretreatment biopsy specimens, reverse transcription, and qPCR analysis and quality control were performed as previously described.^{23,24} The *RPLPO*, TATA box-binding protein (*TBP*), transferrin receptor (*TFR*), beta-actin, beta-glucuronidase (*GUS*), and *GAPDH* genes were used as endogenous reference genes. Target quantities were normalized relative to the median value for the six reference genes. No consensus threshold has been defined for RT-qPCR analyses, so *PTGS2* gene expression was classified on the basis of tertiles (low, intermediate, and high). The odds ratios (ORs) for pathologic complete response of tertiles 1 (OR, 1; four [7.7%] of 52); and 2 (OR, 0.77; three [6%] of 50) were essentially similar (*v* OR, 4.22; 13 [26%] of 50 for tertile 3), so we chose to merge those two tertiles (*PTGS2*-low) and compare them with the third tertile (*PTGS2*-high), as previously described.²⁴

Statistical Analysis

To investigate if tumors were different between the celecoxib and noncelecoxib arms, we performed a differential expression analysis between the two groups of treatment (Appendix). Event-free survival (EFS) was defined as the time from random assignment to progression, locoregional recurrence, distant recurrence, or death, whichever occurred first. Distant relapse-free survival (DRFS) was defined as the time from random assignment to first distant metastasis or death; overall survival (OS) was defined as the time from random assignment to death. Patients for whom none of these events was recorded were censored at the date of last known contact. The cutoff date for the analysis was May 1, 2015. Predictive effects were evaluated with a test of interaction between treatment group and *PTGS2* expression and ER status. EFS and OS were estimated using the Kaplan-Meier method, and survival curves were compared using a log-rank test. Univariable Cox proportional hazard models were performed to determine the variables associated with survival. Covariables selected for the multivariable analysis were those with *P* values no greater than .15 after univariable analysis. A multivariable

TABLE 1. Patient and Tumor Characteristics at Baseline by Treatment Arm in the Intention-to-Treat Population

Characteristic	Standard Treatment Arm (n = 78)	Celecoxib Arm (n = 78)	P
Age, years			
< 40	17 (21.8)	17 (21.8)	.55
40 to 49	30 (38.5)	36 (46.2)	
≥ 50	31 (39.7)	25 (32.1)	
Menopausal status			
Pre	51 (66.2)	55 (70.5)	.69
Post	26 (33.8)	23 (29.5)	
Mean BMI, kg/m ²	25.8 (5.0)	24.5 (4.7)	.09
Tumor size			
T2	40 (51.3)	49 (62.8)	.2
T3 and T4	38 (48.7)	29 (37.2)	
Clinical nodal status			
N0	30 (39.0)	29 (37.2)	.95
N1, N2, N3	47 (61.0)	49 (62.8)	
Histology			
Ductal	67 (85.9)	64 (82.1)	.77
Lobular	7 (9.0)	8 (1.3)	
Other	4 (5.1)	6 (7.7)	
Grade			
1	9 (12.2)	5 (6.6)	.22
2	25 (33.8)	35 (46.1)	
3	40 (54.1)	36 (47.4)	
LVI			
No	67 (85.9)	64 (83.1)	.8
Yes	11 (14.1)	13 (16.9)	
ER status			
Negative	27 (34.6)	29 (37.2)	.87
Positive	51 (65.4)	49 (62.8)	
PR status			
Negative	42 (54.5)	44 (57.9)	.8
Positive	35 (45.5)	32 (42.1)	
TNBC			
Yes	26 (33.3)	29 (37.2)	.74
No	52 (66.7)	49 (62.8)	
p53			
WT	20 (54.1)	24 (61.5)	.67
Mutated	17 (45.9)	15 (38.5)	
Surgery			
No	2 (2.6)	2 (2.6)	.99
Yes	76 (97.4)	76 (97.4)	
Adjuvant chemotherapy			
No	54 (69.2)	55 (70.5)	.99
Yes	24 (3.8)	23 (29.5)	

(continued in next column)

TABLE 1. Patient and Tumor Characteristics at Baseline by Treatment Arm in the Intention-to-Treat Population (continued)

Characteristic	Standard Treatment Arm (n = 78)	Celecoxib Arm (n = 78)	P
Endocrine therapy			
No	23 (31.1)	25 (34.2)	.82
Yes	51 (68.9)	48 (65.8)	
Radiotherapy			
No	1 (1.3)	3 (4.1)	.59
Yes	74 (98.7)	70 (95.9)	

NOTE. Data are presented as No. (%). The following data are missing: menopausal status (n = 1), BMI (n = 1), clinical nodal status (n = 1), grade (n = 6), LVI (n = 1), PR (n = 3), p53 (n = 80), endocrine therapy (n = 9), radiotherapy (n = 8), and pCR (n = 4).

Abbreviations: BMI, body mass index; ER, estrogen receptor; LVI, lymphovascular invasion; PR, progesterone receptor; TNBC, triple-negative breast cancer; WT, wild type.

model was then implemented using a forward stepwise selection procedure. Analyses were performed with R software, version 3.1.2.

Experimental Validation and Independent Human Validation Cohort

We performed an experimental validation on two *PTGS2*-low breast cancer cell lines (MDA-MB-231 and MDA-MB-157), and two *PTGS2*-high cell lines (BT549 and MDA-MB-436; Appendix). Cell lines were treated with increasing concentrations of docetaxel with or without celecoxib 25 μ M. Cellular viability was assessed at 72 hours. Statistical analyses were performed using GraphPad Prism 5 software (GraphPad Software, San Diego, CA). The data were expressed as the mean and standard error of the mean (SEM). One-way analyses of variance followed by Bonferroni post hoc comparison tests were performed in all statistical analyses. The results were considered statistically significant at a $P < .05$, $P < .01$, or $P < .001$. To confirm our results, we also performed a post hoc reanalysis of the CALGB 30801 trial,²⁵ in which 312 patients with advanced NSCLC were randomly assigned to receive celecoxib or placebo in addition to standard chemotherapy. We stratified the analyses by the expression levels of the urinary after they were stratified by the expression levels of the urinary metabolite of prostaglandin E2 (PGE-M; Appendix).

RESULTS

Analyses of the REMAGUS02 Trial

Patient population. In total, 156 patients from the REMAGUS02 trial were included in this study; 78 were randomly assigned to the celecoxib arm, and 78 were randomly assigned to the arm with standard treatment only. Patient and tumor baseline characteristics were similar in the celecoxib and standard treatment arms (Table 1). In addition, no gene of 19,965 was differentially expressed

TABLE 2. EFS and OS HRs by Celecoxib Use for the Whole Study Population, the ER-Negative Subpopulation, and the ER-Positive Subpopulation of the Intention-to-Treat Analyses

Survival by Population	Whole Population (n = 156)				ER Negative (n = 56)				ER Positive (n = 100)						
	No. of Patients	No. of Events	HR	95% CI	<i>P</i> _{Log-Rank}	No. of Patients	No. of Events	HR	95% CI	<i>P</i> _{Log-Rank}	No. of Patients	No. of Events	HR	95% CI	<i>P</i> _{Log-Rank}
EFS															
Whole population															
Standard treatment arm	77	23	1		.046	27	7	1		.027	50	16	1		.523
Celecoxib arm	78	35	1.7	1 to 2.88		29	15	2.69	1.08 to 6.71		49	20	1.24	0.64 to 2.39	
<i>PTGS2</i> low (n = 104)															
Standard treatment arm	49	10	1		.002	12	1	1		.002	37	9	1		.121
Celecoxib arm	54	27	3.01	1.45 to 6.24		12	9	13.45	1.68 to 107.44		42	18	1.87	0.84 to 4.16	
<i>PTGS2</i> high (n = 52)															
Standard treatment arm	28	13	1		.52	15	6	1		.971	13	7	1		.331
Celecoxib arm	24	8	0.75	0.3 to 1.83		17	6	0.98	0.32 to 3.04		7	2	0.46	0.09 to 2.29	
OS															
Whole population															
Standard treatment arm	77	14	1		0.108	27	6	1		.027	50	8	1		.97
Celecoxib arm	78	23	1.71	0.88 to 3.33		29	14	2.84	1.08 to 7.47		49	9	1.02	0.39 to 2.64	
<i>PTGS2</i> low (n = 104)															
Standard treatment arm	49	5	1		.012	12	1	1		.001	37	4	1		.434
Celecoxib arm	54	17	3.32	1.23 to 9.01		12	9	13.64	1.71 to 108.87		42	8	1.61	0.48 to 5.35	
<i>PTGS2</i> high (n = 52)															
Standard treatment arm	28	9	1		.668	15	5	1		.931	13	4	1		.38
Celecoxib arm	24	6	0.8	0.28 to 2.24		17	5	0.95	0.27 to 3.27		7	1	0.39	0.04 to 3.48	

Abbreviations: EFS, event-free survival; ER, estrogen receptor; HR, hazard ratio; OS, overall survival.

between the celecoxib arm and the standard treatment arm, consistent with the random allocation of patients to the celecoxib arm.

Notable differences in tumor characteristics according to *PTGS2* status were observed. The frequencies of grade III, *p53*-mutated, ER-negative and progesterone receptor-negative tumors were higher in the *PTGS2*-high population than in the *PTGS2*-low population (Appendix Table A1, online only).

The Effect of Celecoxib on Survival is Modified by *PTGS2* Expression and ER Status

EFS analysis. In the full study cohort of patients with *HER2*-negative disease (n = 156), celecoxib use was significantly associated with shorter EFS (hazard ratio [HR], 1.7; 95%

CI, 1 to 2.88; $P = .046$; Table 2). There was a significant interaction between *PTGS2* expression and celecoxib for EFS ($P_{\text{interaction}} = .01$), which meant that the effect of celecoxib on EFS differed significantly between the *PTGS2*-low and *PTGS2*-high groups.

In the *PTGS2*-low group, celecoxib use was associated with shorter EFS (HR, 3.01; 95% CI, 1.45 to 6.24; $P = .002$; Fig 1A), and the obtained results differed by ER status. In ER-negative tumors, celecoxib use was strongly associated with shorter EFS (HR, 13.45; 95% CI, 1.68 to 107.44; $P = .002$; Fig 1B), whereas celecoxib had no effect on EFS in ER-positive tumors (HR, 1.87; 95% CI, 0.84 to 4.16; $P = .121$; $P_{\text{interaction}} = .02$; Fig 1C). In the *PTGS2*-high group, celecoxib use did not affect EFS (Fig 1D) in either the ER-negative (Fig 1E) or ER-positive (Fig 1F) population.

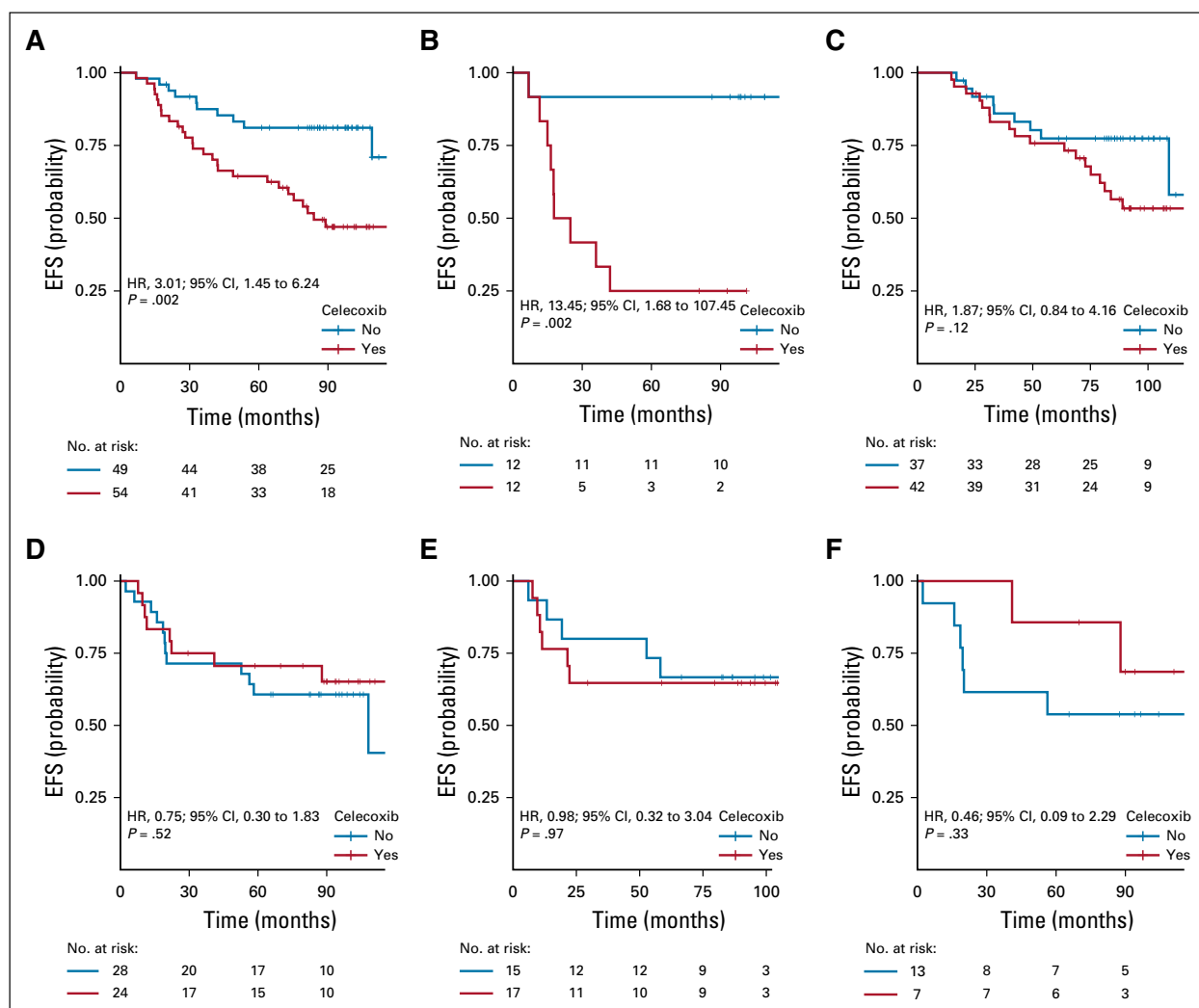


FIG 1. Kaplan-Meier curves for association between treatment arm and event-free survival (EFS), according to *PTGS2* and estrogen receptor (ER) status: (A) *PTGS2*-low population; (B) *PTGS2*-low/ER-negative subpopulation; (C) *PTGS2*-low/ER-positive subpopulation; (D) *PTGS2*-high population; (E) *PTGS2*-high/ER-negative subpopulation; and (F) *PTGS2*-high/ER-positive subpopulation. HR, hazard ratio.

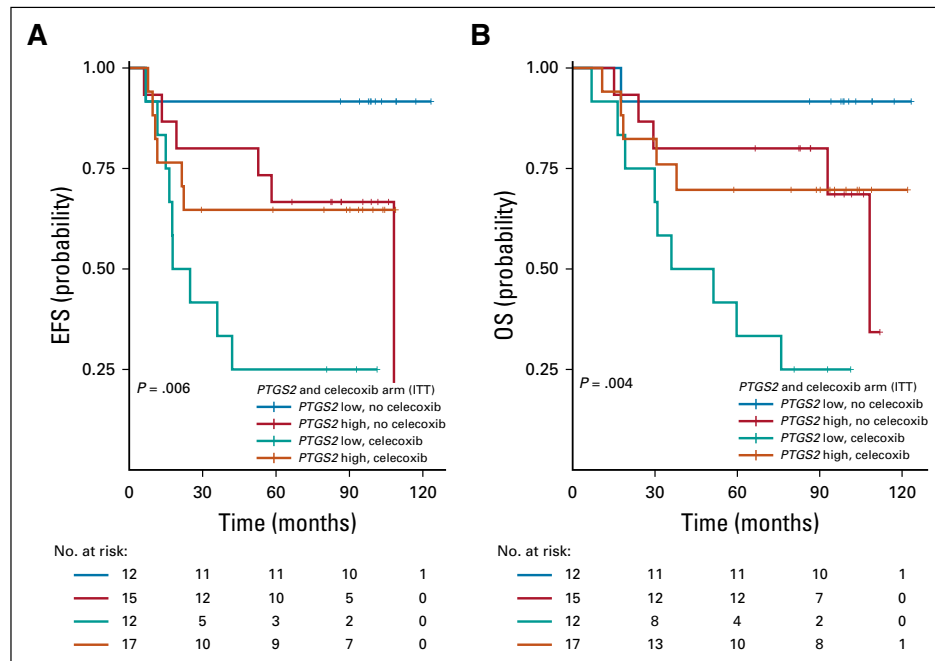


FIG 2. Kaplan-Meier combined survival curves for the association between *PTGS2* expression and treatment arm in the estrogen receptor (ER)-negative population. (A) Event-free survival (EFS) by *PTGS2* expression and celecoxib use; (B) overall survival (OS) by *PTGS2* expression and celecoxib use. ITT, intention to treat.

The association between celecoxib use and impaired EFS ($P < .001$), the interactions between celecoxib use and *PTGS2* expression ($P = .008$), and the interactions between celecoxib use and ER status ($P = .005$) were highly significant after multivariable analysis (Appendix Table A2, online only). Similar results were also found for DRFS (data not shown).

OS analyses. Similar results were obtained for OS (Table 2). In the *PTGS2*-low group, celecoxib use was associated with a shorter OS (HR, 3.32; 95% CI, 1.23 to 9.01; $P = .012$; Appendix Fig A2A, online only), and its effects differed according to ER status ($P_{\text{interaction}} = .05$). Celecoxib use was associated with a shorter OS in ER-negative tumors (HR, 13.64; 95% CI, 1.71 to 108.87; $P = .001$; Appendix Fig A2B) but had no significant effect on OS in ER-positive tumors (HR, 1.61; 95% CI, 0.48 to 5.35; $P = .434$; Appendix Fig A2C).

In the *PTGS2*-high group, celecoxib use had no effect on OS (Appendix Fig A2D) in the ER-negative population (Appendix Fig A2E) or in the ER-positive population (Appendix Fig A2F).

The association between celecoxib use and impaired OS ($P = .001$), the interactions between celecoxib use and *PTGS2* expression ($P = .03$), and the interactions between celecoxib use and ER status ($P = .02$) were again significant after multivariable analysis (Appendix Table A3, online only). The combined Kaplan-Meier curves for EFS and OS

as a function of *PTGS2* expression and celecoxib use are shown for ER-negative tumors in Figure 2.

Per-protocol analyses. Analyses of this study on a per-protocol basis showed comparable results that are provided in the Appendix (Appendix Table A4, online only; Appendix Figs A3, A4, and A5, online only).

Experimental validation. The addition of celecoxib to docetaxel enhances cell viability in *PTGS2*-low but not in *PTGS2*-high breast cancer cell lines. To assess whether preclinical models would mimic the clinical observations, we performed translational research by studying a panel of four ER-negative and *HER2*-negative breast cancer cell lines. *PTGS2* expression was very low in MDA-MB-231 and MDA-MB-157, whereas it was high in BT549 and MDA-MB-436 (Appendix Fig A6, online only). In all four triple-negative breast cancer cell lines, celecoxib alone (5 to 200 μM) had no effect on cellular viability (data not shown).

In the *PTGS2*-low cell lines (MDA-MB-231 and MDA-MB-157), addition of celecoxib enhanced cellular viability compared with docetaxel treatment alone (Figs 3A and 3B). In *PTGS2*-high cell lines (BT549 and MDA-MB-436), celecoxib in association with docetaxel had no effect on cellular viability (Figs 3C and 3D). These cell culture results therefore match the clinical observations and suggest the following: (1) The effect of celecoxib in addition to chemotherapy varies with the expression levels of *PTGS2*, and this effect is restricted to *PTGS2*-low cell lines. (2) In

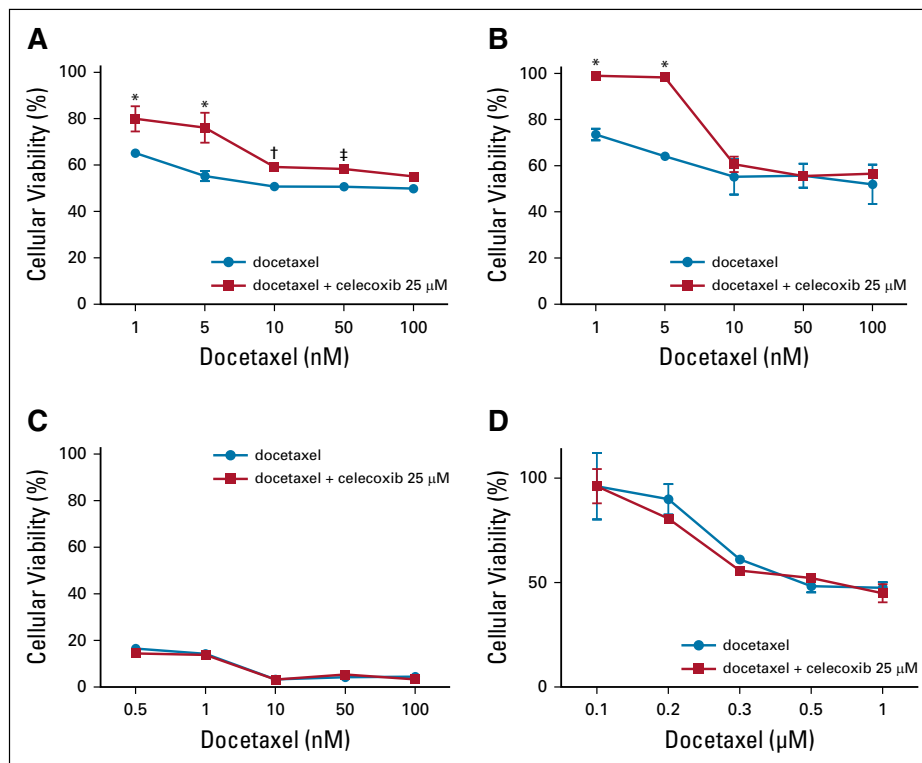


FIG 3. Effect of docetaxel alone or in combination with celecoxib on cellular viability in *PTGS2*-low cell lines (A) MDA-MB-231 and (B) MDA-MB-157 as well as *PTGS2*-high cell lines (C) BT549 and (D) MDA-MB-436. (*) $P < .001$; (†) $P < .01$; (‡) $P < .05$.

PTGS2-low cell lines, the addition of celecoxib to taxanes enhances cellular viability compared with taxanes alone.

Analyses of the CALGB 30801 trial. The effect of celecoxib in addition to chemotherapy is associated with a trend toward an impaired progression-free survival in patients with NSCLC who have low values of PGE-M.

In the population of the CALGB 30801 trial with metabolite of prostaglandin E2 (PGE-M) data available, the addition of celecoxib to chemotherapy had no impact on PFS (celecoxib v no celecoxib: HR, 1.08; 95% CI, 0.85 to 1.36; $P = .53$). In the population with PGE-M values less than quartile 1 (Q1), celecoxib in addition to chemotherapy was associated with a trend toward impaired progression-free survival (PFS) compared with chemotherapy alone (HR, 1.57; 95% CI, 0.87 to 2.84; $P = .13$). In contrast, for the population with PGE-M values of Q1 or greater, the addition of celecoxib to chemotherapy was not associated with differences in PFS (HR, 0.91; 95% CI, 0.66 to 1.26; $P = .57$; Appendix Figs A7A and A7B, respectively, online only).

DISCUSSION

In this exploratory analysis of the REMAGUS02 trial, we report an adverse effect of celecoxib use during NAC on survival in patients with breast cancer. The magnitude of this effect was greater in patients with either *PTGS2*-low tumors or ER-negative tumors, and it was particularly dramatic in the subgroup of patients with ER-negative and

PTGS2-low tumors. One might have expected COX-2 inhibitors to act preferentially on tumors cells that express COX-2. Instead, we identified a paradoxical effect on cells with a low expression of *PTGS2*. The clinical observation was reproduced experimentally by performing translational research in four different breast cancer cell lines. Importantly, this effect was observed only in combination with taxanes and not with celecoxib alone. These results are particularly important because despite the evidence of a potential protective effect of nonsteroidal anti-inflammatory drugs (NSAIDs) against breast cancer in preclinical and epidemiologic data, no randomized trial, to our knowledge, has investigated the addition of any NSAID to NAC in breast cancer. Previous or unpublished randomized trials have been designed using celecoxib alone,²⁶ but evidence is still lacking for the effects of celecoxib in addition to NAC in humans.²⁷ We also found a trend toward a similar effect in an independent cohort derived from a randomized clinical trial, in a different setting, and in another cancer localization (advanced NSCLC).

These results raise concerns about the safety of COX-2 inhibitors during chemotherapy in patients with breast cancer. They are consistent with a recent study¹¹ performed on a cohort of 911 patients with breast cancer, which identified an interaction among COX-2 expression, prognosis, and preoperative NSAID use ($P_{\text{interaction}} = .009$). In that study, patients with preoperative NSAID treatment

TABLE 3. Summary of Randomized Controlled Trials to Evaluate Combinations of COX-2 Inhibitors With Chemotherapy in Patients With Cancer

First Author	Setting and Cancer Type	COX-2 Assessment	No. of Patients in the Analyses	Conclusion of the Authors	Premature or Temporary Discontinuation	Primary End Point	Comments	Interaction/Stratification by COX-2 expression (when assessed)
Maiello ³⁰	First-line locally advanced and/or metastatic colorectal cancer	No	FOLFIRI (n = 38) or FOLFIRI + CBX (n = 39)	FOLFIRI regimen was effective and well tolerated as first-line treatment in patients with advanced colorectal cancer. The addition of CBX to the FOLFIRI regimen did not improve results.		ORR	The ORR was lower in the arm with combined CBX. ORR: FOLFIRI v FOLFIRI + CBX: 45% (95% CI, 29% to 61%) v 36% (95% CI, 21% to 51%)	No
Kohne ³¹	First-line metastatic colorectal cancer	No	FOLFIRI (n = 41) or CAPIRI (n = 44) with CBX (n = 42) or placebo (n = 43)	Because of the small sample size after early termination, no definitive conclusions could be drawn in relation to the noninferiority of CAPIRI compared with FOLFIRI.	Yes	PFS	Median PFS and OS times were shorter for CAPIRI v FOLFIRI (PFS: 5.9 v 9.6 months; OS: 14.8 v 19.9 months) and CBX v placebo (PFS: 6.9 v 7.8 months; OS: 18.3 v 19.9 months).	Assumptions of an absence of interaction between FU v capecitabine and CBX v placebo effects.
Jin ³⁵	First-line metastatic colorectal cancer	Yes (IHC)	FOLFOX4 (n = 30) v FOLFOX4 + CBX (n = 58)	The addition of CBX to the FOLFOX4 regimen increased the short-term efficacy and the 3-year survival rate.		Not reported	RR (CR + PR) was significantly greater in the group with FOLFOX4 + CBX than in the group with FOLFOX4 (<i>P</i> = .022)	No stratification
Lilenbaum ³²	Second-line treatment of stage IIIB or IV NSCLC	No	Irinotecan docetaxel (n = 69) + irinotecan gemcitabine (n = 64) with CBX (n = 67) or without CBX (n = 66)	CBX did not seem to enhance efficacy or improve patient-reported symptoms.	Yes	Median/1-year survival probabilities	Median survival of patients was higher with chemotherapy alone v with CBX: 8.99 months (95% CI, 6.60 to 11.14 months) v 6.31 months (95% CI, 4.53 to 8.57 months).	Study design assumed no interaction between chemotherapy treatment and use of CBX.
Gridelli ³⁶	First-line treatment stage IIIB or IV NSCLC	No	Gemcitabine IV (n = 200) or PCI + cisplatin (n = 200) with rofecoxib (n = 149) or without rofecoxib (n = 251)	Rofecoxib improved RR but did not prolong survival. The trial was closed prematurely because of safety issues.	Yes	OS		The study was not planned to test efficacy interactions in the experimental factors.
Edelman ²⁸	First-line treatment stage IIIB or IV NSCLC	Yes (IHC, n = 83)	Carboplatin + gemcitabine + CBX (n = 44) + zileuton (n = 45) + CBX + zileuton (n = 45)	This study failed to demonstrate the value of dual eicosanoid inhibition or benefit from either agent alone in addition to chemotherapy.		9-month failure-free survival	CBX treatment associated with a trend toward worse OS outcome (HR, 1.59; 95% CI, 0.85 to 2.96; <i>P</i> = .15) after multivariable analysis.	Interaction of receiving CBX and COX-2 expression on OS (<i>P</i> = .0026); analyses stratified by COX-2 expression
Groen ³⁴	First-line treatment stage IIIB or IV NSCLC	Yes (31%)	Docetaxel carboplatin with CBX (n = 281) or placebo (n = 280)	In advanced NSCLC, CBX did not improve survival.		OS		Interaction between COX-2 expression and the impact on CBX/placebo treatment was tested but was not significant. Analyses were stratified by COX-2 expression.

(continued on following page)

TABLE 3. Summary of Randomized Controlled Trials to Evaluate Combinations of COX-2 Inhibitors With Chemotherapy in Patients With Cancer (continued)

First Author	Setting and Cancer Type	COX-2 Assessment	No. of Patients in the Analyses	Conclusion of the Authors	Premature or Temporary Discontinuation	Primary End Point	Comments	Interaction/Stratification by COX-2 expression (when assessed)
Koch ³⁷	First-line treatment stage IIIB or IV NSCLC	No	Palliative chemotherapy with CBX (n = 158) or placebo (n = 158)	This study failed to demonstrate a survival benefit of the addition of CBX to palliative chemotherapy.	Yes	OS	In women, survival was shorter with placebo than with CBX (HR, 1.16; 95% CI, 0.83 to 1.62), whereas the opposite was observed in men (HR, 0.79; 95% CI, 0.57 to 1.09).	No
Edelman ²⁹	Second-line treatment stage IIIB or IV NSCLC	Yes, baseline urinary PGE-M	Docetaxel or pemetrexed with apricoxib (n = 36) or placebo (n = 36)	Apricoxib did not improve PFS, despite biomarker-driven patient selection.		PFS	Patients who received docetaxel + apricoxib (n = 17) had a numerically inferior median PFS of 75 days (95% CI, 47 to 104 days) v 97 days (95% CI, 48 to 216 days) for those who received docetaxel + placebo (n = 20; HR, 1.62; P = .18)	Interaction between baseline PGE-M and chemotherapeutic agents (docetaxel v pemetrexed) for PFS (P = .026).
Edelman ²⁵	Second-line treatment stage IIIB or IV NSCLC	Yes (n = 312; COX-2 IHC and urinary PGE-M)	Carboplatin pemetrexed + gemcitabine with CBX (n = 154) or with placebo (n = 158)	COX-2 expression by IHC failed to select patients who could benefit from selective COX-2 inhibition.	Yes	PFS	Complementary analyses (unpublished, performed for this study): In patients with PGE-M values < Q1 (n = 53), there was a trend toward impaired PFS with CBX compared with CT alone (HR, 1.57; 95% CI, 0.87 to 2.84; P = .13).	Interaction between treatment effect (CBX v placebo) and baseline urinary PGE-M level significant for OS (P = .02) but not for PFS (P = .22)
Reyners ³³	First-line stage IC to IV ovarian cancer	Yes (61%; n = 120)	Carboplatin docetaxel ± CBX	CBX did not influence PFS and OS, but interpretation of results was hampered by premature CBX discontinuation.	Yes	RR and PFS	CBX use was associated with a trend toward worse PFS in the multivariable analysis (HR, 1.28; 95% CI, 0.90 to 1.81; P = .16).	No
This study	Neoadjuvant treatment of locally advanced breast cancers	Yes (PTGS2 RT-qPCR; n = 156)	Epirubicin cyclophosphamide followed by docetaxel (n = 78) + CBX (n = 78)	CBX was associated with impaired EFS (P = .05) and OS (P = .11), particularly in the PTGS2-low and the ER-negative groups.	Yes	pCR	CBX use associated with impaired EFS, metastasis-free survival, OS after multivariable analysis	Significant interactions between PTGS2 expression and CBX use (P = .008) and ER status and CBX use (P = .005) on EFS

Abbreviations: CAPIRI, irinotecan plus capecitabine; CBX, celecoxib; COX-2, cyclooxygenase 2; CR, complete response; EFS, event-free survival; ER, estrogen receptor; FOLFIRI, fluorouracil, leucovorin, and irinotecan; FOLFOX4, folinic acid–fluorouracil–oxaliplatin; FU, fluorouracil; HR, hazard ratio; IHC, immunohistochemistry; IV, intravenously; NSCLC, non–small-cell lung cancer; ORR, objective response rate; OS, overall survival; PCI, prolonged constant infusion; pCR, pathologic complete response; PFS, progression-free survival; PGE-M, prostaglandin E₂ metabolite; PR, partial response; Q1, quartile 1; RR, response rate; RT-qPCR, reverse transcription quantitative polymerase chain reaction.

and COX-2-negative tumors had a significantly higher risk of events (HR, 4.51; $P < .001$) compared with the other patients.

Furthermore, several randomized trials that investigated multiple COX-2 inhibitors in addition to chemotherapy for the treatment of different cancers have reported interactions^{25,28,29} among COX-2 inhibitor use, COX

expression, chemotherapy regimen, and clinical outcome. The findings of these previous studies are listed in Table 3.²⁸⁻³⁷ In eight of these studies^{25,28-33} (including this study), COX-2 inhibitor use during chemotherapy was, or tended to be, associated with a poorer outcome than no COX-2 inhibitor use. Of note, seven trials were temporarily or prematurely discontinued because of safety concerns

or enrollment failure, which may partially explain underpowered definitive analyses. Although several regimens were used, evidence that the combination of COX-2 inhibitor with chemotherapy might be detrimental was reported in four trials (including this study) that evaluated taxane-based chemotherapy regimens.^{29,32,33} Finally, only two of 12 randomized trials were stratified for COX-2 expression.^{28,34} The re-analysis of the 10 remaining trials after stratification by COX-2 expression could unmask a hidden deleterious or beneficial effects in specific subgroups.

This study has limitations. The REMAGUS02 trial was a phase II randomized trial that was only designed to assess the efficacy of celecoxib in the whole population, but analyses stratified by ER or *PTGS2* status were not prespecified. Hence, we cannot strictly infer causality for the negative association we report in the subpopulations. However, two arguments suggest that the relevance of these subgroup analyses is not spurious. First, the interactions between COX-2 inhibitors and COX-2 expression has already been demonstrated by multiple teams.^{25,28,29} Second, both ER status, which is a pivotal biomarker for any breast cancer trial, and *PTGS2* (COX-2 expression), which is the very target of the drug tested (ie, celecoxib), have a strong biologic rationale to justify these subgroup analyses. Finally, we cannot derive any information on the safety profile of celecoxib in *HER2*-positive tumors because of the design of the REMAGUS02 trial (none of the patients with *HER2*-positive disease received celecoxib). The safety data in *HER2*-positive tumors could have been informative, because Subbaramaiah et al³⁸ has reported that celecoxib can interrupt *HER2* downstream signaling.

This study also has several strengths. As the only randomized trial, to our knowledge, to assess celecoxib in association with NAC in patients with breast cancer, the results show independent, significant, negative associations with EFS, DRFS, and OS, after a long follow-up, both in

the intention-to-treat and the per-protocol analyses. A validation phase III trial specifically powered to confirm the deleterious impact of this drug in specified subgroups would be unethical. Thus, these data will remain unique for the foreseeable future. Finally, because there cannot and will not be a confirmatory trial to establish strict causality between celecoxib use during NAC for breast cancer and the risk of adverse outcome, physicians should apply caution and recommend alternatives to prescriptions of celecoxib in patients with ER-negative, *HER2*-negative breast cancer who are being treated with taxane-containing NAC.

This study has several implications: (1) Given the hypothesis-generating value of these findings, additional research should be performed and may include the post hoc reanalysis of randomized trials that evaluated COX-2 inhibitors in addition to chemotherapy after stratification by COX-2 expression. We also strongly recommend that investigators of clinical trials that evaluate COX-2 inhibitors should provide individual patient data that could be pooled into large meta-analyses. Such an effort is critical to reach robust evidence to derive routine recommendations about the avoidance or the safety of the routine prescription of COX-2 inhibitors during chemotherapy. (2) Evidence for synergy between COX inhibitors and checkpoint blockade immunotherapy is emerging.^{39,40} On the basis of our results, we recommend the stratification of all future trials that involve these inhibitors according to COX-2 expression status. (3) In the absence of other evidence, we recommend avoidance of celecoxib use and preference for alternative drugs in patients with ER-negative, *HER2*-negative breast tumors who are receiving docetaxel-containing NAC, unless the expected benefit greatly outweighs the potential risks. Only by carefully addressing these concerns will it be possible to determine the subgroups of patients most likely to benefit from COX inhibitors.

AFFILIATIONS

¹Institut Curie, Université Paris Descartes, Paris, France

²Alliance Statistics and Data Center, Durham, NC

³Hôpital Saint Louis (APHP), Université Paris Diderot, Paris, France

⁴Gustave Roussy, Villejuif, France

⁵Fox Chase Cancer Center, Philadelphia, PA

CORRESPONDING AUTHOR

Fabien Reyat, MD, PhD, Institut Curie, Department of Surgery, 26 rue d'Ulm, 75005 Paris, 00 33 615271980 France; Twitter: @fabienreyat, @institut_curie, @UParisDescartes, @ashampetit; e-mail: fabien.reyat@curie.fr.

SUPPORT

A.-S.H. was supported by an Institut Thématique Multi-Organisme Cancer (ITMO)-Institut National de la Santé et de la Recherche Médicale (INSERM)-Alliance pour les sciences de la vie et de la santé (AVIESAN) cancer translational research grant.

This study was supported by a grant of the French Programme Hospitalier de Recherche Clinique (No. ISRCTN10059974, PHRC: AOM 02 11) and by unrestricted grants from Pfizer France, Roche Pharmaceutical, and Sanofi.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST AND DATA AVAILABILITY STATEMENT

Disclosures provided by the authors and data availability statement (if applicable) are available with this article at DOI <https://doi.org/10.1200/JCO.18.00636>.

AUTHOR CONTRIBUTIONS

Conception and design: Anne-Sophie Hamy, Jean-Yves Pierga, Michel Marty, Bernard Asselain

Collection and assembly of data: Anne-Sophie Hamy, Sandrine Tury, Xiaofei Wang, Junheng Gao, Jean-Yves Pierga, Barbara Pistilli, Marc Espié, Brice Aouchiche

Provision of study material or patients: Jean-Yves Pierga, Sylvie Giacchetti, Etienne Brain, Michel Marty, Marick Laé, Fabien Reyat

Data analysis and interpretation: Anne-Sophie Hamy, Sandrine Tury, Xiaofei Wang, Junheng Gao, Jean-Yves Pierga, Sylvie Giacchetti, Etienne Brain, Gabriel Benchimol, Enora Laas, Marick Laé Bernard Asselain, Martin Edelman, Fabien Reyat

Manuscript writing: All authors

Final approval of manuscript: All authors

Accountable for all aspects of the work: All authors

REFERENCES

- Zhou YY, Hu ZG, Zeng FJ, et al: Clinical profile of cyclooxygenase-2 inhibitors in treating non-small cell lung cancer: A meta-analysis of nine randomized clinical trials. *PLoS One* 11: e0151939, 2016
- Arber N: Cyclooxygenase-2 inhibitors in colorectal cancer prevention: Point. *Cancer Epidemiol Biomarkers Prev* 17:1852-1857, 2008
- Singh-Ranger G, Salhab M, Mokbel K: The role of cyclooxygenase-2 in breast cancer: Review. *Breast Cancer Res Treat* 109:189-198, 2008
- Arun B, Goss P: The role of COX-2 inhibition in breast cancer treatment and prevention. *Semin Oncol* 31:22-29, 2004
- Howe LR: Inflammation and breast cancer: Cyclooxygenase/prostaglandin signaling and breast cancer. *Breast Cancer Res* 9:210, 2007
- Masferrer JL, Leahy KM, Koki AT, et al: Antiangiogenic and antitumor activities of cyclooxygenase-2 inhibitors. *Cancer Res* 60:1306-1311, 2000
- Denkert C, Winzer K-J, Müller B-M, et al: Elevated expression of cyclooxygenase-2 is a negative prognostic factor for disease-free survival and overall survival in patients with breast carcinoma. *Cancer* 97:2978-2987, 2003
- Kim HS, Moon H-G, Han W, et al: COX2 overexpression is a prognostic marker for stage III breast cancer. *Breast Cancer Res Treat* 132:51-59, 2012
- Ristimäki A, Sivula A, Lundin J, et al: Prognostic significance of elevated cyclooxygenase-2 expression in breast cancer. *Cancer Res* 62:632-635, 2002
- Chikman B, Vasyanovich S, Lavy R, et al: COX2 expression in high-grade breast cancer: Evidence for prognostic significance in the subset of triple-negative breast cancer patients. *Med Oncol* 31:989, 2014
- Simonsson M, Björner S, Markkula A, et al: The prognostic impact of COX-2 expression in breast cancer depends on oral contraceptive history, preoperative NSAID use, and tumor size. *Int J Cancer* 140:163-175, 2017
- Xu F, Li M, Zhang C, et al: Clinicopathological and prognostic significance of COX-2 immunohistochemical expression in breast cancer: A meta-analysis. *Oncotarget* 8:6003-6012, 2017
- Evans JF, Kargman SL: Cancer and cyclooxygenase-2 (COX-2) inhibition. *Curr Pharm Des* 10:627-634, 2004
- Regulski M, Regulska K, Prukala W, et al: COX-2 inhibitors: A novel strategy in the management of breast cancer. *Drug Discov Today*, 2015
- Tfayli A, Yang J, Kojouri K, et al: Neoadjuvant therapy with celecoxib to women with early-stage breast cancer. *Neoplasma* 55:122-126, 2008
- Aristarco V, Serrano D, Gandini S, et al: A randomized, placebo-controlled, phase II, presurgical biomarker trial of celecoxib versus exemestane in postmenopausal breast cancer patients. *Cancer Prev Res (Phila)* 9:349-356, 2016
- Lustberg MB, Povoski SP, Zhao W, et al: Phase II trial of neoadjuvant exemestane in combination with celecoxib in postmenopausal women who have breast cancer. *Clin Breast Cancer* 11:221-227, 2011
- Falandry C, Canney PA, Freyer G, et al: Role of combination therapy with aromatase and cyclooxygenase-2 inhibitors in patients with metastatic breast cancer. *Ann Oncol* 20:615-620, 2009
- Gasparini G, Longo R, Sarmiento R, et al: Inhibitors of cyclo-oxygenase 2: A new class of anticancer agents? *Lancet Oncol* 4:605-615, 2003
- Csiki I, Johnson DH: Did targeted therapy fail cyclooxygenase too? *J Clin Oncol* 24:4798-4800, 2006
- Pierga J-Y, Delaloue S, Espié M, et al: A multicenter randomized phase II study of sequential epirubicin/cyclophosphamide followed by docetaxel with or without celecoxib or trastuzumab according to *HER2* status, as primary chemotherapy for localized invasive breast cancer patients. *Breast Cancer Res Treat* 122:429-437, 2010
- Giacchetti S, Hamy A-S, Delaloue S, et al: Long-term outcome of the REMAGUS 02 trial, a multicenter randomised phase II trial in locally advanced breast cancer patients treated with neoadjuvant chemotherapy with or without celecoxib or trastuzumab according to *HER2* status. *Eur J Cancer* 75:323-332, 2017
- de Cremoux P, Valet F, Gentien D, et al: Importance of pre-analytical steps for transcriptome and RT-qPCR analyses in the context of the phase II randomised multicentre trial REMAGUS02 of neoadjuvant chemotherapy in breast cancer patients. *BMC Cancer* 11:215, 2011
- Hamy AS, Bieche I, Lehmann-Che J, et al: BIRC5 (survivin): A pejorative prognostic marker in stage III/IV breast cancer with no response to neoadjuvant chemotherapy. *Breast Cancer Res Treat* 159:499-511, 2016
- Edelman MJ, Wang X, Hodgson L, et al: Phase III randomized, placebo-controlled, double-blind trial of celecoxib in addition to standard chemotherapy for advanced non-small-cell lung cancer with cyclooxygenase-2 overexpression: CALGB 30801 (Alliance). *J Clin Oncol* 35:2184-2192, 2017
- Strasser-Weippl K, Higgins MJ, Chapman JW, et al: Effects of celecoxib and low-dose aspirin on outcomes in adjuvant aromatase inhibitor-treated patients: CCTG MA.27. *J Natl Cancer Inst* 110:1003-1008, 2018
- Hawk E, Maresso KC, Brown P: NSAIDs to prevent breast cancer recurrence? An unanswered question. *J Natl Cancer Inst* 110:927-928, 2018
- Edelman MJ, Watson D, Wang X, et al: Eicosanoid modulation in advanced lung cancer: Cyclooxygenase-2 expression is a positive predictive factor for celecoxib + chemotherapy—Cancer and Leukemia Group B Trial 30203. *J Clin Oncol* 26:848-855, 2008
- Edelman MJ, Tan MT, Fidler MJ, et al: Randomized, double-blind, placebo-controlled, multicenter phase II study of the efficacy and safety of aprioxicib in combination with either docetaxel or pemetrexed in patients with biomarker-selected non-small-cell lung cancer. *J Clin Oncol* 33:189-194, 2015
- Maiello E, Giuliani F, Gebbia V, et al: FOLFIRI with or without celecoxib in advanced colorectal cancer: A randomized phase II study of the Gruppo Oncologico dell'Italia Meridionale (GOIM). *Ann Oncol* 17:vii55-vii59, 2006
- Köhne C-H, De Greve J, Hartmann JT, et al: Irinotecan combined with infusional 5-fluorouracil/folinic acid or capecitabine plus celecoxib or placebo in the first-line treatment of patients with metastatic colorectal cancer: EORTC study 40015. *Ann Oncol* 19:920-926, 2008
- Lilenbaum R, Socinski MA, Altorki NK, et al: Randomized phase II trial of docetaxel/irinotecan and gemcitabine/irinotecan with or without celecoxib in the second-line treatment of non-small-cell lung cancer. *J Clin Oncol* 24:4825-4832, 2006
- Reyners AKL, de Munck L, Erdkamp FLG, et al: A randomized phase II study investigating the addition of the specific COX-2 inhibitor celecoxib to docetaxel plus carboplatin as first-line chemotherapy for stage IC to IV epithelial ovarian cancer, fallopian tube or primary peritoneal carcinomas: The DoCaCel study. *Ann Oncol* 23:2896-2902, 2012
- Groen HJM, Sietsma H, Vincent A, et al: Randomized, placebo-controlled phase III study of docetaxel plus carboplatin with celecoxib and cyclooxygenase-2 expression as a biomarker for patients with advanced non-small-cell lung cancer: The NVALT-4 study. *J Clin Oncol* 29:4320-4326, 2011

35. Jin C-H, Wang A-H, Chen J-M, et al: Observation of curative efficacy and prognosis following combination chemotherapy with celecoxib in the treatment of advanced colorectal cancer. *J Int Med Res* 39:2129-2140, 2011
 36. Gridelli C, Gallo C, Ceribelli A, et al: Factorial phase III randomised trial of rofecoxib and prolonged constant infusion of gemcitabine in advanced non-small-cell lung cancer: the GEmcitabine-COxib in NSCLC (GECO) study. *Lancet Oncol* 8:500-512, 2007
 37. Koch A, Bergman B, Holmberg E, et al: Effect of celecoxib on survival in patients with advanced non-small cell lung cancer: a double blind randomised clinical phase III trial (CYCLUS study) by the Swedish Lung Cancer Study Group. *Eur J Cancer Oxf Engl* 1990 47:1546-1555, 2011
 38. Subbaramaiah K, Norton L, Gerald W, et al: Cyclooxygenase-2 is overexpressed in *HER2/neu*-positive breast cancer: Evidence for involvement of AP-1 and PEA3. *J Biol Chem* 277:18649-18657, 2002
 39. Hennequart M, Pilotte L, Cane S, et al: Constitutive IDO1 expression in human tumors is driven by cyclooxygenase-2 and mediates intrinsic immune resistance. *Cancer Immunol Res* 5:695-709, 2017
 40. Zelenay S, van der Veen AG, Böttcher JP, et al: Cyclooxygenase-dependent tumor growth through evasion of immunity. *Cell* 162:1257-1270, 2015
-

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST**Celecoxib With Neoadjuvant Chemotherapy for Breast Cancer Might Worsen Outcomes Differentially by COX-2 Expression and ER Status: Exploratory Analysis of the REMAGUS02 Trial**

The following represents disclosure information provided by authors of this manuscript. All relationships are considered compensated. Relationships are self-held unless noted. I = Immediate Family Member, Inst = My Institution. Relationships may not relate to the subject matter of this manuscript. For more information about ASCO's conflict of interest policy, please refer to www.asco.org/twc or ascopubs.org/jco/site/ffc.

Jean-Yves Pierga

Honoraria: Amgen, Illumina

Consulting or Advisory Role: Roche, Novartis, Pfizer, Lilly, AstraZeneca, Puma Biotechnology, Celltrion, Sandoz, Ipsen

Research Funding: Roche (Inst), Servier (Inst), Janssen Diagnostics (Inst)

Travel, Accommodations, Expenses: MSD Oncology, Roche, AstraZeneca, Pfizer

Sylvie Giacchetti

Consulting or Advisory Role: Roche, Eisai

Travel, Accommodations, Expenses: Roche, AstraZeneca

Etienne Brain

Honoraria: AstraZeneca, Roche, Pfizer, Mylan, Bristol-Myers Squibb

Consulting or Advisory Role: Puma Biotechnology, Pfizer, Clinigen Group, Bristol-Myers Squibb, Samsung

Research Funding: HalioDx (Inst)

Travel, Accommodations, Expenses: Roche, Pfizer, Mylan, Pierre Fabre, AstraZeneca

Barbara Pistilli

Consulting or Advisory Role: Puma Biotechnology

Research Funding: Pfizer (Inst), Puma Biotechnology (Inst), Merus (Inst)

Michel Marty

Honoraria: Sanofi, Roche, Genentech, Pfizer, Pierre Fabre

Consulting or Advisory Role: Sanofi, Roche, Pfizer, Pierre Fabre

Travel, Accommodations, Expenses: Roche, Genentech

Bernard Asselain

Honoraria: Bristol-Myers Squibb, Roche, AstraZeneca, Ipsen

Consulting or Advisory Role: Bristol-Myers Squibb, Roche, AstraZeneca, Ipsen

Brice Aouchiche

Employment: AbbVie (I)

Martin Edelman

Stock and Other Ownership Interests: Biomarker Strategies

Consulting or Advisory Role: Lilly, Bristol-Myers Squibb, Boehringer Ingelheim, WindMIL, ARMO BioSciences, BerGenBio, Syndax

Research Funding: Bristol-Myers Squibb (Inst), Apexigen (Inst), Nektar (Inst)

Travel, Accommodations, Expenses: Bristol-Myers Squibb, ARMO BioSciences, BerGenBio

Other Relationship: AstraZeneca, MedImmune, Lilly, Boehringer Ingelheim, Takeda

Fabien Reyat

Honoraria: Agendia

Consulting or Advisory Role: Agendia

Travel, Accommodations, Expenses: Roche

No other potential conflicts of interest were reported.

APPENDIX

Analysis of the REMAGUS02 Trial

Patients. All of the patients included in this study were informed about the study in advance and gave written consent for participation in the trial and ancillary studies (ISRCTN Registry No. 10059974, French ethics committee Paris-Bicêtre, No. 03-55). The primary outcome measure of the trial was pathologic complete response (pCR), evaluated according to Chevallier criteria.²¹ The secondary outcome measures were the definition of genomic profiles of success (ie, pCR) or failure for each type of treatment, and these results have been published elsewhere, together with quality-control data.²³

Samples. In total, 156 samples from the 220 patients with breast cancer were available for transcriptomic analyses. The subgroup of 156 patients with available reverse transcription quantitative polymerase chain reaction (RT-qPCR) data did not differ from the remaining 64 patients of the *HER2*-negative population in terms of age, menopausal status, clinical tumor size, or nodal involvement. However, lobular and grade 1 and 2 tumors were overrepresented in the population without available transcriptome data relative to the population with available transcriptome available (26.6% v 9.6% [$P = .004$] and 58.3% v 49.3% [$P = .03$], respectively). Raw data for the patients are provided in the Data Supplement.

Statistical Analysis

Differential expression analysis. Of 156 patients with RT-qPCR available for *PTGS2* expression, 139 had Affymetrix U133A chips (Thermo Fisher Scientific, Waltham, MA) available for analysis. We performed a differential analysis by comparing the mean gene expression of each group according to treatment arm (celecoxib v no celecoxib) using a linear model (limgma R package) and retained as differentially expressed genes those for which the mean expression was different with a P value of .05 or lower. The analysis was performed in the whole population and after stratification by *PTGS2* status (*PTGS2*-low, $n = 93$; *PTGS2*-high, $n = 46$).

Experimental Validation

Cell lines. Human breast cancer cell lines BT-549, MDA-MB-436, MDA-MB-231, and MDA-MB-157 were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The cell lines were authenticated every 20 passages using the GenePrint 10 system kit (B9510; Promega, Madison, WI). All cell lines were cultured in RPMI-1640 medium or DMEM (Thermo Fisher Scientific, Waltham, MA) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific) and 1% antibiotics (penicillin 50 $\mu\text{g}/\text{mL}$, streptomycin 50 $\mu\text{g}/\text{mL}$, neomycin 100 $\mu\text{g}/\text{mL}$; Thermo Fisher Scientific), at 37°C in a humidified atmosphere that contained 5% CO_2 .

Drugs. Docetaxel was purchased from Téva laboratory (Courbevoie, France). Celecoxib was purchased from Biogaran laboratory (Colombes, France) and was dissolved in phosphate-buffered saline.

Viability assay. A total of 8,000 cells per well were seeded in P96 plates and allowed to adhere for 24 hours at 37°C. Cells were then treated with various concentrations of chemotherapeutic agents and/or celecoxib for 72 hours. Cellular proliferation was measured using the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay according to the manufacturer's instructions (Promega, Madison, WI). Absorbance was measured at

490 nm on a 96-well microplate reader (Dynatech Laboratories MRX, Chantilly, VA).

Experimental plan. We assessed the in vitro antitumor activity of celecoxib in combination with chemotherapeutic agents on the triple-negative breast cancer cell lines. MDA-MB-231 and MDA-MB-157 were defined by qRT-PCR as *PTGS2*-low cell lines; BT549 and MDA-MB-436, as *PTGS2*-high cell lines. To this end, we evaluated cellular viability under increasing concentrations of docetaxel in combination or not with celecoxib 25 μM .

Analyses of the Cancer and Leukemia Group P 30801

trial. To confirm our results, we performed a post hoc reanalysis of the Cancer and Leukemia Group B 30801 Alliance trial.²¹ In this trial, 312 patients with advanced non-small-cell lung cancer—stage IIIB with pleural effusion or stage IV—were randomly assigned to receive celecoxib or placebo in addition to standard chemotherapy. Only patients with a COX-2 index of two or greater were registered and randomly assigned to treatment. Urinary metabolite of prostaglandin E₂ (PGE₂), hereafter designated PGE-M, was evaluated at the baseline and on day 8 of the first cycle in 211 patients in the study. Patients were evenly divided into four groups (quartiles) that were based on the quantity of urinary PGE-M at baseline (Q1, 10.09; Q2, 15.38; and Q3, 27.86 ng/mg creatinine). Progression-free survival was analyzed according to celecoxib addition, and the Q1 cutoff was used for PGE-M (PGE-M < Q1 v PGE-M \geq Q1). Kaplan-Meier curves were used for survival analysis, and the log-rank test was used to assess differences in progression-free survival between PGE-M-defined patient groups.

Per-Protocol Analyses Results: Survival Analyses—The Effect of Celecoxib on Survival is Modified by *PTGS2* Expression and Estrogen Receptor Status

Event-free survival analysis. In the *PTGS2*-low group, celecoxib use was associated with poorer event-free survival (EFS; hazard ratio [HR], 1.96; 95% CI, 1.02 to 3.76; $P = .039$; 8-year EFS: 50.5% [95% CI, 37.3% to 68.4%] v 73.1% [95% CI, 62.3% to 85.8%]; Appendix Table A4, online only; Appendix Fig A3A, online only), but the obtained results differed according to ER status ($P_{\text{interaction}} = .011$). In ER-negative tumors, celecoxib use was associated with poor EFS (HR, 7.18; 95% CI, 1.5 to 34.3; $P = .004$, Appendix Fig A3B, online only), whereas it had no such effect on EFS in ER-positive tumors ($P = .65$; Appendix Fig A3C, online only). In the *PTGS2*-high group, celecoxib use was not associated with EFS (Appendix Fig A3D, online only), in either the ER-negative (Appendix Fig A3E, online only) or the ER-positive (Appendix Fig A3F, online only) population.

Overall survival analysis. Similar results were obtained for overall survival (OS; Appendix Table A3, online only; Appendix Figs A4A through A4F, online only). Celecoxib use was associated with poor OS in the *PTGS2*-low/ER-negative subgroup (HR, 6.81; 95% CI, 1.43 to 32.33; $P = .005$; 8-year OS, 27.3% [95% CI, 10.4% to 71.6%] v 84.6% [95% CI, 67.1% to 100%]; Appendix Fig A3B online only) but not in the *PTGS2*-low/ER-positive subgroup (HR, 0.81; 95% CI, 0.26 to 2.56; $P = .72$; $P_{\text{interaction(celecoxib/ER status)}} = .02$; Appendix Fig A4C online only). Finally, Appendix Figure A5 shows the Kaplan-Meier curves for EFS and OS according to *PTGS2* expression and the effect of celecoxib in ER-negative tumors in per-protocol analyses.

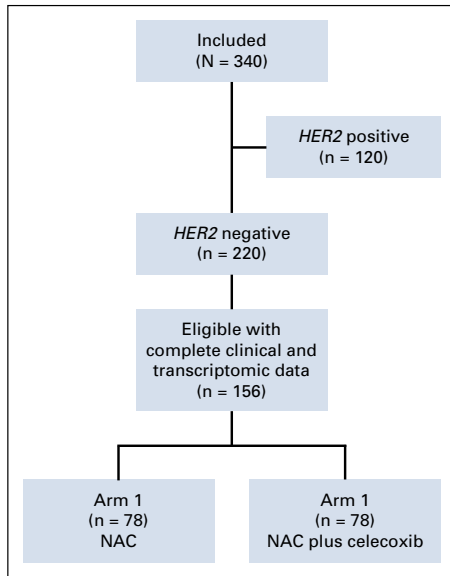


FIG A1. Study flow diagram of included patients and tumors samples available for reverse transcription quantitative polymerase chain reaction analysis in the REMAGUS02 (ISRCTN Registry No. 10059974) biologic trial. NAC (neoadjuvant chemotherapy [epirubicin + cyclophosphamide followed by docetaxel]); *HER2*, human epidermal growth factor receptor 2.

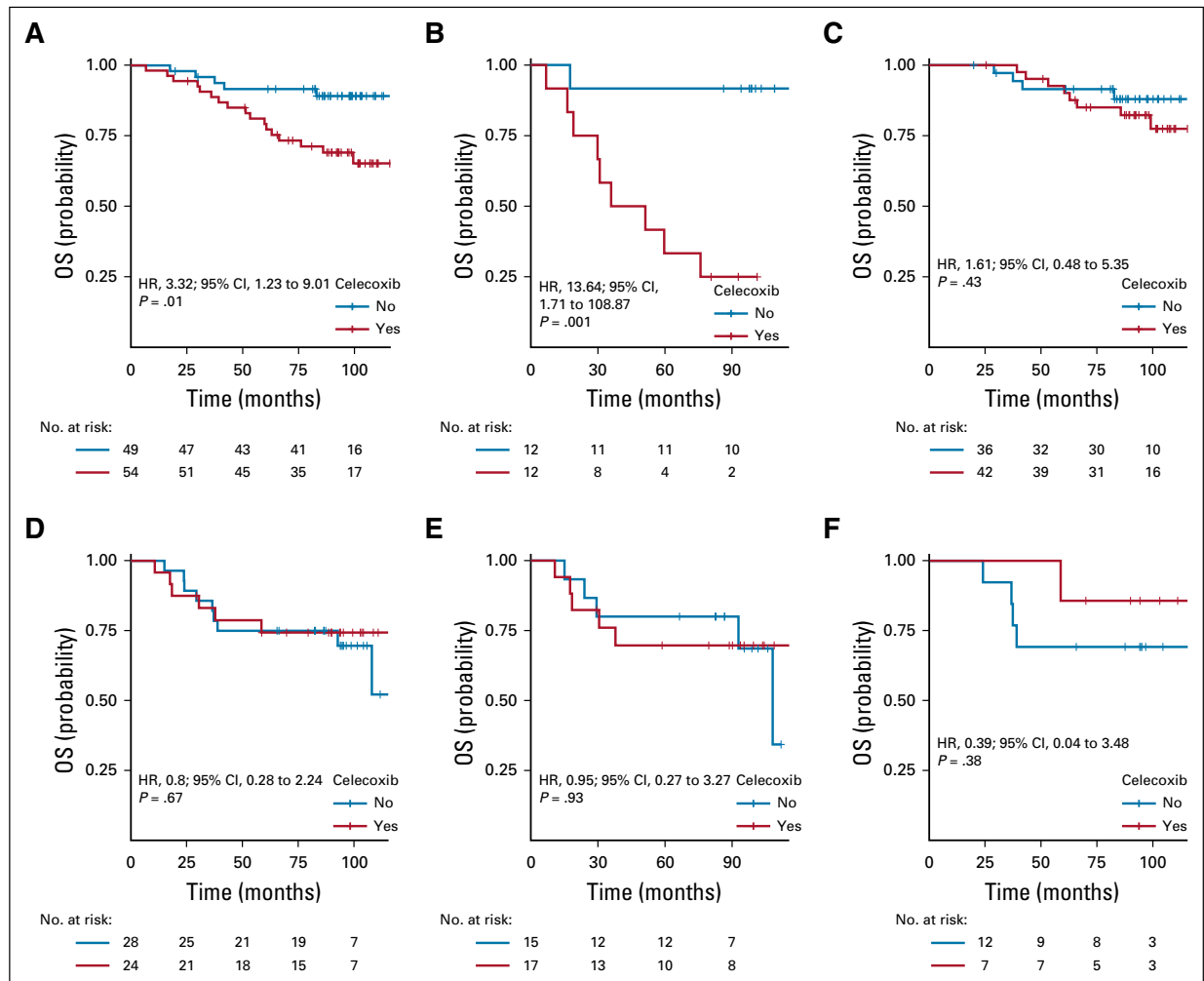


FIG A2. Kaplan-Meier curves for association between treatment arm (intention-to-treat analyses) and overall survival (OS) according to *PTGS2* expression and estrogen receptor (ER) status: (A) *PTGS2*-low population; (B) *PTGS2*-low/ER-negative subpopulation; (C) *PTGS2*-low/ER-positive subpopulation; (D) *PTGS2*-high population; (E) *PTGS2*-high/ER-negative subpopulation; and (F) *PTGS2*-high/ER-positive subpopulation. HR, hazard ratio.

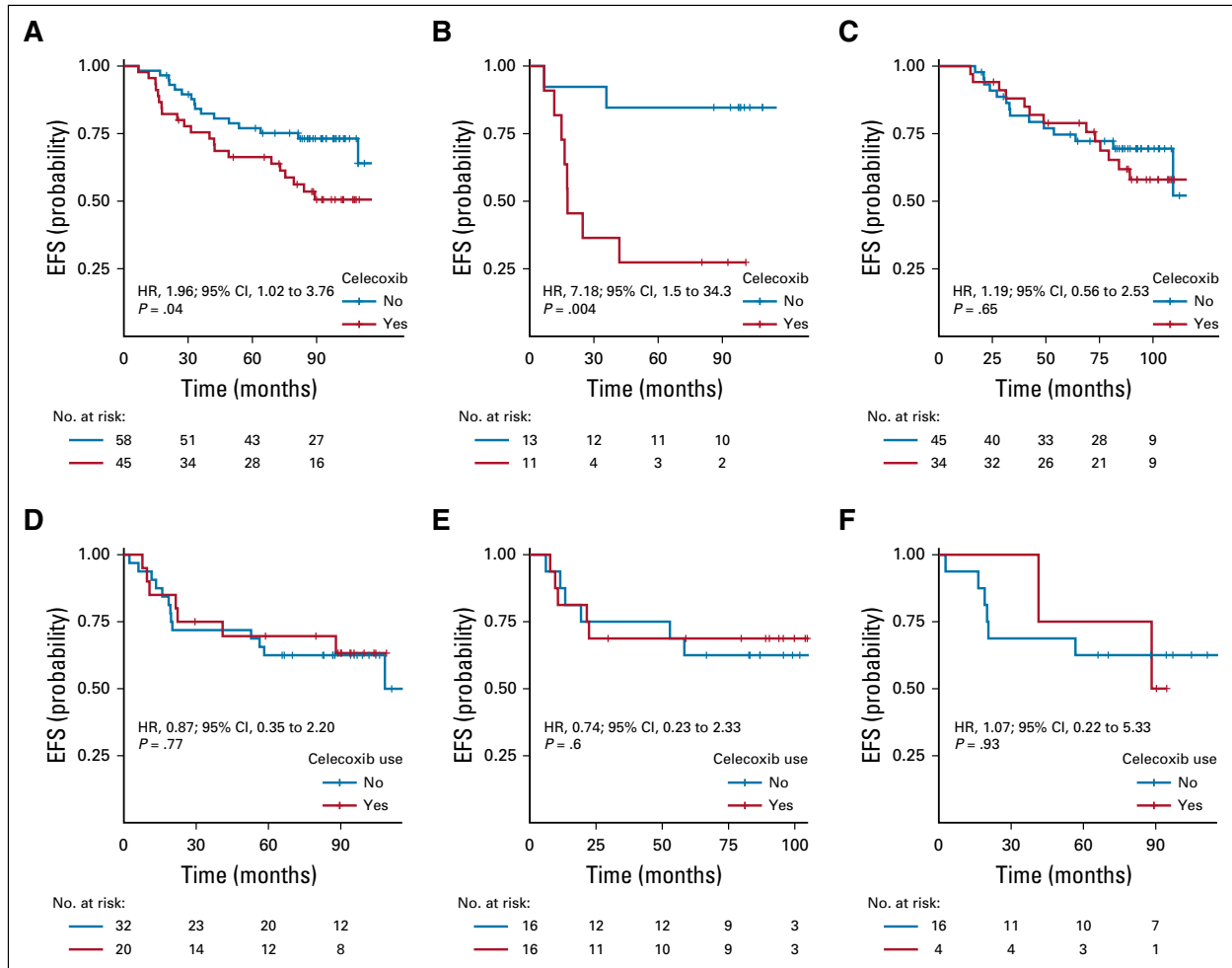


FIG A3. Kaplan-Meier curves for association between treatment arm (per-protocol analyses) and event-free survival (EFS) according to *PTGS2* and estrogen receptor (ER) status: (A) *PTGS2*-low population; (B) *PTGS2*-low/ER-negative subpopulation; (C) *PTGS2*-low/ER-positive subpopulation; (D) *PTGS2*-high population; (E) *PTGS2*-high/ER-negative subpopulation; and (F) *PTGS2*-high/ER-positive subpopulation. HR, hazard ratio.

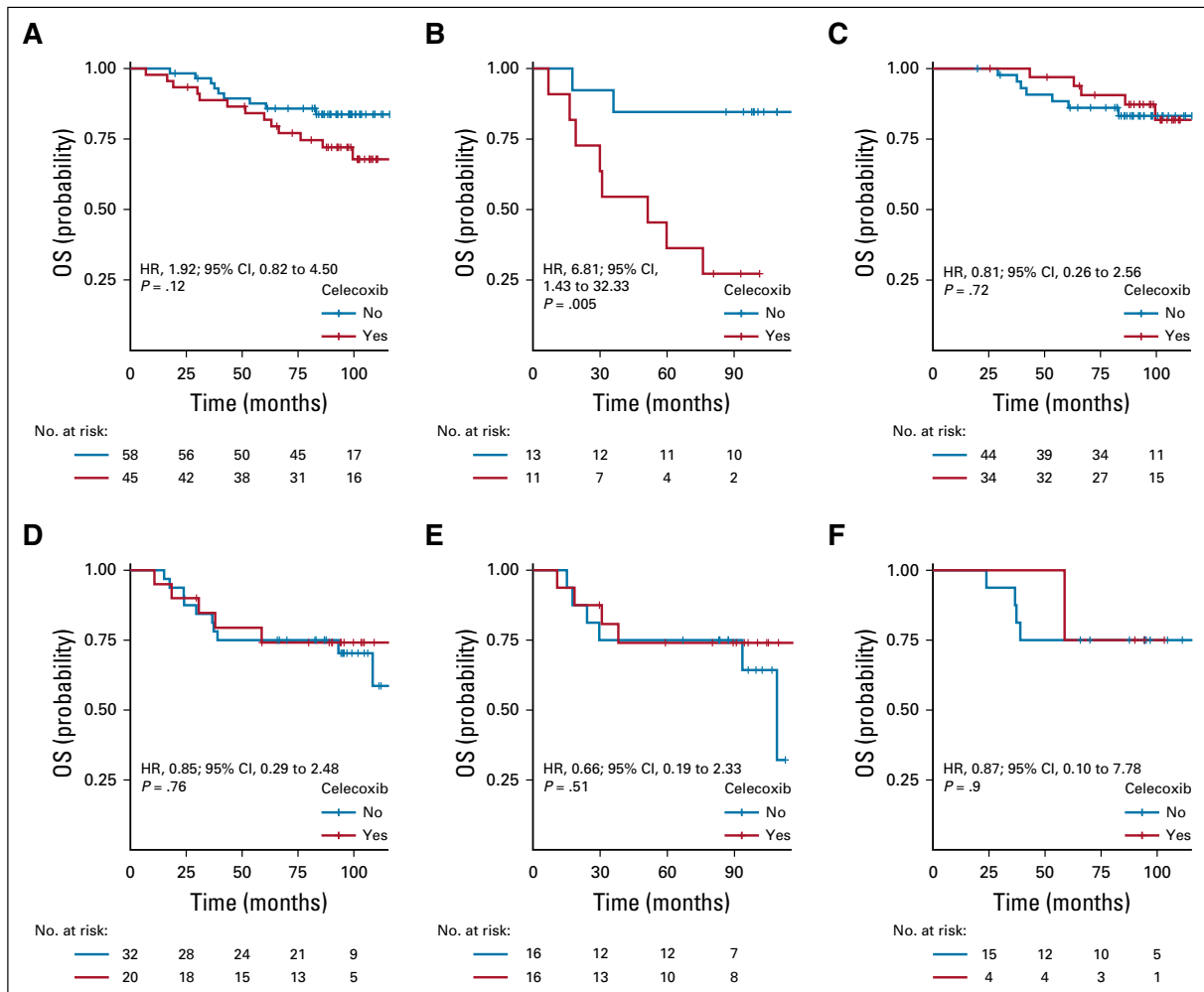


FIG A4. Kaplan-Meier curves for association between treatment arm (per-protocol analyses) and overall survival (OS) according to *PTGS2* expression and estrogen receptor (ER) status: (A) *PTGS2*-low population; (B) *PTGS2*-low/ER-negative subpopulation; (C) *PTGS2*-low/ER-positive subpopulation; (D) *PTGS2*-high population; (E) *PTGS2*-high/ER-negative subpopulation; and (F) *PTGS2*-high/ER-positive subpopulation. HR, hazard ratio.

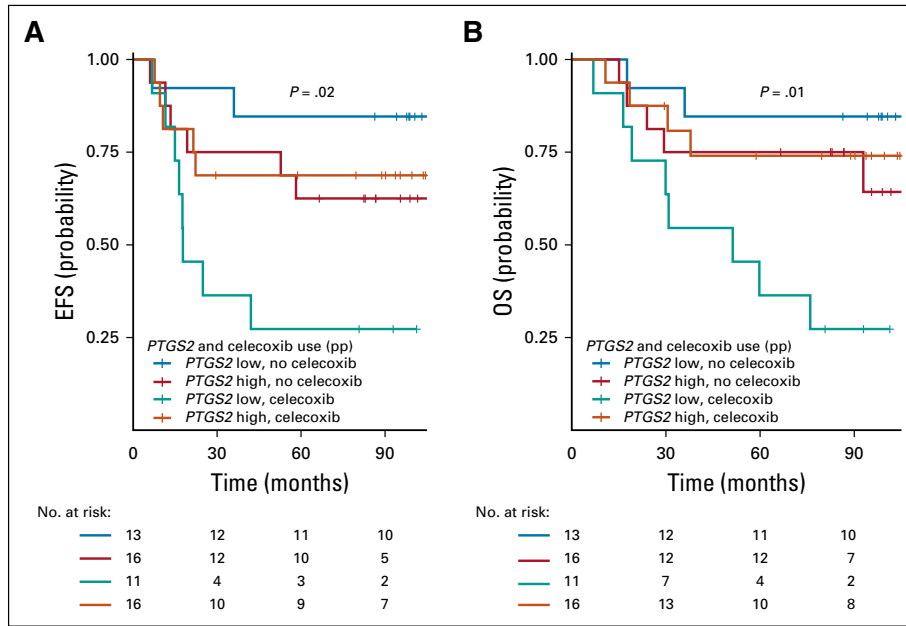


FIG A5. Kaplan-Meier combined survival curves for the association between *PTGS2* expression and treatment arm (per-protocol analyses) in the estrogen receptor (ER)-negative population: (A) event-free survival (EFS) by *PTGS2* expression and celecoxib use; (B) overall survival (OS) by *PTGS2* expression and celecoxib use. pp, per protocol.

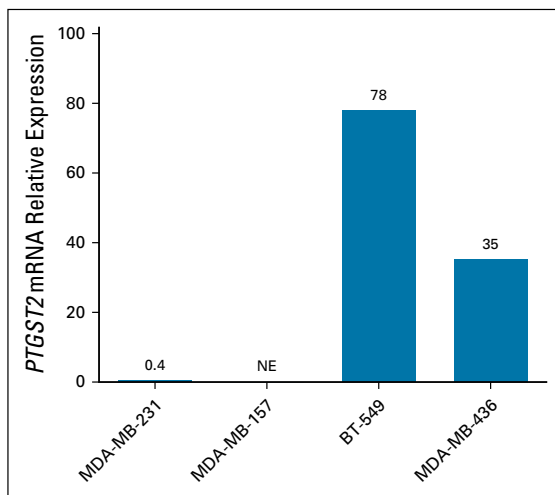


FIG A6. *PTGS2* expression by reverse transcription quantitative polymerase chain reaction analysis in four triple-negative breast cancer cell lines (MDA-MB-231; MDA-MB-157; BT549; and MDA-MB-436). NE, not expressed.

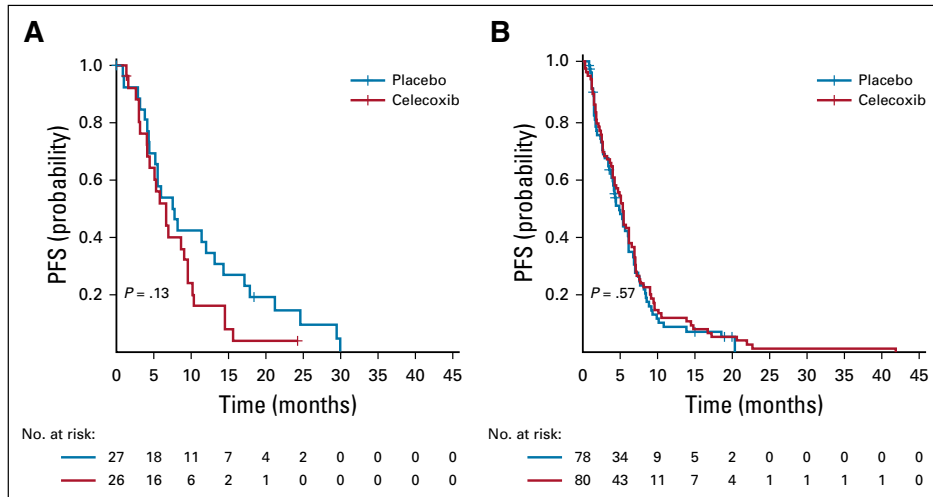


FIG A7. Kaplan-Meier curves for association between treatment arm and progression-free survival (PFS) in the Cancer and Leukemia Group B 30801 Alliance trial: (A) population with metabolite prostaglandin E_2 (PGE-M) values less than Q1 (less than first quartile); and (B) population with PGE-M values of Q1 or greater (\geq first quartile).

TABLE A1. Patient and Tumor Characteristics by *PTGS2* Expression in the Intention-to-Treat Population

Characteristic	No. (%) of Patients		P
	<i>PTGS2</i> Low (n = 104)	<i>PTGS2</i> High (n = 52)	
Age, years			
< 40	23 (22.1)	11 (21.2)	.99
40 to 49	44 (42.3)	22 (42.3)	
≥ 50	37 (35.6)	19 (36.5)	
Menopausal status			
Pre	68 (66.0)	38 (73.1)	.48
Post	35 (34.0)	14 (26.9)	
BMI, kg/m ²			
≤ 25	60 (57.7)	28 (54.9)	.88
> 25	44 (42.3)	23 (45.1)	
Tumor size			
T2	62 (59.6)	27 (51.9)	.46
T3 and T4	42 (4.4)	25 (48.1)	
Clinical nodal status			
N0	37 (35.6)	22 (43.1)	.46
N1, N2, N3	67 (64.4)	29 (56.9)	
Histology			
Ductal	88 (84.6)	43 (82.7)	.11
Lobular	12 (11.5)	3 (5.8)	
Other	4 (3.8)	6 (11.5)	
Grade			
1	12 (11.7)	2 (4.3)	< .01
2	48 (46.6)	12 (25.5)	
3	43 (41.7)	33 (70.2)	
LVI			
No	88 (85.4)	43 (82.7)	.83
Yes	15 (14.6)	9 (17.3)	
ER status			
Negative	24 (23.1)	32 (61.5)	< .01
Positive	80 (76.9)	20 (38.5)	
PR status			
Negative	47 (46.1)	39 (76.5)	< .01
Positive	55 (53.9)	12 (23.5)	
TNBC			
Yes	23 (22.1)	32 (61.5)	< .01
No	81 (77.9)	20 (38.5)	
<i>p53</i>			
WT	35 (71.4)	9 (33.3)	< .01
Mutated	14 (28.6)	18 (66.7)	
Celecoxib (pp)			
No	59 (56.7)	32 (61.5)	.69
Yes	45 (43.3)	20 (38.5)	

Abbreviations: BMI, body mass index; ER, estrogen receptor; LVI, lymphovascular invasion; pp, per protocol; PR, progesterone receptor; TNBC, triple-negative breast cancer; WT, wild type.

TABLE A2. Univariable and Multivariable Analyses of Clinical and Pathologic Factors on EFS

Variable Comparison	Univariable			Multivariable		
	HR	95% CI	P	HR	95% CI	P
Age, years						
40 to 49 v < 40	0.73	0.37 to 1.42	.428			
≥ 50 v < 40	1.05	0.54 to 2.06				
Menopausal status						
Post v pre	0.91	0.52 to 1.61	.755			
Tumor size						
T3 and T4 v T2	1.97	1.17 to 3.31	.009			
Clinical nodal status						
N1, N2, and N3 v N0	1.49	0.84 to 2.62	.169			
Histology						
Lobular v ductal or other	2.13	1.16 to 3.92	.013			
Grade						
3 v 2	1.19	0.69 to 2.06	.524			
ER status						
Positive v negative	0.79	0.46 to 1.35	.386	1.04	0.4 to 2.69	.931
PR status						
Positive v negative	0.54	0.31 to 0.94	.026			
LVI						
Yes v no	1.93	1.04 to 3.6	.035	2.26	1.14 to 4.48	.02
Treatment allocation						
Celecoxib arm v standard arm	1.7	1 to 2.88	.046	9.17	2.88 to 29.15	< .001
<i>PTGS2</i> expression						
High v low	1.24	0.73 to 2.13	.425	2.50	1.05 to 5.95	.038
pCR status						
Yes v no	0.33	0.1 to 1.06	.051	0.21	0.06 to 0.75	.016
Interaction: celecoxib with <i>PTGS2</i>			.011			.008
Interaction: celecoxib with ER			.106			.005

Abbreviations: EFS, event-free survival; ER, estrogen receptor; HR, hazard ratio; LVI, lymphovascular invasion; pCR, pathologic complete response PR, progesterone receptor.

TABLE A3. Univariable and Multivariable Analyses of Clinical and Pathologic Factors on OS

Variable Comparison	Univariable			Multivariable		
	HR	95% CI	P	HR	95% CI	P
Age, years						
40 to 49 v < 40	0.79	0.33 to 1.92	.379			
≥ 50 v < 40	1.33	0.57 to 3.1				
Menopausal status						
Post v pre	0.94	0.46 to 1.91	.866			
Tumor size						
T3 and T4 v T2	2.8	1.43 to 5.5	.002	2.7653	1.35 to 5.67	.006
Clinical nodal status						
N1, N2, and N3 v N0	1.46	0.72 to 2.96	.286			
Histology						
Lobular v ductal or other	2.39	1.18 to 4.86	.013			
Grade						
3 v 2	1.51	0.76 to 3.02	.239			
ER status						
Positive v negative	0.41	0.21 to 0.78	.005	1.86	0.6 to 5.81	.283
PR status						
Positive v negative	0.25	0.11 to 0.57	< .001	0.3049	0.11 to 0.81	.017
LVI						
Yes v no	2.04	0.96 to 4.34	.058			
Treatment allocation						
Celecoxib arm v standard arm	1.71	0.88 to 3.33	.108	9.75	2.41 to 39.45	.001
<i>PTGS2</i> expression						
High v low	1.47	0.76 to 2.83	.25	2.32	0.73 to 7.35	.154
pCR status						
Yes v no	0.37	0.09 to 1.55	.158			
Interaction: celecoxib with <i>PTGS2</i>			.045			.03
Interaction: celecoxib with ER			.124			.023

Abbreviations: ER, estrogen receptor; HR, hazard ratio; LVI, lymphovascular invasion; OS, overall survival; pCR, pathologic complete response; PR, progesterone receptor.

TABLE A4. EFS and OS HRs in Per-Protocol Analyses by Celecoxib Use and *PTGS2* Expression in the Whole Study Population, the ER-Negative Subpopulation, and the ER-Positive Subpopulation

Survival by Population	Whole Population (n = 156)					ER Negative (n = 56)					ER Positive (n = 100)				
	No. of Patients	No. of Events	HR	95% CI	<i>P</i> _{Log-Rank}	No. of Patients	No. of Events	HR	95% CI	<i>P</i> _{Log-Rank}	No. of Patients	No. of Events	HR	95% CI	<i>P</i> _{Log-Rank}
EFS															
Whole population															
Standard treatment arm	90	30	1		.168	29	9	1		.116	61	21	1		.815
Celecoxib arm	65	28	1.44	0.86 to 2.41		27	13	1.97	0.83 to 4.66		38	15	1.08	0.56 to 2.1	
<i>PTGS2</i> low (n = 104)															
Standard treatment arm	58	16	1		.039	13	2	1		.004	45	14	1		.65
Celecoxib arm	45	21	1.96	1.02 to 3.76		11	8	7.18	1.5 to 34.3		34	13	1.19	0.56 to 2.53	
<i>PTGS2</i> high (n = 52)															
Standard treatment arm	32	14	1		.773	16	7	1		.605	16	7	1		.933
Celecoxib arm	20	7	0.87	0.35 to 2.2		16	5	0.74	0.23 to 2.33		4	2	1.07	0.22 to 5.33	
OS															
Whole population															
Standard treatment arm	90	19	1		.349	29	8	1		.138	61	11	1		.596
Celecoxib arm	65	18	1.36	0.71 to 2.59		27	12	1.95	0.79 to 4.81		38	6	0.76	0.28 to 2.07	
<i>PTGS2</i> low (n = 104)															
Standard treatment arm	58	9	1		.125	13	2	1		.005	45	7	1		.72
Celecoxib arm	45	13	1.92	0.82 to 4.5		11	8	6.81	1.43 to 32.33		34	5	0.81	0.26 to 2.56	
<i>PTGS2</i> high (n = 52)															
Standard treatment arm	32	10	1		.759	16	6	1		.511	16	4	1		.899
Celecoxib arm	20	5	0.85	0.29 to 2.48		16	4	0.66	0.19 to 2.33		4	1	0.87	0.1 to 7.78	

Abbreviations: EFS, event-free survival; ER, estrogen receptor; HR, hazard ratio; OS, overall survival.

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.ejcancer.com

Clinical Trial

Long-term outcome of the REMAGUS 02 trial, a multicenter randomised phase II trial in locally advanced breast cancer patients treated with neoadjuvant chemotherapy with or without celecoxib or trastuzumab according to HER2 status



Sylvie Giacchetti ^{a,*}, Anne-Sophie Hamy ^b, Suzette Delaloge ^c,
 Etienne Brain ^d, Frédérique Berger ^e, Brigitte Sigal-Zafrani ^f,
 Marie-Christine Mathieu ^g, Philippe Bertheau ^h, Jean Marc Guinebretière ^f,
 Mahasti Saghatchian ^c, Florence Lerebours ^d, chafouny mazouni ⁱ,
 Olivier Tembo ^k, Marc Espié ^a, Fabien Reyat ^{b,j}, Michel Marty ^{a,k},
 Bernard Asselain ^l, Jean-Yves Pierga ^{d,m}

^a AP-HP, Hôpital Saint-Louis, Breast Disease Unit, University Paris Diderot, 75475 Paris, France

^b Institut Curie, PSL Research University, Translational Research Department, INSERM, U932 Immunity and Cancer, Residual Tumor & Response to Treatment Laboratory (RT2Lab), Paris, France

^c Medical Oncology Department, Gustave Roussy, Cancer Center Villejuif, France

^d Medical Oncology Department, Institut Curie, Saint Cloud, Paris, France

^e Biostatistics Department, Institut Curie, Paris, France

^f Tumor Biology Department, Institut Curie, Saint Cloud, Paris, France

^g Pathology Department, Gustave Roussy, Cancer Center Villejuif, France

^h AP-HP, Hôpital Saint-Louis, Pathology Department, University Paris Diderot, Paris, France

ⁱ Department of Surgery, Gustave Roussy, Cancer Center Villejuif, France

^j Department of Surgery, Institut Curie, Paris, France

^k Center for Therapeutic Innovations in Oncology and Haematology (CITOH), APHP, Hôpital Saint-Louis, Paris, France

^l UMR 8081 'IR4M', Université Paris-Sud, 91400 Orsay, France

^m Université Paris Descartes, Sorbonne Paris Cite, Paris, France

Received 22 December 2016; accepted 11 January 2017

Available online 7 March 2017

* Corresponding author.

E-mail address: sylvie.giacchetti@aphp.fr (S. Giacchetti).

<http://dx.doi.org/10.1016/j.ejca.2017.01.008>

0959-8049/© 2017 Elsevier Ltd. All rights reserved.

KEYWORDS

Neoadjuvant chemotherapy;
Celecoxib;
Trastuzumab;
Breast cancer;
Long-term outcome

Abstract Background: The REMAGUS-02 multicenter randomised phase II trial showed that the addition to neoadjuvant chemotherapy (NAC) of trastuzumab in patients with localised HER2-positive breast cancer (BC) increased the pathological complete response (pCR) rate and that the addition of celecoxib in HER2-negative cases did not increase the pCR rate. We report here the long-term follow-up results for disease-free survival (DFS) and overall survival (OS).

Patients and methods: From 2004 to 2007, 340 stage II–III BC patients were randomly assigned to receive neoadjuvant EC-T (four cycles of epirubicin–cyclophosphamide followed by four cycles of docetaxel) +/- celecoxib in HER2-negative cases (n = 220) and ± trastuzumab in HER2-positive cases (n = 120). From September 2005, all patients with HER2-positive BC received adjuvant T (n = 106).

Results: Median follow-up was nearly 8 years (94.4 months, 20–127 m). In the HER2-negative subgroup, addition of celecoxib was not associated with a DFS benefit. Favourable factors were smaller tumour size, expression of progesterone receptor status (PgR) and pCR. In the HER2-positive population, neoadjuvant trastuzumab was not associated with a DFS benefit. Axillary pCR was the only prognostic factor associated with DFS in this group [HR = 0.44, 95% CI = 0.2–0.97], p = 0.035]. To note, DFS and OS were significantly higher in the HER2-positive than in HER2-negative BC patients (HR = 0.58 [0.36–0.92], p = 0.021).

Conclusion: Celecoxib combined with NAC provided neither pCR nor survival benefit in patients with HER2-negative BC. Absence of PgR is a major prognostic factor. Neoadjuvant trastuzumab increased pCR rates without translation into a DFS or OS benefit compared with adjuvant trastuzumab only. Axillary pCR could be a more relevant surrogate of survival than in the breast in HER2-positive population. A retrospective comparison shows that patients with HER2-positive tumours have a better outcome than HER2-negative BC patients showing the impact of trastuzumab on the natural history of BC.

© 2017 Elsevier Ltd. All rights reserved.

Key message: Celecoxib, an anti-Cox2, neither improves pCR nor the outcome in HER2-negative BC patients receiving sequential NAC. The long-term outcome of luminal B BC can be worse than triple-negative BC, whereas HER2-positive BC patients have a better prognostic than other subtypes with the addition of trastuzumab in neoadjuvant or adjuvant setting.

1. Introduction

Neoadjuvant chemotherapy (NAC) was initially developed for non-resectable breast cancers, but is now widely used in localised breast cancer not eligible for breast-conserving surgery [1]. However, randomised trials and meta-analyses have not shown any improvement in disease-free survival (DFS) or overall survival (OS) compared with adjuvant approaches [1–4]. Despite the lack of demonstrated survival gain with the neoadjuvant strategy, several advantages are recognised. Tumour regression induced by NAC allows breast conservation in a proportion of patients with initially large tumours non-accessible to immediate conservative surgery. In patients with possible breast conservation from the beginning, preoperative treatment may also result in a better cosmetic outcome [5]. In particular, neoadjuvant therapy offers the advantage of rapidly

assessing the response to both standard treatment and novel therapies and a research platform to evaluate the predictive factors of response to treatment [6,7]. Such frontline treatment defined pathological complete response (pCR, i.e. absence of infiltrating tumour in breast and lymph nodes) as the key end-point in predicting the long-term survival especially in patients with oestrogen receptor (ER)-negative and triple-negative (TN) tumours [7–9].

We previously reported the pCR results of this multicenter randomised phase II study aiming to determine the impact of adding celecoxib or trastuzumab to NAC in stage II–III breast cancers [10]. We showed that celecoxib did not improve the pCR rates in HER2-negative population, whereas trastuzumab added to NAC was associated with an increased pCR rate in patients with HER2-positive tumours [10]. Here, we report the long-term outcome of the patients treated in

this study and evaluate the prognostic factors associated with DFS and OS.

2. Patients and methods

From May 2004 to October 2007, 340 patients were randomly assigned to receive NAC ± celecoxib in HER2-negative patients (Stratum A, n = 220), +/- trastuzumab in HER2-positive patients (Stratum B, n = 120) [10] (Fig. 1).

2.1. Patients selection

Main eligibility criteria were female patients over the age of 18 and under the age of 65 with histologically proven non-metastatic invasive breast carcinoma (Stage II and III), not amenable to breast-conserving surgery (diameter > 3 cm, central) or with risk factors making NAC the preferred treatment (i.e. N2–N3, rapid growth rate). Inflammatory breast cancers and clinical T4 stage were allowed. HER2 status was centrally reviewed for all patients and HER2 positivity confirmed by FISH in all cases.

2.2. Treatment

All patients were to receive epirubicin (75 mg/m²)–cyclophosphamide (750 mg/m²) intravenously every 3 weeks for four cycles followed by docetaxel (100 mg/m²)

every 3 weeks for four cycles. During neoadjuvant docetaxel sequence, HER2-negative tumour patients (stratum A) were randomised between without (arm1) or with (arm 2) celecoxib 400 mg twice daily orally. In HER2-positive tumour, patients were randomised between without (arm 3) or with (arm 4) trastuzumab every 3 weeks. Surgery was performed 21–45 d after cycle eight, based on initial and post-chemotherapy assessment. Surgery was followed by local and regional radiotherapy when indicated. The administration of adjuvant trastuzumab for a total of 18 3-weekly i.v. infusions was amended for HER2-positive cancer patients from September 2005, when adjuvant trastuzumab was available in France. Trastuzumab was thus started after surgery for patients randomised to control and pursued for patients randomised to neoadjuvant arm. Patients with hormone receptor-positive tumours received adjuvant tamoxifen or aromatase inhibitors according to their menopausal status. Adjuvant chemotherapy according to centres' preferences in patients with residual axillary nodal involvement (pN+) could be delivered based on 5-fluorouracil and vinorelbine combination (four cycles), concomitantly or not with radiotherapy.

2.3. Objectives

The primary end-point of the study was pCR rates. pCR was defined as absence of residual invasive cancer cells

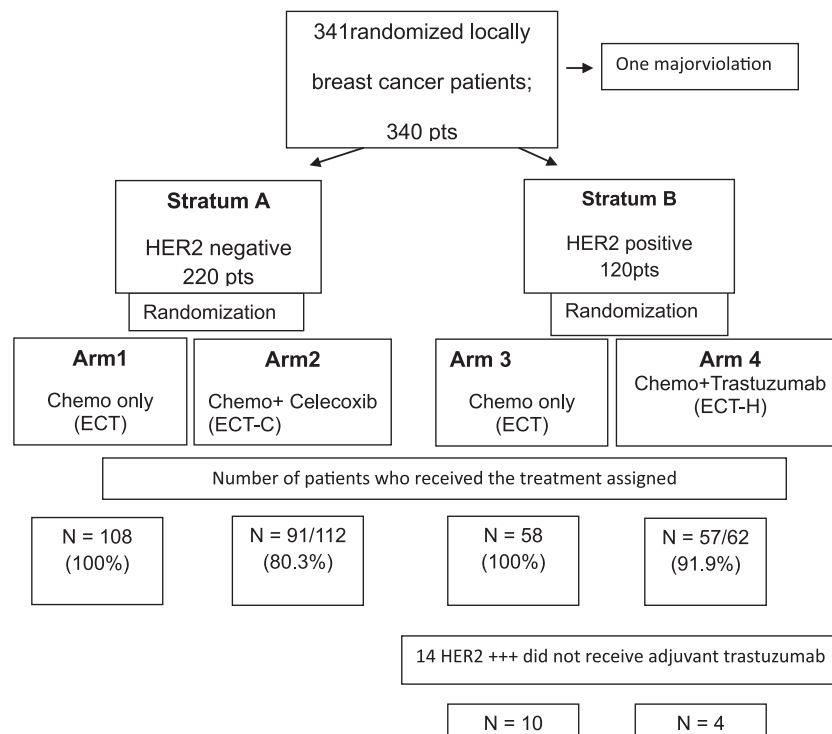


Fig. 1. Consort diagram.

in the breast and axillary lymph nodes (grade 1 and 2 of Chevallier's classification) [11]. Both DFS and OS were predefined secondary objectives. DFS was defined as the time from surgery to death, loco-regional or distant recurrence, or contralateral cancer, whichever occurred first. OS was defined as the time from surgery to death. Patients for whom none of these events were recorded were censored at the date of their last known contact. Results are described in the whole population, and in each stratum.

2.4. Statistical analysis

The study population was described in terms of frequencies for qualitative variables or medians and associated ranges for quantitative variables. The cutoff date for the analysis was May 1st 2015. Hazard ratios (HRs) and their associated 95% confidence intervals (CIs) were calculated with the Cox proportional hazard model. Age class, menopausal status, clinical tumour size, clinical nodal status, histological type, SBR Grade, lymphovascular invasion, ER, PgR, HER2 status, p53 and randomised treatment were included in the univariate analysis. Variables with a *p*-value for the likelihood ratio test lower than 0.15 in univariate analysis were included in the multivariate model. Backward selection was used to establish the final multivariate model. The significance threshold was 5%. Survival probabilities were estimated by the Kaplan–Meier method, and survival curves were compared with log-rank tests. Analyses were performed with R software, version 3.1.2 (R Development Core Team, 2011).

3. Results

Among 340 patients included in the study, 220 had HER2-negative tumours (stratum A) and 120 had HER2-positive tumours (stratum B). All the patients received the first four courses of epirubicin–cyclophosphamide. In the stratum A, 21 patients who were assigned to celecoxib arm (arm 2) did not receive it (19.6%). In the stratum B, five patients who were assigned in the neoadjuvant trastuzumab arm (arm 4) did not receive it (8%), whereas two patients in the arm without trastuzumab (arm 3) received neoadjuvant trastuzumab. All patients but 14 received adjuvant trastuzumab (Fig. 1), 10 in arm 3 (17.2%) and four in arm 4 (6.5%), *p* = 0.07. A total of 14 patients were not included in the DFS analysis [no surgery *n* = 9; distant metastases during NAC (*n* = 4); protocol violation (*n* = 1)].

Patient's characteristics are described in Table 1. Overall, the population had poor prognostic factors. Near half of the patients had T3–T4 (47.4%) and grade SBR 3 tumours (49.8%); 60% had lymph nodes involved clinically at baseline (N1, N2 and N3). In the HER2-

negative population, almost 40% of the patients had TN tumours. The median follow-up was 94.5 months [range: 19.9–126.9].

3.1. Whole population

A total of 111 patients experienced relapse and 74 patients died. The median DFS and the median OS are not reached for the whole population. The 8-year DFS and OS were 67.9% [62.8–73.6] and 77.5% [72.7–82.6], respectively. After univariate analysis, ER expression was not (Fig. 2A) significantly associated with DFS, whereas clinical tumour size, clinical nodal status, histological type, lymphovascular invasion, progesterone receptor (PgR) (Fig. 2B), HER2 status (Fig. 2C) and pCR were prognostic for DFS. Tumour size (T2 versus T3 and T4; HR = 1.75 [1.15–2.66], *p* = 0.009); PgR status (positive versus negative; HR = 0.47 [0.3–0.72], *p* = 0.001), HER2 status (positive versus negative; HR = 0.58 [0.36–0.92], *p* = 0.021) and pCR (yes versus no; HR = 0.38 [0.17–0.84], *p* = 0.016) remained significantly associated with DFS after multivariate analysis (Table 2). All four factors were also associated with OS (clinical tumour size HR = 2.69 [1.61–4.49], *p* < 0.001, PgR, HR = 0.3 [0.17–0.53], *p* < 0.001, HER2; HR = 0.58 [0.34–0.99], *p* = 0.046, pCR; HR = 0.38 [0.15–0.94], *p* = 0.037).

3.2. Stratum A: HER2-negative tumours

Of 220 patients with HER2-negative tumours, 82 patients experienced recurrences and 53 patients died. Median DFS and OS were not reached. The 8-year DFS and OS are, respectively, 64.4% [57.7–71.7] and 76.5% [70.7–82.9]. No effect of the addition of celecoxib to NAC on DFS was observed, neither in intent to treat (ITT) (celecoxib versus no, HR = 1.23 [0.77–1.96], *p* = 0.38) nor in the per protocol analysis (HR = 1.27 [0.8–2.02], *p* = 0.32).

Due to the differences of the shapes of the Kaplan Meier curves for ER and PgR status, the heterogeneity of luminal breast cancer [12,13], we combined ER and PgR status into three classes (ER+/PgR+, ER+/PgR-, ER-/PgR-) (Fig. 3). Only two patients were ER-/PgR+ in the stratum A and were pooled with the ER+/PgR+ population into a PgR+ group in the analysis. Overall, ER+/PgR-status was associated with a worse long-term DFS when compared with ER+/PgR+ (reference class, HR = 1); ER+/PgR-: HR: 2.3 [1.28–4.12], *p* = 0.005, whereas TN status was not HR = 1.49 (95% CI (0.86–2.6), *p* = 0.16).

After univariate analysis, clinical tumour size, histological type, PgR and pCR were significantly associated with DFS and all factors, but histological type remained associated with DFS after multivariate analysis (Table 2). Histological type, ER status, tumour size, PgR status and pCR were significantly associated with

Table 1
Patients' characteristics.

Variable		Stratum A: HER2 negative		Stratum B: HER2 positive		Total	p Value
		CT alone (arm 1)	CT + celocoxib (arm 2)	CT alone (arm 3)	CT + trastuzumab (arm 4)		
		**n = 108 (%)	n = 112 (%)	n = 58 (%)	n = 62 (%)		
Age (years)	<40	23 (21.3)	25 (22.3)	13 (22.4)	15 (24.2)	76 (22.4)	0.8191
	40–49	42 (38.9)	49 (43.8)	25 (43.1)	20 (32.3)	136 (40.0)	
	≥50	43 (39.8)	38 (33.9)	20 (34.5)	27 (43.5)	128 (37.6)	
Menopausal status*	No	69 (65.1)	76 (67.9)	41 (70.7)	40 (64.5)	226 (66.9)	0.8645
	Yes	37 (34.9)	36 (32.1)	17 (29.3)	22 (35.5)	112 (33.1)	
Clinical tumour size	T2	55 (50.9)	65 (58)	27 (46.6)	32 (51.6)	179 (52.6)	0.508
	T3 and T4	53 (49.1)	47 (42)	31 (53.4)	30 (48.4)	161 (47.4)	
Clinical nodal status*	N0	42 (39.6)	45 (40.2)	18 (31.6)	25 (40.3)	130 (38.6)	0.699
	N1, N2, N3	64 (60.4)	67 (59.8)	39 (68.4)	37 (59.7)	207 (61.4)	
Histological type	Ductal	83 (76.9)	90 (80.4)	56 (96.6)	57 (91.9)	286 (84.1)	0.0059
	Lobular	19 (17.6)	13 (11.6)	1 (1.7)	2 (3.2)	35 (10.3)	
	Others	6 (5.6)	9 (8)	1 (1.7)	3 (4.8)	19 (5.6)	
SBR grade*	I	13 (12.6)	7 (6.4)	0 (0)	0	20 (6.1)	0.0042
	II	41 (39.8)	56 (50.9)	22 (40.7)	25 (41.7)	144 (44%)	
	III	49 (47.6)	47 (42.7)	32 (59.3)	35 (58.3)	163 (49.8)	
LVI	No	90 (84.9)	93 (85.3)	41 (71.9)	43 (74.1)	267 (80.9)	0.0668
	Yes	16 (15.1)	16 (14.7)	16 (28.1)	15 (25.9)	63 (19.1)	
ER	Negative	35 (32.4)	45 (40.2)	23 (39.7)	30 (48.4)	133 (39.1)	0.2271
	Positive	73 (67.6)	67 (59.8)	35 (60.3)	32 (51.6)	207 (60.9)	
PgR*	Negative	55 (51.4)	64 (58.2)	37 (63.8)	41 (67.2)	197 (58.6)	0.1867
	Positive	52 (48.6)	46 (41.8)	21 (36.2)	20 (32.8)	139 (41.4)	
HR	Negative	34 (31.5)	44 (39.3)	21 (36.2)	28 (45.2)	127 (37.4)	0.3316
	Positive	74 (68.5)	68 (60.7)	37 (63.8)	34 (54.8)	213 (62.6)	
Triple negative	Yes	34 (31.5)	44 (39.3)				
	No	74 (68.5)	68 (60.7)				
HR (4 classes)	ER–/PgR–	34 (31.8)	44 (40)	21 (36.2)	28 (45.9)	127 (37.8)	0.303
	ER+/PgR–	21 (19.6)	20 (18.2)	16 (27.6)	13 (21.3)	70 (20.8)	
	ER–/PgR+	1 (0.9)	1 (0.9)	2 (3.4)	2 (3.3)	6 (1.8)	
	ER+/PgR+	51 (47.7)	45 (40.9)	19 (32.8)	18 (29.5)	133 (39.6)	
p53*	WT	20 (54.1)	26 (63.4)	10 (47.6)	9 (39.1)	65 (53.3)	0.2815
	Mutated	17 (45.9)	15 (36.6)	11 (52.4)	14 (60.9)	57 (46.7)	
Per protocol neoadjuvant Treatment	No celocoxib	108 (100)	21 (18.8)				
	Celocoxib	0	91 (81.2)				
Surgery	No trastuzumab			56 (96.6)	5 (8.1)		
	Trastuzumab			2 (3.4)	57 (91.9)		
Adjuvant chemotherapy	No	4 (3.7)	4 (3.6)	1 (1.7)	0	9 (2.6)	0.4374
	Yes	104 (96.3)	108 (96.4)	57 (98.3)	62 (100)	331 (97.4)	
Adjuvant trastuzumab	No	77 (71.3)	82 (73.2)	46 (79.3)	49 (79)	254 (74.7)	0.5598
	Yes	31 (28.7)	30 (26.8)	12 (20.7)	13 (21)	86 (25.3)	
Hormonotherapy*	No			48 (82.8)	58 (93.5)		0.0658
	Yes	28 (27.7)	38 (36.5)	26 (48.1)	32 (51.6)	124 (38.6)	
Radiotherapy*	No	73 (72.3)	66 (63.5)	28 (51.9)	30 (48.4)	197 (61.4)	0.0276
	Yes	1 (1)	3 (2.9)	0	5 (8.1)	9 (2.8)	
pCR*	No	101 (99)	101 (97.1)	54 (100)	57 (91.9)	313 (97.2)	0.0458
	Yes	93 (89.4)	94 (87)	46 (80.7)	46 (74.2)	279(84.3)	
	Yes	11 (10.6)	14 (13)	11 (19.3)	16 (25.8)	52 (15.7)	
pCR (breast)	No	89 (85.6)	91 (85)	42 (73.7)	46 (74.2)	268 (81.2)	0.0917
	Yes	15 (14.4)	16 (15)	15 (26.3)	16 (25.8)	62 (18.8)	
pCR (axilla)	No	69 (67)	64 (60)	30 (52.6)	28 (45.2)	191 (58.2)	0.0361
	Yes	34 (33)	42 (39.6)	27 (47.4)	34 (54.8)	137 (41.8)	
Relapses		37	45	15	14	111	
Deaths		23	30	10	11	74	

*Missing data: menopausal status (n = 2), nodal status (n = 3), grade (n = 13), LVI (n = 10), RP (n = 4), p53 (n = 218), hormonotherapy (n = 19), radiotherapy (n = 18), pCR (n = 9).

**n = number of patients.

Bold values are the values which are statistically significant.

Abbreviations: LVI, lymphovascular invasion; pCR, pathological complete response.

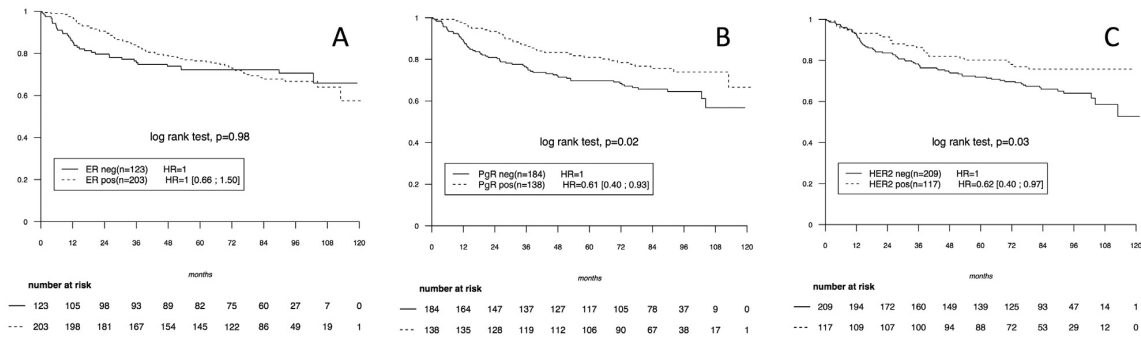


Fig. 2. DFS in the whole population. A: ER+ versus ER–, B: PgR+ versus PgR–, C: HER2-positive versus HER2 negative.

OS after univariate analysis. As for DFS, three of these factors remained significantly associated with OS after multivariate analysis: clinical tumour size (T3 and T4: HR = 2.64 [1.43–4.86] versus T2, p = 0.002); PgR positive (HR = 0.25 [0.12–0.49], p < 0.001) and pCR (HR = 0.23 [0.06–0.97], p = 0.046).

3.3. Stratum B: HER2-positive tumours

Of the 120 patients with HER-positive tumours, 29 patients experienced recurrences and 21 patients died. There was no association between neoadjuvant trastuzumab and DFS neither in intent to treat nor in the per

Table 2
Multivariate analysis for DFS in stratum A and B.

Variable	Stratum A (HER2 neg)			DFS			Stratum B (HER2+++)			DFS		
	Univariate analysis			Multivariate analysis			Univariate analysis			Multivariate analysis		
	HR	IC	p	HR	IC	p	HR	IC	p	HR	IC	p
Age	>40	1										
	40–49	0.81	[0.45–1.47]	0.78			1.4	[0.48–4.09]	0.583			
	≤50	0.91	[0.5–1.67]	0.78			1.71	[0.61–4.8]	0.583			
Menopausal status	No	1					1					
	Yes	1.07	[0.66–1.75]	0.787			1.42	[0.67–3.04]	0.36			
Clinical tumour size	T2	1		1	–	–	1					
	T3/T4	1.92	[1.21–3.07]	0.005	1.96	[1.2–3.19]	0.007	1.55	[0.72–3.3]	0.256		
Clinical nodal status	N0	1					1					
	N1/N2/N3	1.42	[0.86–2.34]	0.165			1.98	[0.8–4.9]	0.134			
Histological type	Ductal	1					1					
	Lobular	2	[1.14–3.52]	0.048								
	Others	1.25	[0.5–3.15]	0.048			1.3	[0.31–5.5]	0.718			
SBR Grade	I	1					1					
	II	1.79	[0.63–5.04]	0.41								
	III	2	[0.71–5.66]	0.41			1.99	[0.84–4.73]	0.113			
LVI	No	1					1					
	Yes	1.67	[0.93–3]	0.084			1.58	[0.72–3.46]	0.246			
ER	negative	1					1					
	positive	0.97	[0.59–1.58]	0.888			0.94	[0.44–1.99]	0.872			
PgR	negative	1		1	–	–	1					
	positive	0.56	[0.35–0.92]	0.019	0.41	[0.25–0.68]	0.001	0.65	[0.29–1.49]	0.307		
HR	negative	1					1					
	positive	0.91	[0.56–1.48]	0.698			0.8	[0.38–1.69]	0.557			
p53	WT	1					1					
	mutated	1.25	[0.57–2.77]	0.573			0.42	[0.1–1.78]	0.227			
pCR (breast and axilla)	No	1		1	–	–	1					
	Yes	0.28	[0.09–0.9]	0.023	0.24	[0.07–0.76]	0.016	0.53	[0.18–1.52]	0.229		
pCR (breast)	No	1					1					
	Yes	0.3	[0.11–0.81]	0.012			0.77	[0.31–1.9]	0.569			
pCR (axilla)	No	1					1			1		
	Yes	0.4	[0.22–0.72]	0.001			0.44	[0.2–0.97]	0.035	0.44	[0.2–0.97]	0.035

Bold values are the values which are statistically significant.

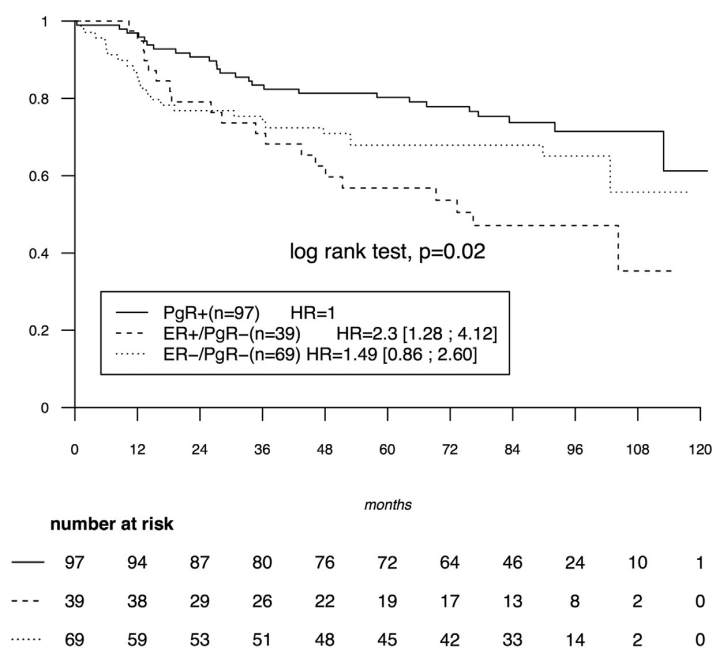
DFS as a function of HR status, 3 classes (HER2 negative population)

Fig. 3. DFS as a function of hormonal receptors status in patients with HER2-negative tumours.

protocol analysis in the HER2-positive stratum. Eight years DFS was 73.8% [62.9–86.8%] in the group randomised to receive NAC alone, versus 76.2% [65.9–88.1%] in the group randomised to receive NAC plus trastuzumab. After univariate analysis, none of the classical factors were significantly associated with DFS except axillary pCR: HR = 0.44 [0.2–0.97], $p = 0.035$. No factor was significantly associated with the OS after univariate analysis, although a trend to a better OS was observed when axillary pCR was reached [HR = 0.34 (0.11–1.07), $p = 0.053$].

4. Discussion

We report here the long-term outcome of patients with locally advanced breast cancer included in a randomised phase II trial studying the impact of adding NAC to celecoxib in HER2-negative population and trastuzumab in HER2-positive population. We showed that the addition of celecoxib was not associated with an increased DFS nor OS in HER2-negative patients irrespective of hormone receptors status. We previously published that the adjunction of celecoxib to NAC was not associated with higher pCR rates [10]. However, this study will provide the opportunity to study predictive genomic factors of celecoxib response as *PTGS2* overexpression.

In the HER2-positive population, neoadjuvant trastuzumab was associated with a higher pCR rate but this did not translate into a DFS or OS benefit. The REMAGUS 02 trial is the only neoadjuvant randomised trial including patients with HER2-positive tumours that compared neoadjuvant trastuzumab with no neoadjuvant trastuzumab in a population that received adjuvant trastuzumab in both arms. In the NOAH trial [14], patients received either trastuzumab given as neoadjuvant and adjuvant treatment or no trastuzumab at all. In other neoadjuvant trials as the GeparQuinto [15], NeoALTT0 [16] or Neosphere [17,18] trials, all control arms included neoadjuvant anti-HER2-targeted therapy. We acknowledge some weaknesses in our trial, possible lack of statistical power due to randomised phase II design, absence of adjuvant trastuzumab in a small subset of patients and a few discrepancies between treatment randomization and treatment allocation. However, these data arguing for no effect on DFS of an early introduction of trastuzumab in addition to NAC may ever remain the only trial to address such a question as neoadjuvant trastuzumab has become a standard of care in HER2-positive breast cancers.

In our study, pCR was not a surrogate of DFS and OS, regardless of the hormonal receptors in patients with HER2-positive tumours. pCR has been proposed as a surrogate end-point of long-term benefit such as event-free survival (EFS), DFS and OS. However, the association between pCR and long-term outcome is not

clearly demonstrated. The German Breast Group reported that in HER-positive patients, reaching pCR was associated with a DFS advantage in patients with ER negative, but not ER positive tumours [9]. Cortazar *et al.* on behalf of the FDA established an international working group (CTNeoBC) which aims to investigate the relation between pCR and long-term outcome via a pooled analysis of neoadjuvant trials [7]. They showed that pCR was positively associated with EFS and OS in all subgroups of breast cancers. However, at a trial level, the authors recorded little or no association between increases of pCR and the treatment's effect on EFS or OS. In a recent meta-analysis [19], only the NOAH trial has demonstrated that pCR achievement could be a surrogate end-point for EFS and OS [14]. The effect of pCR gain on survival in the NOAH trial could be seen only because the control group lacked an effective targeted therapy for HER2-positive breast cancer. In our trial, trastuzumab was given in both arms but earlier in the experimental group. The introduction of adjuvant trastuzumab could have compensated the use of neoadjuvant trastuzumab in terms of long-term DFS or OS. Our study, as well as other studies, emphasises that in future neoadjuvant studies in HER2-positive population, all patients will receive HER2-targeted therapy, and thus the gain of a new drug on pCR gain must be large enough to have an effect on EFS and survival [19].

The other point underlined in our series is the major role of post-NAC lymph nodes' involvement in patients with HER2-positive tumours. The analysis of the pathological response in the axilla is not or rarely studied independently of the breast, although we know that axilla involvement is a major predictor of DFS and/or OS in adjuvant. Patients with extensive nodal involvement after NAC have a very poor outcome [20–22]. The population included in our study has large locally advanced tumours with almost half of the population having T3/T4 tumours. The size of the tumours (T3 and T4 versus T2) is one of the major predictive factor in our series with a HR = 2.96, $p = 0.002$. Other authors have reported that the clinical size of the tumours is a major prognostic factor in breast cancers treated with NAC [23–26].

Another key observation from this study is the major impact of PgR receptors. Indeed, the long follow-up (nearly 8 years) allowed to report such mature data and to highlight the long-lasting risk of hormone-responsive breast cancers, as opposed to hormone receptor--negative BC. We already published that there is a non-constant effect in time for TN and non-TN breast cancers. Overall, TN status had the worst initial risk of recurrence that gradually decreased during the first 24 months of follow-up to disappear after 48 months of follow-up [27]. Here, ER+/PgR-status was associated with a worse DFS when compared with ER+/PgR+ (reference class, HR = 1); ER+/PgR-: HR: 2.3 [1.28–4.12], $p = 0.005$, whereas TN status was not

HR = 1.49 (95% CI (0.86–2.6), $p = 0.16$). With a long follow-up, the prognosis of TNBC was not significantly different from ER+/PgR--positive patients, whereas ER+/PgR-negative BC patients had a nearly two-fold increased risk of relapse. These results are very important as chemotherapy is increasingly avoided for patients with ER+ tumours. At the last San Gallen conference, in patients with 'luminal B-like' (HER2-negative) tumours, the Panel was more closely divided, but only a minority would recommend chemotherapy for most of the cases [28]. In the European Organisation for Research and Treatment of Cancer recommendations [29], the luminal B HER2-negative cancers were considered as a population of the highest uncertainty regarding chemotherapy indications. Other authors explored the significance of PgR negativity [30–32]. Bae *et al.* [32] showed that patients with single HR+, negative HER2 tumours were associated with poorer survival than ER + PgR + tumours and had comparable poor survival than TN tumours. It has been recently shown that PgR is not merely an ER α -induced gene target, but is also an ER α -associated protein that modulates its behaviour [33]. PgR functions as a molecular rheostat to control ER α chromatin binding and transcriptional activity. Our findings call for the necessity of PgR determination which has important implications for prognosis and therapeutic interventions, as considering chemotherapy in the Luminal B BC population.

In conclusion, the addition of celecoxib to NAC was not associated with an increase of DFS nor OS in patients with HER2-negative locally advanced tumours. Our study emphasised the role of progesterone receptors and their importance in distinguishing luminal A from luminal B breast cancers with worst prognostic.

Patients with HER2-positive tumours treated with trastuzumab had a statistically higher DFS and OS than patients with HER2-negative tumours, highlighting how targeted anti-HER2 treatment has improved the initial poor prognostic of these cancers. This is the first study comparing neoadjuvant to adjuvant trastuzumab. Both DFS and OS were similar in the patients who received neoadjuvant versus adjuvant trastuzumab. It is not unlikely that adjuvant trastuzumab, compensate the lack of an early trastuzumab. Finally, in HER2-positive patients, only the pCR in the axilla was associated with a poor DFS. These patients with no pCR after NAC remain at high risk of relapse despite adjuvant trastuzumab treatment and should be considered for specific 'adjuvant-post neoadjuvant' studies.

Conflict of interest statement

jean Yves Pierga declared "Honoraria and research funding from Roche".the other authors declared no conflict of interest.

Funding

This work was funded by the Programme Hospitalier de Recherche Clinique: ISRCTN10059974, PHRC: AOM 02 11.

Sponsor

This work was sponsored by Roche Pharmaceutical, Pfizer Inc, France, and Sanofi Aventis.

Role of the funding sources

Academic fundings: Data manager and research technician salaries.

Industrial fundings: Provided drugs free of charge (Roche: trastuzumab, Pfizer: celecoxib, and Sanofi: taxotere).

Acknowledgement

This work has been presented as a poster at SABCS 2015 (Abstract number: P1-14-09).

Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.ejca.2017.01.008>.

References

- [1] Rastogi P, Anderson SJ, Bear HD, et al. Preoperative chemotherapy: updates of National Surgical Adjuvant Breast and Bowel Project Protocols B-18 and B-27. *J Clin Oncol* 2008;26:778–85.
- [2] Mauri D, Pavlidis N, Ioannidis JP. Neoadjuvant versus adjuvant systemic treatment in breast cancer: a meta-analysis. *J Natl Cancer Inst* 2005;97:188–94.
- [3] Mieog JS, van der Hage JA, van de Velde CJ. Neoadjuvant chemotherapy for operable breast cancer. *Br J Surg* 2007;94:1189–200.
- [4] Fisher CS, Ma CX, Gillanders WE, et al. Neoadjuvant chemotherapy is associated with improved survival compared with adjuvant chemotherapy in patients with triple-negative breast cancer only after complete pathologic response. *Ann Surg Oncol* 2012;19:253–8.
- [5] Ademuyiwa FO, Ellis MJ, Ma CX. Neoadjuvant therapy in operable breast cancer: application to triple negative breast cancer. *J Oncol* 2013;2013:219869.
- [6] Loibl S, Denkert C, von Minckwitz G. Neoadjuvant treatment of breast cancer—clinical and research perspective. *Breast* 2015;24(Suppl. 2):S73–7.
- [7] Cortazar P, Zhang L, Untch M, et al. Pathological complete response and long-term clinical benefit in breast cancer: the CTNeoBC pooled analysis. *Lancet* 2014;384:164–72.
- [8] Symmans WF, Peintinger F, Hatzis C, et al. Measurement of residual breast cancer burden to predict survival after neoadjuvant chemotherapy. *J Clin Oncol* 2007;25:4414–22.
- [9] von Minckwitz G, Untch M, Blohmer JU, et al. Definition and impact of pathologic complete response on prognosis after neoadjuvant chemotherapy in various intrinsic breast cancer subtypes. *J Clin Oncol* 2012;30:1796–804.
- [10] Pierga JY, Delaloge S, Espie M, et al. A multicenter randomized phase II study of sequential epirubicin/cyclophosphamide followed by docetaxel with or without celecoxib or trastuzumab according to HER2 status, as primary chemotherapy for localized invasive breast cancer patients. *Breast Cancer Res Treat* 2010;122:429–37.
- [11] Chevallier B, Roche H, Olivier JP, Chollet P, Hurteloup P. Inflammatory breast cancer. Pilot study of intensive induction chemotherapy (FEC-HD) results in a high histologic response rate. *Am J Clin Oncol* 1993;16:223–8.
- [12] Skibinski A, Kuperwasser C. The origin of breast tumor heterogeneity. *Oncogene* 2015;34:5309–16.
- [13] Ignatiadis M, Sotiriou C. Luminal breast cancer: from biology to treatment. *Nat Rev Clin Oncol* 2013;10:494–506.
- [14] Gianni L, Eiermann W, Semiglazov V, et al. Neoadjuvant and adjuvant trastuzumab in patients with HER2-positive locally advanced breast cancer (NOAH): follow-up of a randomised controlled superiority trial with a parallel HER2-negative cohort. *Lancet Oncol* 2014;15:640–7.
- [15] Untch M, Loibl S, Bischoff J, et al. Lapatinib versus trastuzumab in combination with neoadjuvant anthracycline-taxane-based chemotherapy (GeparQuinto, GBG 44): a randomised phase 3 trial. *Lancet Oncol* 2012;13:135–44.
- [16] de Azambuja E, Holmes AP, Piccart-Gebhart M, et al. Lapatinib with trastuzumab for HER2-positive early breast cancer (Neo-ALTTO): survival outcomes of a randomised, open-label, multicentre, phase 3 trial and their association with pathological complete response. *Lancet Oncol* 2014;15:1137–46.
- [17] Gianni L, Pienkowski T, Im YH, et al. Efficacy and safety of neoadjuvant pertuzumab and trastuzumab in women with locally advanced, inflammatory, or early HER2-positive breast cancer (NeoSphere): a randomised multicentre, open-label, phase 2 trial. *Lancet Oncol* 2012;13:25–32.
- [18] Gianni L, Pienkowski T, Im YH, et al. 5-year analysis of neoadjuvant pertuzumab and trastuzumab in patients with locally advanced, inflammatory, or early-stage HER2-positive breast cancer (NeoSphere): a multicentre, open-label, phase 2 randomised trial. *Lancet Oncol* 2016;17:791–800.
- [19] Broglio KR, Quintana M, Foster M, et al. Association of pathologic complete response to neoadjuvant therapy in HER2-positive breast cancer with long-term outcomes: a meta-analysis. *JAMA Oncol* 2016;2:751–60.
- [20] Guarneri V, Broglio K, Kau SW, et al. Prognostic value of pathologic complete response after primary chemotherapy in relation to hormone receptor status and other factors. *J Clin Oncol* 2006;24:1037–44.
- [21] Pierga JY, Mouret E, Dieras V, et al. Prognostic value of persistent node involvement after neoadjuvant chemotherapy in patients with operable breast cancer. *Br J Cancer* 2000;83:1480–7.
- [22] Bonsang-Kitzis H, Chaltier L, Belin L, et al. Beyond axillary lymph node metastasis, BMI and menopausal status are prognostic determinants for triple-negative breast cancer treated by neoadjuvant chemotherapy. *PLoS One* 2015;10:e0144359.
- [23] Gonzalez-Angulo AM, McGuire SE, Buchholz TA, et al. Factors predictive of distant metastases in patients with breast cancer who have a pathologic complete response after neoadjuvant chemotherapy. *J Clin Oncol* 2005;23:7098–104.
- [24] Bodilsen A, Offersen BV, Christiansen P, Overgaard J. Pattern of relapse after breast conserving therapy, a study of 1519 early breast cancer patients treated in the Central Region of Denmark 2000–2009. *Acta Oncol* 2016;55:964–9.
- [25] Fei F, Messina C, Slaets L, et al. Tumour size is the only predictive factor of distant recurrence after pathological complete response to neoadjuvant chemotherapy in patients with large operable or locally advanced breast cancers: a sub-study of EORTC 10994/BIG 1-00 phase III trial. *Eur J Cancer* 2015;51:301–9.
- [26] Hamy-Petit AS, Belin L, Bonsang-Kitzis H, et al. Pathological complete response and prognosis after neoadjuvant chemotherapy

- for HER2-positive breast cancers before and after trastuzumab era: results from a real-life cohort. *Br J Cancer* 2016;114:44–52.
- [27] Giacchetti S, Porcher R, Lehmann-Che J, et al. Long-term survival of advanced triple-negative breast cancers with a dose-intense cyclophosphamide/anthracycline neoadjuvant regimen. *Br J Cancer* 2014;110:1413–9.
- [28] Coates AS, Winer EP, Goldhirsch A, et al. Tailoring therapies—improving the management of early breast cancer: St. Gallen international expert consensus on the primary therapy of early breast cancer 2015. *Ann Oncol* 2015;26:1533–46.
- [29] Senkus E, Kyriakides S, Penault-Llorca F, et al. Primary breast cancer: ESMO clinical practice guidelines for diagnosis, treatment and follow-up. *Ann Oncol* 2013;24(Suppl. 6). vi7–23.
- [30] Prat A, Cheang MC, Martin M, et al. Prognostic significance of progesterone receptor-positive tumor cells within immunohistochemically defined luminal a breast cancer. *J Clin Oncol* 2013; 31:203–9.
- [31] Sun JY, Wu SG, Li FY, et al. Progesterone receptor loss identifies hormone receptor-positive and HER2-negative breast cancer subgroups at higher risk of relapse: a retrospective cohort study. *Onco Targets Ther* 2016;9:1707–13.
- [32] Bae SY, Kim S, Lee JH, et al. Poor prognosis of single hormone receptor-positive breast cancer: similar outcome as triple-negative breast cancer. *BMC Cancer* 2015;15:138.
- [33] Mohammed H, Russell IA, Stark R, et al. Progesterone receptor modulates ERalpha action in breast cancer. *Nature* 2015;523:313–7.

Comedications influence immune infiltration and pathological response to neoadjuvant chemotherapy in breast cancer.

Anne-Sophie Hamy^{1,2†}, Lisa Derosa^{3-5 †}, Constance Valdelièvre⁶, Satoru Yonekura³⁻⁵, Paule Opolon³⁻⁵, Maël Priour⁷, Julien Guerin⁷, Jean-Yves Pierga², Bernard Asselain⁸, Diane de Croze⁹, Alice Pinheiro¹, Marick Lae⁹, Laure-Sophie Talagrand⁶, Enora Laas⁶, Lauren Darrigues⁶, Elisabetta Marangoni¹⁰, Elodie Montaudon¹⁰, Guido Kroemer¹¹⁻¹⁷, Laurence Zitvogel^{3-5,18‡}, Fabien Reyat^{1,6,14‡*}.

Affiliations:

1. *Residual Tumor & Response to Treatment Laboratory, RT2Lab, Translational Research Department, U932, Immunity and Cancer, Institut Curie, PSL Research University, Institut Curie, F-75248, France.*
2. *Department of Medical Oncology, Institut Curie, Saint-Cloud, France.*
3. *Gustave Roussy Cancer Campus (GRCC), 94800 Villejuif, France*
4. *Institut National de la Santé Et de la Recherche Medicale (INSERM) U1015, Equipe Labellisée—Ligue Nationale contre le Cancer, 94800 Villejuif, France.*
5. *Univ. Paris-Sud, Université Paris-Saclay, Gustave Roussy, Villejuif, France.*
6. *Department of Surgery, Institut Curie, Paris, F-75248, France.*
7. *Informatics Department, Institut Curie, 75005 Paris, France.*
8. *Biostatistics Department, Institut Curie, 75005 Paris, France.*
9. *Tumor Biology, Institut Curie, 75005 Paris, France.*
10. *Preclinical investigation laboratory, Translational Research Department, Institut Curie, PSL Research University, Paris,*
11. *Equipe 11 labellisée par la Ligue contre le Cancer, Centre de Recherche des Cordeliers; Paris, France;*

12. *Cell Biology and Metabolomics platforms, Gustave Roussy Cancer Campus; Villejuif, France;*
13. *INSERM, U1138, Paris, France;*
14. *Université Paris Descartes, Sorbonne Paris Cité; Paris, France;*
15. *Université Pierre et Marie Curie, Paris, France;*
16. *Pôle de Biologie, Hôpital Européen Georges Pompidou, AP-HP; Paris, France;*
17. *Karolinska Institute, Department of Women's and Children's Health, Karolinska University Hospital, Stockholm, Sweden*
18. *Center of Clinical Investigations in Biotherapies of Cancer (CICBT) 1428, Gustave Roussy and Institut Curie, France.*

† and ‡ : contributed equally to this work.

Running Title: Comedications may modulate immunity in breast cancer.

Keywords: Breast Cancer, immunomodulation, comedication, pCR, TILs

Additional information:

- **Corresponding author(*):** Pr Fabien REYAL, Institut Curie, Department of Surgery, 26 rue d'Ulm, 75005 Paris; 00 33 615271980; fabien.reyal@curie.fr
- **Declaration of Interests:** LZ and GK are cofounders of EverImmune, a biotech company devoted to the use of commensal bacteria for the treatment of cancers. The authors declare no potential conflicts of interest.
- **Manuscript:** Abstract: 233 words - Text: 4782 words – Number of figures: 7

Abstract (234 words)

Immunosurveillance plays an important role in breast cancer (BC) prognosis and progression, and can be geared by immunogenic chemotherapy. In a cohort of 1023 BC patients treated with neoadjuvant chemotherapy (NAC), 40% of the individuals took comedications mostly linked to aging and comorbidities. We systematically analyzed the off-target effects of 1178 concurrent comedications (classified according to the Anatomical Therapeutic Chemical (ATC) Classification System) on the density of tumor infiltrating lymphocytes (TILs) and pathological complete responses (pCR). At level 1 of the ATC system, the main anatomical classes of drugs were those targeting the nervous system (class N, 39.1%), cardiovascular disorders (class C, 26.6%), alimentary and metabolism (class A, 16.9%), or hormonal preparations (class H, 6.5%). At level 2, the most frequent therapeutic classes were psycholeptics (N05), analgesics (N02), and psychoanaleptics (N06). Pre-NAC TIL density in triple negative BC (TNBC) was influenced by medications from class H, N and A, while TIL density in $HER2^+$ BC was associated with the use of class C. Psycholeptics (N05) and agents acting on the renin-angiotensin system (C09) were independently associated with pCR in the whole population of BC or TNBC, and in *HER2*-positive BC respectively. Importantly, level 3 hypnotics (N05C) alone were able to reduce tumor growth in BC bearing mice and increased the anti-cancer activity of cyclophosphamide in a T cell-dependent manner. These findings prompt for prospective drug repositioning to improve the efficacy of NAC in BC.

Introduction

Breast cancer (BC) incidence increases with age, as does the prevalence of many other chronic diseases, such as diabetes, hypertension, and cardiovascular disease. Molecular BC subtypes and the density of tumor-infiltrating immune cells are both considered as important predictive and prognostic factors for optimal risk stratification and treatment individualization of BC patients. Denkert *et al.* first evidenced that the amount of stromal immune infiltration was positively associated with pathological complete response (pCR) after neoadjuvant chemotherapy (NAC) (1). These results were recently confirmed on a pooled analysis of large cohort of 3771 patients receiving NAC from German Breast Group(2), showing that the relationships between TIL levels and pCR translates into improved disease-free survival in *HER2*-positive and triple negative BC (TNBC).

The drivers of immunosurveillance have largely been studied in the past decade, and derive from both (i) tumor-intrinsic characteristics; and/or (ii) extrinsic factors related to the host or the environment(3)(4). Among endogenous tumor characteristics, molecular features (BC subtype, proliferative patterns), expression of human leukocyte antigen (HLA)-class I, tumor mutational burden(3), activation of cellular pathways(5), or induction of autophagy(5) have been found to be associated with immune infiltration. Extrinsic factors including host characteristics (gender(6), age(7), body mass index), environment (tobacco, alcohol), nutritional factors, diet, commensal microbiota, physical activity, hormonal exposure(8) have been studied less extensively.

There is growing interest in chronically used medications that may influence the risk for and the progression of cancer (9). Some medications such as aspirin or non-steroidal anti-inflammatory drugs (NSAID) have been reported to decrease BC risk or BC recurrence (statins(10), NSAIDs (11), beta blockers (BB) (12) and metformin(13)). So far, the impact of chronic comedication on immune infiltration has not been investigated. A few studies

suggest that drugs that do not fall into the class of antineoplastics may have an impact on immunosurveillance through various mechanisms. For instance, metformin may increase CD8⁺ TILs (14) or potentiate PD-1 blockade through reduction of tumor hypoxia(15); propranolol and etodolac modulate tumor infiltration(16); zoledronic acid(17) targets tumor-associated macrophages; proton pump inhibitors(18) reverse T cell anergy; and tadalafil inhibits myeloid derived-suppressor cells(19).

In the current study, we hypothesized that some comedications might be associated with TIL levels in BC. We evaluated the interactions between comedications, immune infiltration at diagnosis and pCR rates in a cohort of 1023 non-metastatic BC patients treated with NAC. Here, we report on epidemiological associations between distinct classes of comedications, TIL density and pCR rates, as we exemplify the T lymphocyte-dependent anticancer effects of the psycholeptic zolpidem in preclinical mouse models. Altogether, our results indicate that comedication may represent a confounding factor in BC clinical trials, prompting for prospective studies aimed at validating their beneficial role in drug repositioning for BC treatment.

Material and methods

Patients, tumors and cancer treatments

We analyzed a cohort of 1023 T1-3N_xM0 patients with invasive breast carcinoma (NEOREP Cohort, CNIL declaration number 1547270) treated with NAC at Institut Curie, Paris, between 2002 and 2012. We included only unilateral, non-recurrent, non-inflammatory, non-metastatic tumors, and excluded T4 tumors. All patients received NAC, followed by surgery and all but 21 patients received radiotherapy. The study was approved by the Breast Cancer Study Group of Institut Curie and was conducted according to institutional and ethical rules regarding research on tissue specimens and patients. Written informed consent from the patients was not required by French regulations. Patients were treated according to national guidelines. NAC regimens changed over time (anthracycline-based regimen or sequential anthracycline-taxane regimen), with trastuzumab used in an adjuvant and/or neoadjuvant setting since the mid 2000's. Surgery was performed four to six weeks after the end of the chemotherapy. Endocrine therapy (tamoxifen, aromatase inhibitor, and/or GnRH agonists) was prescribed when indicated.

Tumor samples and pathology review

ER, PR and *HER2* positivity determination is detailed in the supplemental material. BC subtypes were defined as follows: tumors positive for either ER or PR, and negative for *HER2* were classified as luminal; tumors positive for *HER2* were considered *HER2*-positive BC; tumors negative for ER, PR, and *HER2* were considered as triple negative BC (TNBC).

For a subset of patients (n=615), stromal TILs were retrospectively reviewed on pathological specimens of pre-NAC core needle biopsy. Pretreatment core needle biopsies were evaluated independently by two expert breast pathologists for the presence of a mononuclear cells infiltrate (including lymphocytes and plasma cells, excluding polymorphonuclear leukocytes)

following the recommendations of the international TILs Working Group(43). TILs were scored continuously on hematoxylin and eosin-stained sections without additional staining as the average percentage of stromal area occupied by mononuclear cells.

Comedications

Chronic concomitant therapies - designed throughout the manuscript as comedications - were extracted retrospectively from medical charts, as any chronic treatment declared by the patient at initial or anaesthetics consultation. Intercurrent treatments lasting less than one week were excluded, as well as medications prescribed around chemotherapy (anti vomiting drugs, granulocytes-colony stimulating factors, steroids), as they are systematically prescribed to all patients. As the information on drug dosing, schedule, or date of introduction was not constantly available, comedication use was coded as a binary variable (yes: at least one drug declaration; no: no comedication mentioned). They were classified according to the Anatomical Therapeutic Chemical (ATC) Classification System controlled by the World Health Organization Collaboration (available at the URL https://www.whooc.no/atc_ddd_index/). ATC is used for the classification of active ingredients of drugs according to the organ or system on which they act and their therapeutic, pharmacological and chemical properties. The first level of the code indicates the anatomical main group (14 main groups) and consists of one letter; the second level of the code indicates the therapeutic subgroup; the third level and the fourth levels of the code indicate the chemical / therapeutic / pharmacological subgroup, and the fifth level indicates the chemical substance. The complete classification of metformin illustrates the structure of the code:

A	Alimentary tract and metabolism (1st level, anatomical main group)
A10	Drugs used in diabetes (2nd level, therapeutic subgroup)
A10B	Blood glucose lowering drugs, excl. insulins (3rd level, pharmacological subgroup)
A10BA	Biguanides (4th level, chemical subgroup)
A10BA02	Metformin (5th level, chemical substance)

Drugs from categories D (dermatologicals), P (antiparasitic products, insecticides and repellents), L (antineoplastic and immunomodulating agents), S (sensory organs) were excluded from the analyses. Only chronic antivirals for systemic use (J05) and oral drugs for respiratory system (antihistamines for systemic use (R06)) were included in drugs from category J (anti-infectives for systemic use) and R (respiratory system) respectively. Anatomical classes with less than 50 comedications were grouped into the category “Others”.

Comorbidities

Comorbidities, defined as any chronic condition declared by the patient at initial or anaesthetics consultation were extracted retrospectively from medical charts. Comorbidities were regrouped into 6 classes: hypertension / heart disease, depression/anxiety, dyslipidemia, diabetes, ulcer/gastritis, thyroid disorders, and the category “Others” regrouped the remaining chronic conditions.

Gene expression

Total RNA extraction from frozen pretreatment biopsies was previously performed for 140 patients who participated in the clinical trials REMAGUS02 and REMAGUS04. Human Genechip U133 plus 2.0 microarray hybridization and quality controls have already been

described in details elsewhere(44). For each dataset, batch effects were eliminated by the median centering of each probe set across arrays and the quantile normalization of all arrays separately for each set. The expression levels of 6 immune genes (*IFNG*, *IDO1*, *CXCL9*, *CXCL10*, *HLA-DRA*, *STAT1*) from the previously published Interferon- γ signature (20) were extracted from the pooled gene expression matrix. We assessed Interferon- γ metagene expression by calculating the mean normalized expression value for all the genes considered together and we generated a heatmap of the metagene expression profile using the *gplot* package.

Study endpoints

ypTN stage was defined according to the American Joint Committee on Cancer/Union for International Cancer Control staging(45). A pathological complete response (pCR) was defined as an absence of invasive residual tumor in the breast, and of invasive disease in the axillary nodes (ypT0/is+ ypN0) (46).

Animal models

All animal experiments were carried out in compliance with French and European laws and regulations. The local institutional animal ethics board and French Ministère de la Recherche approved all mouse experiments (permission numbers: 2014-071-1124, 2016-049-4646).

Immunodeficient xenograft model

The PDX HBCx-8 xenograft was established from a triple-negative negative breast cancer as previously described(47). The *in vivo* efficacy study was conducted by transplanting HBCx-8 tumor fragments into female 8-week-old Swiss nude mice that were randomly assigned to the control or treated groups (6 mice per group) when tumors reached a volume of 60 to 200 mm³. Adriamycin, 2 mg/kg (Doxorubicin, Teva Pharmaceuticals) and cyclophosphamide, 100

mg/kg (Endoxan, Baxter), or docetaxel, 20 mg/kg (Taxotere, Sanofi-Aventis) were given as single injection at day 1 by intraperitoneal (i.p.) and intravenous (i.v.) injections. Bromazepam was given orally at 0.6 mg/kg 5 days/week until ethical sacrifice. Tumor growth was evaluated by measurement of two perpendicular diameters of tumors with a caliper twice per week. Individual tumor volumes were calculated as $V = \frac{a \times b^2}{2}$, a being the largest diameter, b the smallest. Mice were ethically sacrificed when the tumor volume reached 1500 mm³.

Immunocompetent mice model

The C57BL/6 mice were injected intraperitoneally (i.p.) for 14 consecutive days with Zolpidem (5 mg/kg twice a day) or Pantoprazole (100 mg/kg once a day) or vehicle (NaCl). On day 14, 10⁶ AT3 cells were inoculated and mice continued their treatment with Zolpidem or Pantoprazole or vehicle. When tumors reached 20 to 35 mm² in size, mice received either NaCl or Cyclophosphamide (100 mg/kg of body weight) every 7 days x 3-4 injections. Tumor size was routinely monitored every 3 days by means of a caliper. In experiments using anti-CD4 mAb (clone GK1.5, 200 μg per mouse) or anti-CD8 mAb (clone 53-6.72, 200 μg per mouse) or their isotype controls (clone LTF-3 or clone 2A3), mAb were injected i.p. 2 days before Cyclophosphamide injection and then continued every 7 days starting from day 0 until the final Cyclophosphamide injection. All antibodies were purchased from BioXcell, NH, USA.

Immunohistochemistry

Immunofluorescence staining, scanning and analysis were performed for Foxp3, CD4 and CD3 expression in AT3 tumor from treated mice. For multiplexed staining, 3 μm-thick sections of formalin-fixed, paraffin-embedded AT3 tumor from treated mouse were stained by

means of an automated immunostainer (DISCOVERY ULTRA, Ventana, IGR). Heat-induced antigen retrieval in EDTA buffer (pH 8.0) for 48 minutes at 95°C was performed. The primary polyclonal Rabbit anti-human Foxp3 antibody (Thermo Fisher Scientific, #PA-1-46126, 1mg/mL) was applied on the slides for 1hour at 37°C, followed by detection using the biotin-free peroxydase system of detection, Discovery UltraMap anti-Rabbit HRP (Ventana, #760-4315). The Visualization of Foxp3 was accomplished using TSA fluorophore system, Discovery Rhodamine 6G kit (Ventana, #760-244). Heat-induced antigen retrieval in Citrate buffer (pH 6.0) for 10 minutes at 100°C was performed. Then, the slides were incubated on primary monoclonal Rabbit anti-human CD4 antibody (Abcam, EPR19514, 0.623mg/mL) for 1 hour at 37°C, detected by Discovery UltraMap anti-rabbit HRP and visualized by Discovery Cy5 kit 360 (Ventana, #760-238). Heating step with Citrate Buffer was carried out, as described above. Next, the slides were incubated on primary polyclonal rabbit anti- human CD3 antibody (DAKO, #IS503, ready to use) for 32 minutes at RT, detected by Discovery UltraMap anti rabbit HRP and visualized by Discovery FAM kit (Ventana, # 760-364 243). After the heating step with Citrate Buffer, nuclei were subsequently visualized with Spectral DAPI (Perkin Elmer, FP1490, 1:10). Images displayed in the figures were acquired as whole slide images (WSI) with a slide scanner Zeiss Axio Scan.Z1 (objective Plan-Apochromat 20x/0.8, 3CCD camera Hitachi HV-F202SCL) and exported from the Zeiss Zen 2 lite software as TIFF images. Image analysis of WSIs was performed using QuPath(48). Regions of Interest were defined for tumor in each WSI by hand. Cells were detected based on the DAPI intensity. Next, CD3, CD4, and Foxp3 positive cells were determined by thresholds of each fluorescence intensity on QuPath.

Statistical analysis

Clinical cohort

The study population was described in terms of frequencies for qualitative variables, or medians and associated ranges for quantitative variables. All the analyses were performed on the whole population and after stratification by BC subtype. The association between TIL levels, qualitative variables, and comedications (ATC level 1,2,3) in classes were compared by student's/ANOVA tests, or in Mann Whitney U/Kruskall-Wallis tests where indicated. Interactions tests were performed when a differential effect between TILs levels and comedication was suspected across BC subtypes. The relationships between pCR and comedications are reported according to the levels 1,2,3 of the ATC. Factors predictive of pCR (clinical, pathological variables, and comedication according to ATC level 1,2,3) were introduced into a univariate logistic regression model. The covariates selected for the multivariate analysis were the clinical, pathological variables, and comedications according to ATC level 2 classes with a likelihood ratio test p -value 0.05 or lower in univariate analysis. A multivariate logistic model was then implemented using a forward stepwise selection procedure. Analyses were performed with R software, version 3.1.2 (49), with the ggplot2, dplyr, cowplot, tableone, and survival libraries.

Animal experiments

Data analyses were performed with the statistical environment Prism 6 (GraphPad, San Diego, CA, USA). Tumor size differences were calculated using Anova, Student's t-test or dedicated software (<https://kroemerlab.shinyapps.io/TumGrowth/>). Briefly, tumor growth was subjected to a linear mixed effect modeling applied to log pre-processed tumor surfaces. P -values were calculated by testing jointly whether both tumor growth slopes and intercepts (on a log scale) were different between treatment groups of interest. All reported tests are two-tailed and were considered significant at $p < 0.05$.

Results

Up to 40% BC patients at diagnosis take drugs affecting nervous, cardiovascular systems or alimentary tract

Overall, 1023 patients with different BC subtypes (luminal: 44.6% (n=456); TNBC: 31.2% (n=319), *HER2*-positive: 24.2% (n=248)) were included in the analyses. 482 patients (47.1%) took at least one comedication (total number of comedications: n=1178) and 421 (41.1%) had at least one comorbidity. The five main anatomical classes (level 1) were drugs for nervous system (Class N, n=460, 39.1%), cardiovascular diseases (class C, n=313, 26.6%), alimentary and metabolism (class A, n=199, 16.9%), and hormonal preparations (class H, n=76, 6.5%), whereas 130 comedications were grouped in the category “others” (11.0 %) (Supplementary Fig. S1, Supplementary Table S1). At level 2, the most frequent therapeutic classes were psycholeptics (N05, n=199), analgesics (N02, n=118), and psychoanaleptics (N06, n=114).

The more frequent comorbidity was hypertension / heart disease, (n=177), followed by ulcer / gastritis (n=109) (Supplementary Fig. S2A). The number of comedications was strongly associated with the number of comorbidities ($p < 0.001$) (Supplementary Fig. S2B). The majority of patients with a given comorbidity took at least one comedication from the corresponding class (57% of patients with depression/anxiety taking drugs for nervous system (N), 69% of patients with hypertension/heart disease taking cardiovascular drugs (C), 70% of patients with thyroid disorders taking drugs from class H mainly composed of thyroid therapy) (Supplementary Fig. S2C). However, the class of the comedication was not always related to the very indication (Supplementary Fig. S2D). Indeed, the use of compounds affecting the nervous system was more frequently related to self-medication than to the diagnosis of a psychiatric disease.

Patients with comedications were older, and/or more likely to be post-menopausal, and/or obese, and to have comorbidity than patients without comedication (Supplementary Table S2). Intrinsic tumor characteristics (tumor size, nodal status, grade, BC subtype, mitotic index) were not significantly associated with comedication use of any class (except for a lower tumor size in patients using a class A comedication, and a lower proportion of histologies of the non-specific type (NST) in *HER2*-positive BC patients using class N drugs). We conclude that a sizeable proportion (approximately 40%) of BC patients took a medication that may be related or not to aging and distinct classes of comorbidity. However, as an aggregate, comedication does not impact on disease presentation at diagnosis.

Some comedications are associated with pre-NAC TIL levels, mostly in TNBC.

Information on pre-NAC TIL levels was available for 615 patients (60%). The TIL density was increased in BC patients taking drugs from class H (systemic hormonal preparations (H), Fig. 1A). After stratification by BC subtype, TILs were higher in TNBC patients taking class N (nervous System), class A (alimentary tract) or class (H) drugs (Fig. 1, A to C) whereas in *HER2*-positive patients, TILs were higher in patients taking drugs from class C (cardiovascular system, Fig. 1D). Conversely, TIL levels were not different according to any comorbidity (Supplementary Fig. S3).

At the ATC level 2 (Supplementary Table S3), pre-NAC TILs were increased in patients with diuretics (C03) or thyroid therapy (H03) (Fig. 2, A to D). TIL levels were increased specifically in TNBC patients taking analgesics (N02) and drugs for [gastric] “acid related disorders” (A02) (Fig. 2, B and C). This was not found in the other BC subtypes and the interactions tests were statistically significant ($P_{\text{interaction comedication/BC subtype}} = 0.019$ and 0.027 for N02 and A02, respectively), meaning that the association of the comedication use

and TIL levels differed by BC subtype. Conversely, in TNBC patients, TILs tended to be decreased ($p=0.175$) in patients taking lipid-modifying agents (C10) and were significantly ($p=0.044$) reduced in individuals consuming anti-inflammatory and anti-rheumatic products (M01) (Fig. 2, E and F).

We next analyzed gene expression profiles (GEPs) using RNA from baseline tumor samples in pre-NAC BC patients ($n=140$). We focused on immune-related signatures that had been reported to correlate with clinical benefit in different clinical studies using immune checkpoint inhibitors for various cancer types (20). The T cell-inflamed GEP enriched in IFN γ -responsive genes related to antigen presentation, chemokine expression, cytotoxic activity and adaptive immune resistance were found in about 40% specimen (Supplementary Fig. S3). The level of the T cell-inflamed GEP or "IFN metagene" was significantly higher in patients taking hormonal preparations (whole population, luminal, *HER2*-positive, Fig. 3A), and had a differential impact according to the molecular type among patients taking drugs from class A (lower in luminal, higher in TNBC patients) (Fig. 3, B and C).

Altogether, hormonal preparations (mostly targeting thyroid disorders), nervous system-affecting drugs (such as analgesics), medications targeting cardiovascular diseases (such as diuretics) and compounds treating acid-related disorders were associated with increased lymphocytic infiltrates, and T cell inflamed GEP in all and/or triple negative BC at diagnosis. In contrast, anti-inflammatory and anti-rheumatic products were negatively correlated with TIL density.

Comedication influences pCR rates in BC

Bearing in mind the strong correlations between pre-NAC TIL density and pathological responses (1, 21), we next undertook to analyze potential associations between comedications and rates of pathological complete responses (pCR) assessed by pathologists at surgery post-NAC. The use of drugs from the class N (Nervous system) was associated with higher pCR rates than no use (Supplementary Table S4) in the whole population ($p=0.035$) and in TNBC patients ($p=0.026$). At the level 2 (Supplementary Table S5), pCR rates were increased in patients taking psycholeptics (N05), agents acting on the renin-angiotensin system (C09), and TNBC patients taking psychoanaleptics (N06) (Fig. 4, A to C). Conversely, pCR rates tended to be decreased in TNBC patients taking vasoprotective drugs (C05) or anti-inflammatory and anti-rheumatic products (M01) (Fig. 4, D and E).

After multivariate analysis, the association between psycholeptics (N05) and pCR remained statistically significant in the whole population (OR=1.64, 95%CI [1.05 - 2.55], $p=0.027$) and in TNBC patients (OR=2.04, CI [1.06 - 3.97], $p=0.034$). Accordingly, the association between pCR and agents acting on the renin-angiotensin system (C09) in *HER2*-positive BC withheld the multivariate Cox regression model (OR=3.13, CI [1.1 - 9.71], $p=0.037$) (Supplementary Table S6). No comorbidity was significantly associated with pCR after multivariate analysis.

T cell- dependent antitumor effects of zolpidem in mouse breast cancer

We next analyzed cause-effect relationships between comedications taken by patients and natural or chemotherapy-induced cancer immunosurveillance in immunodeficient or immunocompetent mice bearing BC. First, we tested the combination of bromazepam with standard of care (anthracycline based chemotherapy and taxanes) in the PDX model of TNBC

HBCx-8 inoculated in immunosuppressed animals. HBCx-8 xenografts were treated with PBS, AC (adriamycin, 2 mg/kg, and cyclophosphamide (CTX), 100 mg/kg), or docetaxel (TXT), 20 mg/kg, given as single injection at day 1 by i.p. or i.v. injections, respectively, alone or combined with the benzodiazepine bromazepam (class N, ATC level 3, anxiolytics), given orally at 0.6 mg/kg, 5 days/week. Bromazepam alone did not reduce tumor growth. Both the AC or TXT regimens mediated marked antitumor effects, followed by tumor recurrence. The addition of bromazepam to AC and TXT did not delay the time until tumor recurrence (Fig. 5A).

Based on the findings that comedications correlated with the T cell inflamed GEP and TIL densities in tumor beds (Fig. 2 and Fig. 3), and the assumption that an intact immune system is required for long lasting anticancer protective effects induced by cytotoxicants, we challenged immunocompetent mice with the transplantable AT3 triple negative mouse BC(22). AT3 showed a significant albeit minimal response to zolpidem (N05CF) (10mg/kg/day, i.p. for 30 days), an imidazopyridine nonbenzodiazepine hypnotic drug (binding with high affinity to the $\alpha 1$ subunit of the gamma amino butyric acid A receptor) (Fig. 5B, left panel) but not to the proton pump inhibitor pantoprazole (A02BC) (100mg/kg/day), when the medication was initiated 14 days prior to tumor inoculation and pursued for >14 days (Fig. 5B, middle panel). When combined to CTX (100 mg/kg weekly for 3 weeks) alone, Zolpidem (N05CF) (but not pantoprazole (A02BC)) ameliorated the anticancer effects (Fig. 5C). The additive effects of the cytotoxicant CTX and hypnotic drugs were markedly abolished after the depletion of $CD4^+$ and $CD8^+$ T cells by means of suitable antibodies (Fig. 5D), supporting the working hypothesis. Moreover, tissue immunofluorescence stainings revealed that AT3 TNBC were infiltrated with $CD3^+CD4^+$ lymphocytes that were inversely correlated with tumor size across all experiments and animals (Fig. 6A). Similarly the T effector ratio over that of regulator T cells (Treg)

negatively correlated with tumor size across all experiments (Fig. 6B). Importantly, the density of such effectors was increased by concomitant therapy with Zolpidem (N05CF) alone as compared to untreated controls. Zolpidem combined with CTX also yielded a higher density of effector T cells compared to CTX alone (Fig. 6C).

These results illustrate that specific drugs modulating the nervous system can advantageously be combined with chemotherapy to increase tumor infiltration by T lymphocytes and to reduce tumor progression in immunocompetent (but not immunodeficient) TNBC bearing hosts.

Discussion

Comedication may represent an underestimated confounding factor in many clinical trials conducted in oncology. Off-target effects mediated by non-cytotoxic drugs with a satellite role in the oncological armamentarium may have direct or indirect anti-cancer effects through several mechanisms. These mechanisms include reduction of inflammation(16), decrease of invasion and metastasis(16), modulation of angiogenesis and vasculature(23), enhancement of apoptosis, inhibition of epithelia-mesenchymal transition, reversal of hypoxia(15) and acidosis, decrease of proliferation, inhibition of critical growth or tumor suppressor pathways(24). These effects were evidenced either alone(16, 17) and in combination with other anti-cancer treatments (chemotherapy(23) or radiotherapy(25)). The concomitant use of co-medication during NAC may also affect pharmacokinetic-related parameters, in as much as a wide range of medications interfere with cytochromes. For example, cimetidine has been described to modify the pharmacokinetic of epirubicin in advanced BC patients(26). Comedications may also inhibit multi-drug resistance (MDR), involving efflux proteins of the ATP binding cassette transporter family translocating a substrate from the intracellular to the

extracellular compartment. P-glycoproteins and breast cancer resistance protein can indeed be inhibited by atorvastatin, itraconazole, verapamil or PPI(27).

In this hypothesis-generating study performed in 1023 BC patients, we found that the use of several comedication classes was associated with either increased or decreased TIL levels, some of these associations translating into increased pCR rates. While independent from the intrinsic molecular and clinical characteristics of BC at disease presentation, the use of comedications, linked to age-related morbidities, was found to correlate with immune infiltration at diagnosis, knowing that a high TIL density is a prerequisite for optimal pathological response to NAC(1, 21). Our preclinical data also support a mandatory role for T lymphocytes in the additive effects of hypnotic and cytotoxic compounds in immunocompetent tumor bearers while they failed to boost each other in PDX models established in immunodeficient hosts. These findings plead for immune related effects mediated by comedications and their capacity to shape the tumor microenvironment to pave the way to the immunomodulatory role of chemotherapy(28).

While many retro- or prospective studies evaluated the links between aspirin or NSAID and reduced cancer occurrence(29) , no such study has evaluated the potential impact of daily administration of other types of self-medication or prescription by the general practitioner on immune functions and cancer immunosurveillance. In a randomized controlled trial in 38 BC patients, perioperative COX-2 and β -adrenergic blockade by propranolol and etodolac was associated with changes in immune profiles of surgical specimens with notably decreased tumor-infiltrating monocytes and increased tumor-infiltrating B cells(16). Moreover, retrospective clinical data suggest that some anesthetic techniques can attenuate immunosuppression and minimize metastasis after cancer surgery (30). For example, in

patients undergoing breast cancer surgery, propofol anesthesia with postoperative ketorolac analgesia reportedly has a favorable impact on NK cell cytotoxicity compared with sevoflurane anesthesia and postoperative fentanyl analgesia (31).

Several mechanisms have been proposed to account for these off-target effects of distinct compounds, not necessarily annotated as "cytotoxic agents". ER stress response inducers (*i.e.* taspigargin(32) or cardiac glycosides(33) or autophagy inducers (such as aspirin, spermidine, hydroxycitrate (34) could mediate a cellular stress of cancer cells associated with secretion of alarmins or cell surface expression of danger signals igniting the inflammasome and/or pattern recognition receptors(28). These cell autonomous changes of cancerous cells preceding immunogenic cell death pave the way to synergistic anticancer activities when these compounds are combined with conventional chemotherapy, radiotherapy or targeted treatments. Other comedications can reprogram the tumor microenvironment by dampening myeloid suppressor cells. Thus, the anti-diabetic biguanide metformin may yield clinical benefit in ovarian cancer patients through improvement of antitumor T-cell immunity by dampening CD39/CD73-dependent MDSC immunosuppression(35). Metformin may also act on the cognate arm of immunity *i.e.* CD8⁺ TILs and protect them from apoptosis and exhaustion(14), thereby potentiating the efficacy of PD-1 blockade. In addition, proton pump inhibitors could cause reversal of acidity-induced cancer immune escape(18) and modulate myelopoiesis and the polarization of tumor associated macrophages(36). Compounds impacting the nervous system, more specifically hypnotic drugs are broadly prescribed. The hypnotic zolpidem was associated with higher TIL density in our retrospective clinical study and turned out to be immunogenic in combination with CTX in our preclinical AT3 model. These proinflammatory effects are consistent with correlative associations reported in a large population-based study of >59 000 individuals in the National Health Insurance Research Database (NHIRD) of Taiwan performed among patients with sleep disturbance taking

zolpidem for at least 2 years (n=14 000 patients). The authors found positive associations between the use of zolpidem and the risk of ischemic stroke(37), of Parkinson disease after 5 years of follow-up(38) and of cancer occurrence(39) (oral cancer (HR, 2.36; 95% CI, 1.57-3.56), as well as kidney cancer, esophageal cancer, and BC). Conversely, GABAergic modulation with classical benzodiazepines lorazepam and clonazepam, aside from exerting anxiolytic and antidepressant effects, may have therapeutic potential as neuroimmunomodulators during psychosocial stress. Lorazepam and clonazepam as well as the antidepressant imipramine blocked stress-induced accumulation of macrophages in the central nervous system, prevented neuroinflammatory signaling and reversed anxiety-like and depressive-like behavior in mice exposed to repeated social defeat(40). The use of beta-blocker, specifically the selective blockade of β_2 adrenergic receptors, correlated with better overall survival in metastatic melanoma patients and improved the efficacy of anti-PD1 and IL-2-based immunotherapies mobilizing T lymphocytic effectors in mice(41). Conversely, in another experimental study where epinephrine-mobilized NK cells prevented tumor outgrowth following exercise, β -adrenergic signaling blunted training-dependent tumor inhibition and the trafficking of IL-6-dependent NK effectors into the tumor bed(42). These apparent contradictions highlight the need for mechanistic exploration of the synergistic or antagonistic off-target bioactivity of these comedications.

We found that the effect of comedications on TILs and pCR varied by BC subtype. The multiple interactions and the high number of drugs to explore on a single cohort highlights the need for large-scale validation studies to address the immense complexity that likely underlies the interactions between comedication, immune infiltration and chemotherapy outcome. Only very large patient cohorts would provide the sufficient statistical power for meaningful comparisons among tumor and drug subgroups.

Our work opens several thrilling perspectives in drug repositioning. It paves the way to explore the field of comedications as immunomodulators and chemotherapy sensitizers. As nearly half of the patients take one or more comedications, a considerable amount of untapped data is already available for exploitation in electronic health records of patients treated with NAC in cancer centers. We hypothesize that a variety of drugs that are not usual part of the oncological armamentarium may exert off-target effects against BC or other cancer types. Digging into such real-life data could help to identify drugs or life style experiences that improve the response to antineoplastic treatments, followed by the design of clinical trials to quickly validate these hypotheses. In a context where the financial burden of innovative oncologic therapies jeopardizes health systems, repositioning routine prescriptions as anticancer treatments sensitizers could be an exciting strategy.

Acknowledgments: We are thankful to the animal facility team of Gustave Roussy and all the technicians from Centre GF Leclerc. LZ and GK were supported by the Ligue contre le Cancer (équipe labellisée); Agence National de la Recherche (ANR) – Projets blancs; ANR under the frame of E-Rare-2, the ERA-Net for Research on Rare Diseases; Association pour la recherche sur le cancer (ARC); Cancéropôle Ile-de-France; Chancellerie des universités de Paris (Legs Poix), Fondation de France; Fondation pour la Recherche Médicale (FRM); a donation by Elior; the European Commission (ArtForce); the European Research Council (ERC); Fondation Carrefour; Institut National du Cancer (INCa); Inserm (HTE); Institut Universitaire de France; LeDucq Foundation; the LabEx Immuno-Oncology; the RHU Torino Lumière; the Swiss Bridge Foundation; the Seerave and Carrefour Foundation; the SIRIC Stratified Oncology Cell DNA Repair and Tumor Immune Elimination (SOCRATE); the SIRIC Cancer Research and Personalized Medicine (CARPEM); and the Paris Alliance of Cancer Research Institutes (PACRI).

Author Contributions: Conceptualization, F.R. and L.Z.; Experimentation and resources, S.Y., P.O., A.P., E. Marangoni, E.Montlaudon; Formal Analysis, A.-S. H., L. Derosa.; Data acquisition: M.P., J. G., D.d.C., M.L.; Data Curation ; C.V., L.-S. T., L.Darrigues; Methodology, B.A., E.L.; Writing – Original Draft, A.-S. H., L.Derosa. F.R., and L.Z.; Writing – Review & Editing, F.R., G.K. and L.Z.; Supervision, F.R. and L.Z.; Funding Acquisition, F.R., and L.Z.

References

1. C. Denkert, S. Loibl, A. Noske, M. Roller, B. M. Müller, M. Komor, J. Budczies, S. Darb-Esfahani, R. Kronenwett, C. Hanusch, C. von Törne, W. Weichert, K. Engels, C. Solbach, I. Schrader, M. Dietel, G. von Minckwitz, Tumor-associated lymphocytes as an independent predictor of response to neoadjuvant chemotherapy in breast cancer, *J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol.* **28**, 105–113 (2010).
2. C. Denkert, G. von Minckwitz, S. Darb-Esfahani, B. Lederer, B. I. Heppner, K. E. Weber, J. Budczies, J. Huober, F. Klauschen, J. Furlanetto, W. D. Schmitt, J.-U. Blohmer, T. Karn, B. M. Pfitzner, S. Kümmel, K. Engels, A. Schneeweiss, A. Hartmann, A. Noske, P. A. Fasching, C. Jackisch, M. van Mackelenbergh, P. Sinn, C. Schem, C. Hanusch, M. Untch, S. Loibl, Tumour-infiltrating lymphocytes and prognosis in different subtypes of breast cancer: a pooled analysis of 3771 patients treated with neoadjuvant therapy, *Lancet Oncol.* **19**, 40–50 (2018).
3. M. S. Rooney, S. A. Shukla, C. J. Wu, G. Getz, N. Hacohen, Molecular and genetic properties of tumors associated with local immune cytolytic activity, *Cell* **160**, 48–61 (2015).
4. S. Loi, S. Dushyanthen, P. A. Beavis, R. Salgado, C. Denkert, P. Savas, S. Combs, D. L. Rimm, J. M. Giltane, M. V. Estrada, V. Sánchez, M. E. Sanders, R. S. Cook, M. A. Pilkinton, S. A. Mallal, K. Wang, V. A. Miller, P. J. Stephens, R. Yelensky, F. D. Doimi, H. Gómez, S. V. Ryzhov, P. K. Darcy, C. L. Arteaga, J. M. Balko, RAS/MAPK Activation Is Associated with Reduced Tumor-Infiltrating Lymphocytes in Triple-Negative Breast Cancer: Therapeutic Cooperation Between MEK and PD-1/PD-L1 Immune Checkpoint Inhibitors, *Clin. Cancer Res.* **22**, 1499–1509 (2016).
5. S. Ladoire, D. Enot, L. Senovilla, F. Ghiringhelli, V. Poirier-Colame, K. Chaba, M.

- Semeraro, M. Chaix, F. Penault-Llorca, L. Arnould, M. L. Poillot, P. Arveux, S. Delalogue, F. Andre, L. Zitvogel, G. Kroemer, The presence of LC3B puncta and HMGB1 expression in malignant cells correlate with the immune infiltrate in breast cancer, *Autophagy* **12**, 864–875 (2016).
6. E. N. Fish, The X-files in immunity: sex-based differences predispose immune responses, *Nat. Rev. Immunol.* **8**, 737–744 (2008).
 7. L. Lazuardi, B. Jenewein, A. M. Wolf, G. Pfister, A. Tzankov, B. Grubeck-Loebenstein, Age-related loss of naïve T cells and dysregulation of T-cell/B-cell interactions in human lymph nodes, *Immunology* **114**, 37–43 (2005).
 8. H. A. Azim, A. Vingiani, F. Peccatori, G. Viale, S. Loi, G. Pruneri, Tumour infiltrating lymphocytes (TILs) in breast cancer during pregnancy, *Breast Edinb. Scotl.* **24**, 290–293 (2015).
 9. P. J. Goodwin, V. Stambolic, J. Lemieux, B. E. Chen, W. R. Parulekar, K. A. Gelmon, D. L. Hershman, T. J. Hobday, J. A. Ligibel, I. A. Mayer, K. I. Pritchard, T. J. Whelan, P. Rastogi, L. E. Shepherd, Evaluation of metformin in early breast cancer: a modification of the traditional paradigm for clinical testing of anti-cancer agents, *Breast Cancer Res. Treat.* **126**, 215–220 (2011).
 10. T. P. Ahern, L. Pedersen, M. Tarp, D. P. Cronin-Fenton, J. P. Garne, R. A. Silliman, H. T. Sørensen, T. L. Lash, Statin prescriptions and breast cancer recurrence risk: a Danish nationwide prospective cohort study, *J. Natl. Cancer Inst.* **103**, 1461–1468 (2011).
 11. M. L. Kwan, L. A. Habel, M. L. Slattery, B. Caan, NSAIDs and breast cancer recurrence in a prospective cohort study, *Cancer Causes Control CCC* **18**, 613–620 (2007).
 12. D. G. Powe, M. J. Voss, K. S. Zänker, H. O. Habashy, A. R. Green, I. O. Ellis, F. Entschladen, Beta-blocker drug therapy reduces secondary cancer formation in breast cancer and improves cancer specific survival, *Oncotarget* **1**, 628–638 (2010).
 13. J. Haukka, L. Niskanen, A. Auvinen, Risk of Cause-Specific Death in Individuals with Cancer-Modifying Role Diabetes, Statins and Metformin, *Int. J. Cancer* (2017), doi:10.1002/ijc.31016.
 14. S. Eikawa, M. Nishida, S. Mizukami, C. Yamazaki, E. Nakayama, H. Uono, Immune-mediated antitumor effect by type 2 diabetes drug, metformin, *Proc. Natl. Acad. Sci. U. S. A.* **112**, 1809 (2015).
 15. N. E. Scharping, A. V. Menk, R. D. Whetstone, X. Zeng, G. M. Delgoffe, Efficacy of PD-1 Blockade Is Potentiated by Metformin-Induced Reduction of Tumor Hypoxia, *Cancer Immunol. Res.* **5**, 9–16 (2017).
 16. L. Shaashua, M. Shabat-Simon, R. Haldar, P. Matzner, O. Zmora, M. Shabtai, E. Sharon, T. Allweis, I. Barshack, L. Hayman, J. Arevalo, J. Ma, M. Horowitz, S. Cole, S. Ben-Eliyahu, Perioperative COX-2 and β -Adrenergic Blockade Improves Metastatic Biomarkers in Breast Cancer Patients in a Phase-II Randomized Trial, *Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res.* (2017), doi:10.1158/1078-0432.CCR-17-0152.
 17. S. Junankar, G. Shay, J. Jurczyk, N. Ali, J. Down, N. Pocock, A. Parker, A. Nguyen, S. Sun, B. Kashemirov, C. E. McKenna, P. I. Croucher, A. Swarbrick, K. Weilbaecher, T. G. Phan, M. J. Rogers, Real-time intravital imaging establishes tumor-associated macrophages as the extraskelatal target of bisphosphonate action in cancer, *Cancer Discov.* **5**, 35–42 (2015).
 18. A. Calcinotto, P. Filipazzi, M. Grioni, M. Iero, A. D. Milito, A. Ricupito, A. Cova, R. Canese, E. Jachetti, M. Rossetti, V. Huber, G. Parmiani, L. Generoso, M. Santinami, M. Borghi, S. Fais, M. Bellone, L. Rivoltini, Modulation of Microenvironment Acidity Reverses Anergy in Human and Murine Tumor-Infiltrating T Lymphocytes, *Cancer Res.* **72**, 2746–2756 (2012).
 19. J. A. Califano, Z. Khan, K. A. Noonan, L. Rudraraju, Z. Zhang, H. Wang, S. Goodman, C. G. Gourin, P. K. Ha, C. Fakhry, J. Saunders, M. Levine, M. Tang, G. Neuner, J. D. Richmon,

- R. Blanco, N. Agrawal, W. M. Koch, S. Marur, D. T. Weed, P. Serafini, I. Borrello, Tadalafil augments tumor specific immunity in patients with head and neck squamous cell carcinoma, *Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res.* **21**, 30–38 (2015).
20. M. Ayers, J. Lunceford, M. Nebozhyn, E. Murphy, A. Loboda, D. R. Kaufman, A. Albright, J. D. Cheng, S. P. Kang, V. Shankaran, S. A. Piha-Paul, J. Yearley, T. Y. Seiwert, A. Ribas, T. K. McClanahan, IFN- γ -related mRNA profile predicts clinical response to PD-1 blockade, *J. Clin. Invest.* **127**, 2930–2940 (2017).
21. C. Denkert, G. von Minckwitz, J. C. Brase, B. V. Sinn, S. Gade, R. Kronenwett, B. M. Pfitzner, C. Salat, S. Loi, W. D. Schmitt, C. Schem, K. Fisch, S. Darb-Esfahani, K. Mehta, C. Sotiriou, S. Wienert, P. Klare, F. André, F. Klauschen, J.-U. Blohmer, K. Krappmann, M. Schmidt, H. Tesch, S. Kümmel, P. Sinn, C. Jackisch, M. Dietel, T. Reimer, M. Untch, S. Loibl, Tumor-infiltrating lymphocytes and response to neoadjuvant chemotherapy with or without Carboplatin in human epidermal growth factor receptor 2-positive and triple-negative primary breast cancers, *J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol.* **33**, 983–991 (2015).
22. J. Liu, S. J. Blake, M. C. R. Yong, H. Harjunpää, S. F. Ngiew, K. Takeda, A. Young, J. S. O'Donnell, S. Allen, M. J. Smyth, M. W. L. Teng, Improved Efficacy of Neoadjuvant Compared to Adjuvant Immunotherapy to Eradicate Metastatic Disease, *Cancer Discov.* **6**, 1382–1399 (2016).
23. V. P. Chauhan, J. D. Martin, H. Liu, D. A. Lacorre, S. R. Jain, S. V. Kozin, T. Stylianopoulos, A. S. Mousa, X. Han, P. Adstamongkonkul, Z. Popović, P. Huang, M. G. Bawendi, Y. Boucher, R. K. Jain, Angiotensin inhibition enhances drug delivery and potentiates chemotherapy by decompressing tumour blood vessels, *Nat. Commun.* **4**, 2516 (2013).
24. S. Fujihara, A. Morishita, K. Ogawa, T. Tadokoro, T. Chiyo, K. Kato, H. Kobara, H. Mori, H. Iwama, T. Masaki, The angiotensin II type 1 receptor antagonist telmisartan inhibits cell proliferation and tumor growth of esophageal adenocarcinoma via the AMPKa/mTOR pathway in vitro and in vivo, *Oncotarget* **8**, 8536–8549 (2016).
25. J. Cong, Y. Wang, X. Zhang, N. Zhang, L. Liu, K. Soukup, T. Michelakos, T. Hong, A. DeLeo, L. Cai, F. Sabbatino, S. Ferrone, H. Lee, V. Levina, B. Fuchs, K. Tanabe, K. Lillemoe, C. Ferrone, X. Wang, A novel chemoradiation targeting stem and nonstem pancreatic cancer cells by repurposing disulfiram, *Cancer Lett.* (2017), doi:10.1016/j.canlet.2017.08.028.
26. L. S. Murray, D. I. Jodrell, J. G. Morrison, A. Cook, D. J. Kerr, B. Whiting, S. B. Kaye, J. Cassidy, The effect of cimetidine on the pharmacokinetics of epirubicin in patients with advanced breast cancer: Preliminary evidence of a potentially common drug interaction, *Clin. Oncol.* **10**, 35–38 (1998).
27. F. Bertolini, V. P. Sukhatme, G. Bouche, Drug repurposing in oncology—patient and health systems opportunities, *Nat. Rev. Clin. Oncol.* **12**, 732–742 (2015).
28. L. Galluzzi, A. Buqué, O. Kepp, L. Zitvogel, G. Kroemer, Immunological Effects of Conventional Chemotherapy and Targeted Anticancer Agents, *Cancer Cell* **28**, 690–714 (2015).
29. B. Trabert, R. B. Ness, W.-H. Lo-Ciganic, M. A. Murphy, E. L. Goode, E. M. Poole, L. A. Brinton, P. M. Webb, C. M. Nagle, S. J. Jordan, Australian Ovarian Cancer Study Group, Australian Cancer Study (Ovarian Cancer), H. A. Risch, M. A. Rossing, J. A. Doherty, M. T. Goodman, G. Lurie, S. K. Kjær, E. Hogdall, A. Jensen, D. W. Cramer, K. L. Terry, A. Vitonis, E. V. Bandera, S. Olson, M. G. King, U. Chandran, H. Anton-Culver, A. Ziogas, U. Menon, S. A. Gayther, S. J. Ramus, A. Gentry-Maharaj, A. H. Wu, C. L. Pearce, M. C. Pike, A. Berchuck, J. M. Schildkraut, N. Wentzensen, Ovarian Cancer Association Consortium, Aspirin, nonaspirin nonsteroidal anti-inflammatory drug, and acetaminophen use and risk of invasive epithelial ovarian cancer: a pooled analysis in the Ovarian Cancer Association

- Consortium, *J. Natl. Cancer Inst.* **106**, djt431 (2014).
30. X. Chen, P. Lu, L. Chen, S. Yang, H.-Y. Shen, D. Yu, X. Zhang, S. Zhong, J. Zhao, J. Tang, Perioperative propofol-paravertebral anesthesia decreases the metastasis and progression of breast cancer, *Tumour Biol. J. Int. Soc. Oncodevelopmental Biol. Med.* **36**, 8259–8266 (2015).
31. J. S. Cho, M.-H. Lee, S. I. Kim, S. Park, H. S. Park, E. Oh, J. H. Lee, B.-N. Koo, The Effects of Perioperative Anesthesia and Analgesia on Immune Function in Patients Undergoing Breast Cancer Resection: A Prospective Randomized Study, *Int. J. Med. Sci.* **14**, 970–976 (2017).
32. I. Martins, O. Kepp, F. Schlemmer, S. Adjemian, M. Tailler, S. Shen, M. Michaud, L. Menger, A. Gdoura, N. Tajeddine, A. Tesniere, L. Zitvogel, G. Kroemer, Restoration of the immunogenicity of cisplatin-induced cancer cell death by endoplasmic reticulum stress, *Oncogene* **30**, 1147–1158 (2011).
33. L. Menger, E. Vacchelli, S. Adjemian, I. Martins, Y. Ma, S. Shen, T. Yamazaki, A. Q. Sukkurwala, M. Michaud, G. Mignot, F. Schlemmer, E. Sulpice, C. Locher, X. Gidrol, F. Ghiringhelli, N. Modjtahedi, L. Galluzzi, F. André, L. Zitvogel, O. Kepp, G. Kroemer, Cardiac glycosides exert anticancer effects by inducing immunogenic cell death, *Sci. Transl. Med.* **4**, 143ra99 (2012).
34. F. Madeo, T. Eisenberg, F. Pietrocola, G. Kroemer, Spermidine in health and disease, *Science* **359** (2018), doi:10.1126/science.aan2788.
35. L. Li, L. Wang, J. Li, Z. Fan, L. Yang, Z. Zhang, C. Zhang, D. Yue, G. Qin, T. Zhang, F. Li, X. Chen, Y. Ping, D. Wang, Q. Gao, Q. He, L. Huang, H. Li, J. Huang, X. Zhao, W. Xue, Z. Sun, J. Lu, J. J. Yu, J. Zhao, B. Zhang, Y. Zhang, Metformin-Induced Reduction of CD39 and CD73 Blocks Myeloid-Derived Suppressor Cell Activity in Patients with Ovarian Cancer, *Cancer Res.* **78**, 1779–1791 (2018).
36. N. K. Vishvakarma, S. M. Singh, Augmentation of myelopoiesis in a murine host bearing a T cell lymphoma following in vivo administration of proton pump inhibitor pantoprazole, *Biochimie* **93**, 1786–1796 (2011).
37. W.-S. Huang, C.-H. Tsai, C.-C. Lin, C.-H. Muo, F.-C. Sung, Y.-J. Chang, C.-H. Kao, Relationship between zolpidem use and stroke risk: a Taiwanese population-based case-control study, *J. Clin. Psychiatry* **74**, e433-438 (2013).
38. Y.-W. Yang, T.-F. Hsieh, C.-H. Yu, Y.-S. Huang, C.-C. Lee, T.-H. Tsai, Zolpidem and the risk of Parkinson's disease: a nationwide population-based study, *J. Psychiatr. Res.* **58**, 84–88 (2014).
39. C.-H. Kao, L.-M. Sun, J.-A. Liang, S.-N. Chang, F.-C. Sung, C.-H. Muo, Relationship of Zolpidem and Cancer Risk: A Taiwanese Population-Based Cohort Study, *Mayo Clin. Proc.* **87**, 430–436 (2012).
40. K. Ramirez, A. Niraula, J. F. Sheridan, GABAergic modulation with classical benzodiazepines prevent stress-induced neuro-immune dysregulation and behavioral alterations, *Brain. Behav. Immun.* **51**, 154–168 (2016).
41. K. M. Kokolus, Y. Zhang, J. M. Sivik, C. Schmeck, J. Zhu, E. A. Repasky, J. J. Drabick, T. D. Schell, Beta blocker use correlates with better overall survival in metastatic melanoma patients and improves the efficacy of immunotherapies in mice, *Oncoimmunology* **7**, e1405205 (2018).
42. L. Pedersen, M. Idorn, G. H. Olofsson, B. Lauenborg, I. Nookaew, R. H. Hansen, H. H. Johannesen, J. C. Becker, K. S. Pedersen, C. Dethlefsen, J. Nielsen, J. Gehl, B. K. Pedersen, P. Thor Straten, P. Hojman, Voluntary Running Suppresses Tumor Growth through Epinephrine- and IL-6-Dependent NK Cell Mobilization and Redistribution, *Cell Metab.* **23**, 554–562 (2016).
43. R. Salgado, C. Denkert, S. Demaria, N. Sirtaine, F. Klauschen, G. Pruneri, S. Wienert, G.

- Van den Eynden, F. L. Baehner, F. Penault-Llorca, E. A. Perez, E. A. Thompson, W. F. Symmans, A. L. Richardson, J. Brock, C. Criscitiello, H. Bailey, M. Ignatiadis, G. Floris, J. Sparano, Z. Kos, T. Nielsen, D. L. Rimm, K. H. Allison, J. S. Reis-Filho, S. Loibl, C. Sotiropoulos, G. Viale, S. Badve, S. Adams, K. Willard-Gallo, S. Loi, The evaluation of tumor-infiltrating lymphocytes (TILs) in breast cancer: recommendations by an International TILs Working Group 2014, *Ann. Oncol. Off. J. Eur. Soc. Med. Oncol. ESMO* **26**, 259–271 (2015).
44. P. de Cremoux, F. Valet, D. Gentien, J. Lehmann-Che, V. Scott, C. Tran-Perennou, C. Barbaroux, N. Servant, S. Vacher, B. Sigal-Zafrani, M.-C. Mathieu, P. Bertheau, J.-M. Guinebretière, B. Asselain, M. Marty, F. Spyrtos, Importance of pre-analytical steps for transcriptome and RT-qPCR analyses in the context of the phase II randomised multicentre trial REMAGUS02 of neoadjuvant chemotherapy in breast cancer patients, *BMC Cancer* **11**, 215 (2011).
45. S. B. Edge, C. C. Compton, The American Joint Committee on Cancer: the 7th edition of the AJCC cancer staging manual and the future of TNM, *Ann. Surg. Oncol.* **17**, 1471–1474 (2010).
46. E. Provenzano, V. Bossuyt, G. Viale, D. Cameron, S. Badve, C. Denkert, G. MacGrogan, F. Penault-Llorca, J. Boughey, G. Curigliano, J. M. Dixon, L. Esserman, G. Fastner, T. Kuehn, F. Peintinger, G. von Minckwitz, J. White, W. Yang, W. F. Symmans, Residual Disease Characterization Working Group of the Breast International Group-North American Breast Cancer Group Collaboration, Standardization of pathologic evaluation and reporting of postneoadjuvant specimens in clinical trials of breast cancer: recommendations from an international working group, *Mod. Pathol. Off. J. U. S. Can. Acad. Pathol. Inc* **28**, 1185–1201 (2015).
47. E. Marangoni, A. Vincent-Salomon, N. Auger, A. Degeorges, F. Assayag, P. de Cremoux, L. de Plater, C. Guyader, G. De Pinieux, J.-G. Judde, M. Rebucci, C. Tran-Perennou, X. Sastre-Garau, B. Sigal-Zafrani, O. Delattre, V. Diéras, M.-F. Poupon, A new model of patient tumor-derived breast cancer xenografts for preclinical assays, *Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res.* **13**, 3989–3998 (2007).
48. P. Bankhead, M. B. Loughrey, J. A. Fernández, Y. Dombrowski, D. G. McArt, P. D. Dunne, S. McQuaid, R. T. Gray, L. J. Murray, H. G. Coleman, J. A. James, M. Salto-Tellez, P. W. Hamilton, QuPath: Open source software for digital pathology image analysis, *Sci. Rep.* **7**, 16878 (2017).
49. R Development Core Team, R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing. (2011) (available at URL <http://www.R-project.org/>).
50. D. P. Enot, E. Vacchelli, N. Jacquelot, L. Zitvogel, G. Kroemer, TumGrowth: An open-access web tool for the statistical analysis of tumor growth curves, *Oncoimmunology* **7**, e1462431 (2018).

FIGURE LEGENDS

Fig. 1. Pre-NAC TILs densities by comedication use (ATC level 1) in the whole population and by BC subtype.

Fig. 2. Pre-NAC TILs densities by comedication use (ATC level 2) in the whole population and by BC subtype.

Fig. 3. Levels of the T cell–inflamed gene expression profile or "IFN metagene" in the whole population and by BC subtype.

Fig. 4. Pathological complete response (pCR) rates by comedication use (ATC level 2) in the whole population and by BC subtype.

Fig. 5. Immune effects of co-medication in mouse breast cancer models.

Fig. 6. Comedications influence TIL densities in mo

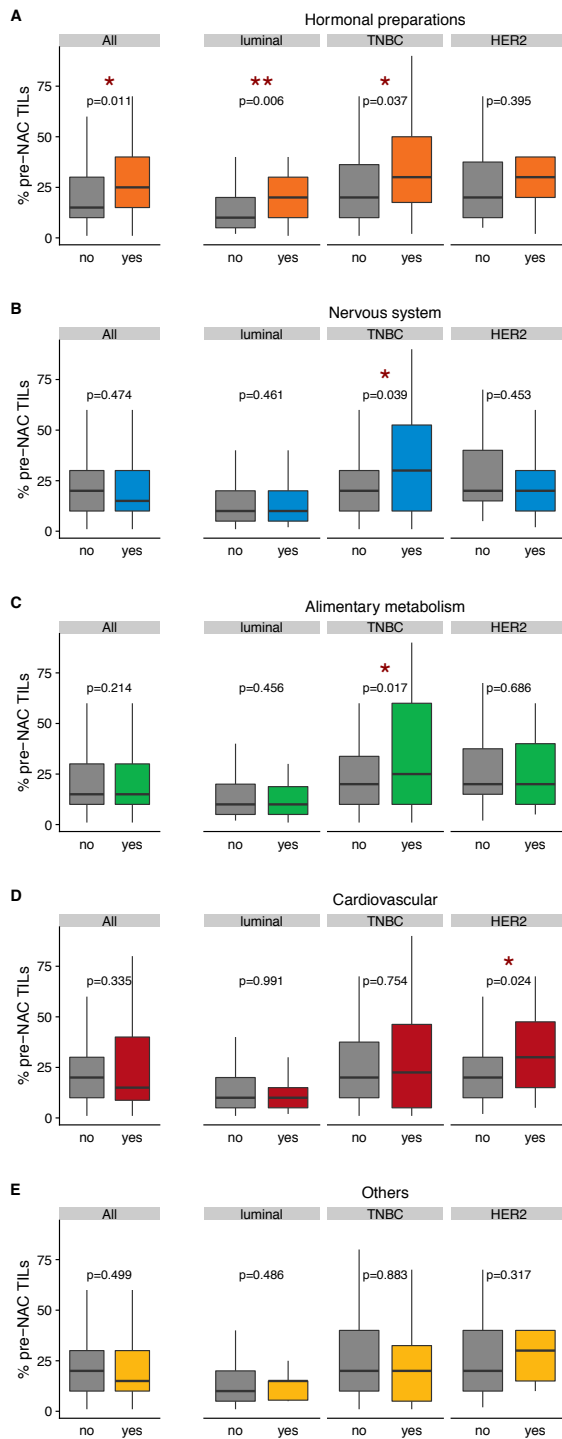


Fig. 1. Pre-NAC TILs densities by comedication use (ATC level 1) in the whole population and by BC subtype. The TIL density (% pre-NAC TILs) was scored continuously as the average percentage of stromal area occupied by mononuclear cells as previously recommended(43). In the x-axis, patients were classified according to their use ("yes") or absence of use ("no") of a comedication. **(A)** Systemic hormonal preparations (class H); **(B)** Nervous system (class N); **(C)** Alimentary and metabolism (class A); **(D)** Cardiovascular (class C); **(E)** Others. In boxplots, lower and upper bars represent the first and third quartile respectively, the medium bar is the median, and whiskers extend to 1.5 times the inter-quartile range. TIL density was compared in Wilcoxon-Mann-Whitney tests (for groups including less than 30 patients) or with student t-test (n ≥30).

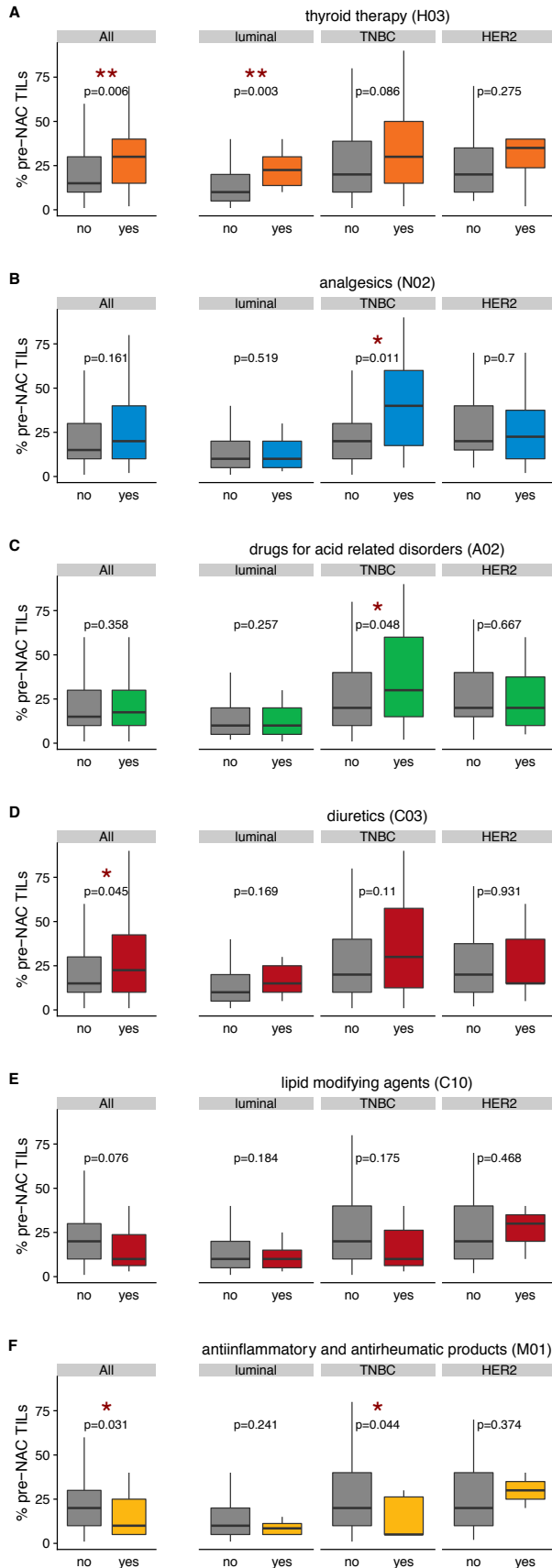


Fig. 2. Pre-NAC TILs densities by comedication use (ATC level 2) in the whole population and by BC subtype.

The TIL density (% pre-NAC TILs) was scored continuously as the average percentage of stromal area occupied by mononuclear cells as previously recommended(43). In the x-axis, patients were classified according to their use ("yes") or absence of use ("no") of a co-medication. **(A)** Thyroid therapy (class H03); **(B)** Analgesics (class N02); **(C)** Drugs for acid related disorders (class A02); **(D)**: Diuretics (C03); **(E)** Lipid modifying agents (C10); **(F)** Anti-inflammatory and anti-rheumatic products (M01). In boxplots, lower and upper bars represent the first and third quartile respectively, the medium bar is the median, and whiskers extend to 1.5 times the inter-quartile range. TIL density was compared in Wilcoxon-Mann-Whitney tests (for groups including less than 30 patients) or with student t-test (n ≥30).

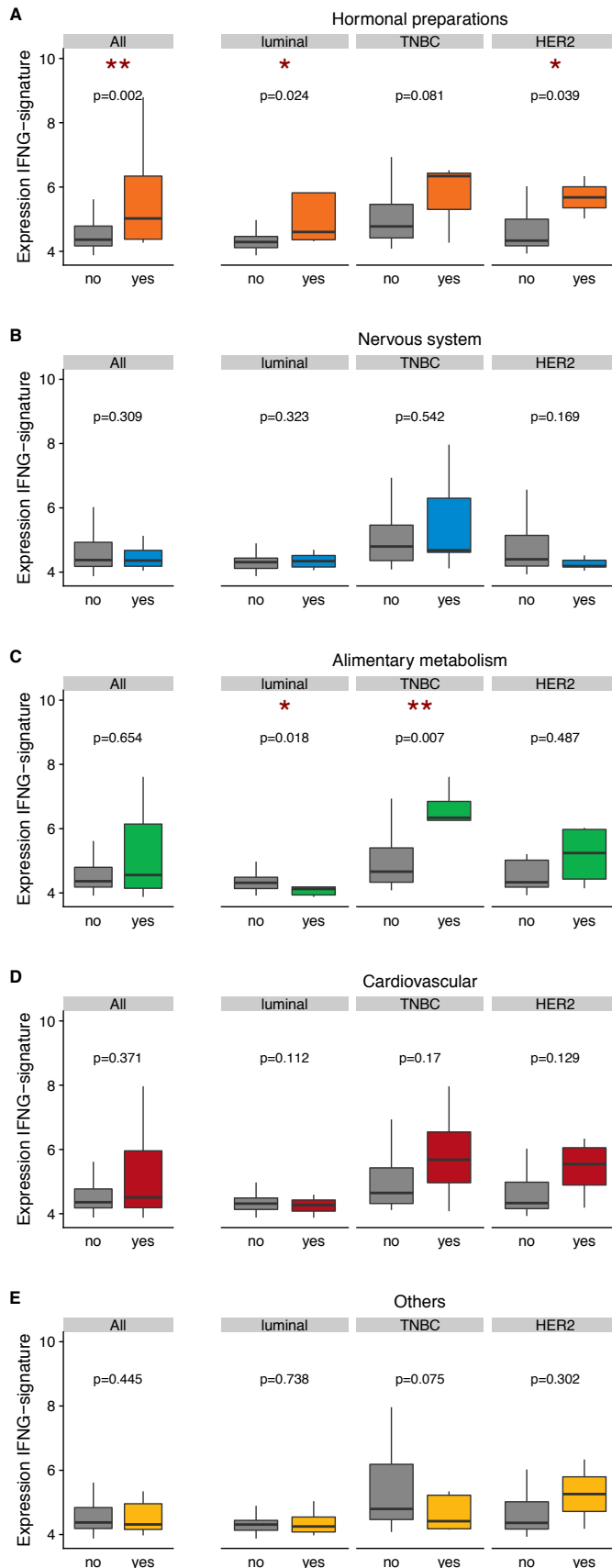


Fig. 3. Levels of the T cell-inflamed gene expression profile or "IFN metagene" in the whole population and by BC subtype.

Gene expression profiling on 140 pre-NAC tumor samples centered around the IFN- γ metagene described in Ayers *et al.*(20) quantified according to the mean normalized expression value for 6 genes (*IFNG*, *IDO1*, *CXCL9*, *CXCL10*, *HLA-DRA*, *STAT1*). In the x-axis, patients were classified according to their use ("yes") or absence of use ("no") of a co-medication. (A) Systemic hormonal preparations (class H); (B) Nervous system (class N); (C) Alimentary and metabolism (class A); (D) Cardiovascular (class C); (E) Others. In boxplots, lower and upper bars represent the first and third quartile respectively, the medium bar is the median, and whiskers extend to 1.5 times the inter-quartile range. IFN-g-metagene levels were compared in Wilcoxon-Mann-Whitney tests (for groups including less than 30 samples) or with student t-test ($n \geq 30$).

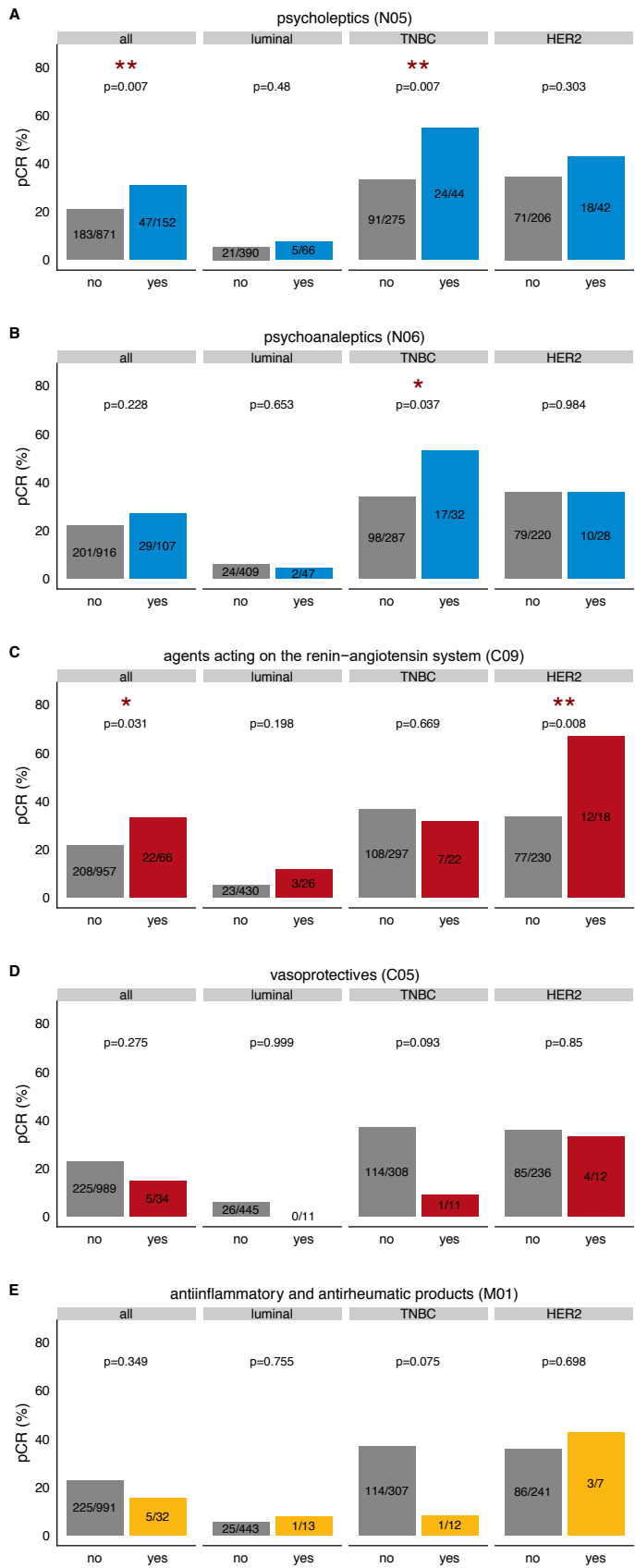


Fig. 4. Pathological complete response (pCR) rates by comedication use (ATC level 2) in the whole population and by BC subtype.

The pCR was assessed according to routine clinical guidelines(46). Effectives mentioned on the barplot represent the number of patients whose tumor reached pCR / total number of patients of the given category. In the x-axis, patients were classified according to their use ("yes") or absence of use ("no") of a comedication. (A) Psycholeptics (N05); (B) Psychoanalectics (N06); (C) agents acting on the renin-angiotensin system (class C09); (D) Vasoprotectives (class C05); (E) Anti-inflammatory and anti-rheumatic products (M01). The association between categorical variables was assessed with chi-square test or with the Fisher's exact test if at least one category showed less than 3 patients.

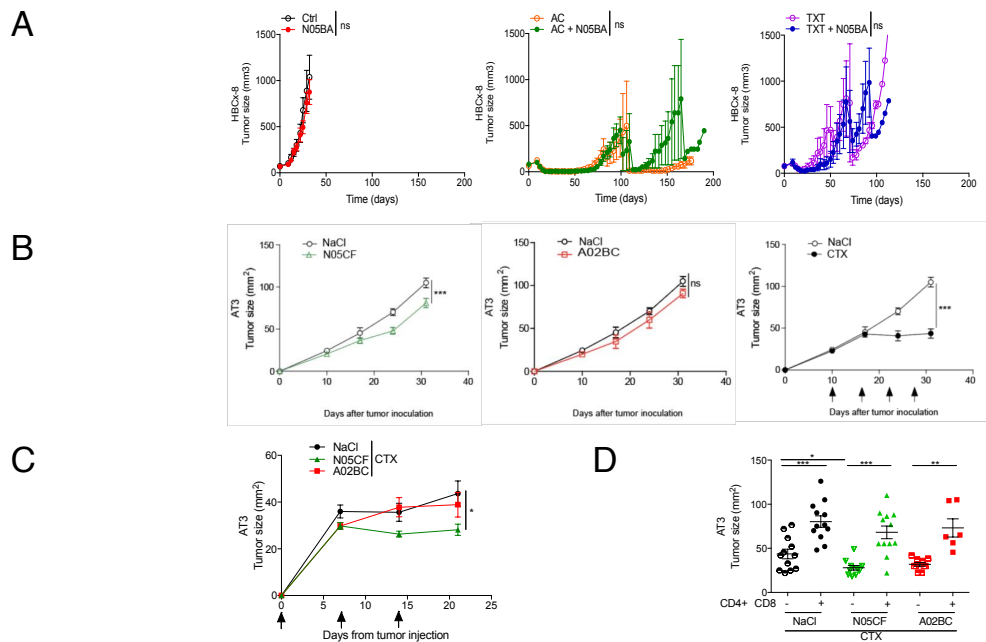


Fig. 5. Immune effects of co-medication in mouse breast cancer models.

(A) The PDX HBCx-8 xenograft established from a TNBC patient was transplanted into female 8-week-old Swiss nude mice and then, randomly assigned to the control or treatment groups (AC versus TXT alone or combined with bromazepam (N05BA)). Tumor growth kinetics with broma alone versus Ctrl, AC versus AC+ N05BA and TXT versus TXT+ N05BA are represented overtime, in six animals/group, in a representative experiment out of two yielding similar conclusions. Statistical analyses(50): * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns=not significant. (B) and (C). Prophylactic and therapeutic i.p. administration of zolpidem (N05CF) or pantoprazole (A02BC) versus NaCl alone (B) or in combination with Cyclophosphamide (CTX) (C) in C57Bl/6 mice bearing the TNBC AT3. (D) Depletion of CD4⁺ or CD8⁺ lymphocytes with specific antibodies in the same setting as in (C). Tumor growth kinetics are depicted for a pool of two independent experiments comprising 6 mice/groups for (B) and (D). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns=not significant.

Anne-Sophie Hamy and Lisa Derosa et al. submitted to Cancer Immunology Research

34

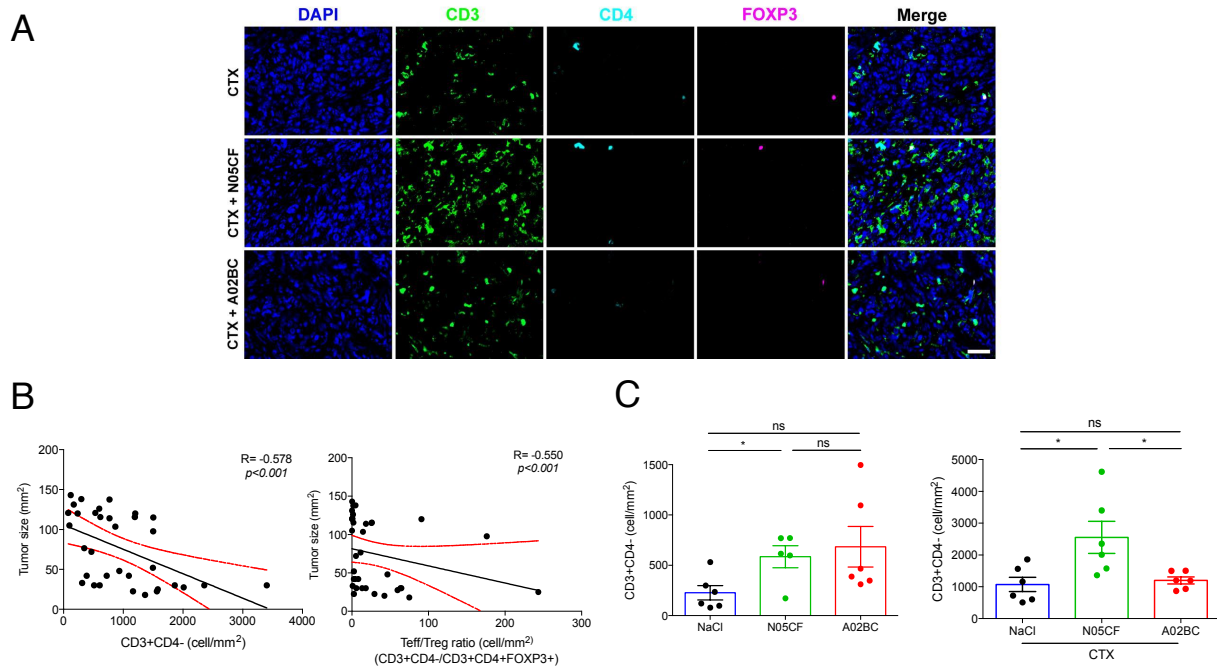


Fig.6. Comedications influence TIL densities in mouse TNBC. Representative micrograph pictures of co-immunofluorescence of CD3 (green), CD4 (cyan), FOXP3 (magenta), and DAPI stain (blue) in AT3 tumors at sacrifice in mice treated with CTX, zolpidem (N05CF) or pantoprazole (A02BC) alone or combined together. Scale bar: 20 μm (A). Spearman correlations between tumor sizes at sacrifice and $\text{CD3}^+\text{CD4}^-$ cell density (B, left) and the ratio of $\text{CD3}^+\text{CD4}^-$ cells/ $\text{CD3}^+\text{CD4}^+\text{FOXP3}^+$ cells across 6 experimental groups comprising 6 mice/group (B, right). Bar graphs showing $\text{CD3}^+\text{CD4}^-$ cell density in AT3-bearing mice treated with NaCl, N05CF or A02BC, (C, left), CTX, CTX + N05CF or CTX + A02BC (C, right). Data are shown as means \pm SEM. P values were obtained using ANOVA test.

Synthèse

La chimiothérapie néoadjuvante (CNA) est utilisée dans les cancers du sein agressifs ou localement avancés (CS). Au delà des bénéfices cliniques, elle représente une opportunité pour monitorer in vivo la sensibilité d'une tumeur à un traitement.

A partir de l'analyse de sets de données de patients traités par CNA, nous souhaitons identifier des mécanismes associés à la résistance ou sensibilité au traitement.

Dans la première partie, nous avons évalué des paramètres, cliniques, anatomopathologiques et transcriptomiques. Nous avons démontré que :

- les voies de la prolifération et les voies de signalisation liées au récepteur à l'estrogène (ER) étaient des marqueurs forts de prédiction de la réponse à la chimiothérapie ;
- les facteurs prédictifs et pronostiques variaient en fonction du sous groupe de cancer du sein : l'atteinte ganglionnaire, le statut ménopausique et l'indice de masse corporelle étaient des éléments déterminants dans les cancers du sein triple négatif; tandis que le traitement par trastuzumab avait un impact pronostique majeur sur les cancers du sein HER2-positif.
- Enfin, des éléments non explorés comme la présence d'embols après CNA revêtaient une information pronostique importante, avec une valeur différente dans les différents sous types de cancer du sein.

Dans une 2ème partie, nous avons démembré l'hétérogénéité des cancers du sein grâce à des analyses bioinformatiques. Quel que soit le sous type de cancer du sein, un sous groupe de patients présentait des tumeurs surexprimant des gènes de l'immunité ; et

cette expression avait une valeur prédictive sur la réponse à la chimiothérapie et/ou pronostique.

Nous avons ensuite analysé l'impact de l'infiltrat immunitaire dans le cancer du sein, et avons décrit les changements observés entre des échantillons avant et après CNA. Nous avons montré que l'impact pronostique des TILs était différent avant et après CNA, et était opposé dans les CS triple négatif (où des niveaux de TILs élevé avaient une tendance à un effet protecteur) ou *HER2*-positif (où des niveaux de TILs élevé avaient une tendance à un effet délétère).

Finalement, dans une 3^{ème} partie, nous avons analysé l'impact des comédications pendant la CNA. Nous avons analysé de manière systématique l'ensemble des comédications chroniques déclarées par les patients pendant la CNA. Nous avons trouvé des effets positifs – via l'augmentation de l'infiltrat immunitaire et la réponse au traitement.

Enfin, nous avons réanalysé en fonction de l'expression du gène *PTGS2* (*COX2*) l'essai clinique REMAGUS02 testant l'adjonction d'un inhibiteur sélectif de *COX2*, le celecoxib, à la chimiothérapie néoadjuvante. Nous avons mis en évidence des effets délétères sur la survie dans certains sous groupes de patients (patients dont la tumeur exprimait faiblement *PTGS2* ; particulièrement en cas de tumeur du sein ER-négative).

En conclusion, la situation néoadjuvante représente une plateforme pour générer et valider des hypothèses de recherche. La mise à disposition de jeux de données de patients traités par chimiothérapie néoadjuvante constituerait une ressource majeure pour accélérer la recherche contre le cancer du sein et doit être encouragée.

Titre : Identification des facteurs prédictifs de sensibilité ou résistance à la chimiothérapie néoadjuvante dans le cancer du sein

Mots clés : Cancer du sein – chimiothérapie néoadjuvante - résistance

Résumé : La chimiothérapie néoadjuvante (CNA) est utilisée dans les cancers du sein agressifs ou localement avancés (CS). Au delà des bénéfices cliniques, elle représente une opportunité pour monitorer *in vivo* la sensibilité d'une tumeur à un traitement.

A partir de l'analyse de sets de données de patients traités par CNA, nous souhaitons identifier des mécanismes associés à la résistance ou sensibilité au traitement.

Dans la première partie, nous avons évalué des paramètres, cliniques, anatomopathologiques et transcriptomiques. Nous avons démontré que des éléments non explorés comme la présence d'embols après CNA revêtaient une information pronostique importante.

Dans une 2ème partie, nous avons analysé l'impact de l'infiltrat immunitaire dans le cancer du sein, et avons décrit les changements

observés entre des échantillons avant et après CNA. Nous avons montré que l'impact pronostique des TILs était différent avant et après CNA, et était opposé dans les CS triple négatif ou *HER2*-positif.

Finally, nous avons analysé l'impact des comédications pendant la CNA. Nous avons trouvé des effets positifs – via l'augmentation de l'infiltrat immunitaire et la réponse au traitement – et des effets négatifs avec des effets délétères dans certains sous groupes de patients.

En conclusion, la situation néoadjuvante représente une plateforme pour générer et potentiellement valider des hypothèses de recherche. La mise à disposition de jeux de données de patients traités par chimiothérapie néoadjuvante constituerait une ressource majeure pour accélérer la recherche contre le cancer du sein.

Title : Identification of factors predicting sensitivity or resistance to neoadjuvant chemotherapy in breast cancer

Keywords : Breast cancer – Neoadjuvant treatment - resistance

Abstract : Neoadjuvant chemotherapy (NAC *i.e.* chemotherapy before surgery) is increasingly being used for aggressive or locally advanced breast cancer (BCs). Beyond clinical benefits, it represents an opportunity to monitor *in vivo* sensitivity to treatment.

Based on the analysis of datasets of BCs patients treated with NAC, we aimed at identifying mechanisms associated with resistance or sensitivity to treatment.

In the first part, we evaluated biological, clinical, pathological and transcriptomic patterns. We demonstrated that unexplored pathological features such as post-NAC lymphovascular invasion may carry an important prognostic information.

In a second part, we analyzed impact of immune infiltration in BC and we described extensively the changes of tumor infiltrating lymphocytes

(TILs) between pre and post-NAC samples. We showed that the prognostic impact of TILs was different before and after NAC, and was opposite in TNBC and *HER2*-positive BCs.

Finally, we investigated the impact of comedication use during NAC. We found both positive effects - while enhancing immune infiltration and response to treatment - and negative effects with deleterious oncologic outcomes in specific patients subgroups.

In conclusion, the neoadjuvant setting represents a platform to both generate and potentially validate research hypotheses aiming at increasing the efficacy of treatment. The public release of real-life datasets of BC patients treated with NAC would represent a major resource to accelerate BC research.

