Genomic Characterization of Primary Invasive Lobular Breast Cancer

Christine Desmedt, Gabriele Zoppoli, Gunes Gundem, Giancarlo Pruneri, Denis Larsimont, Marco Fornili, Debra Fabagalli, David Brown, Françoise Rothé, Delphine Vincent, Naima Kheddaroumi, Ghizlane Rouas, Samira Majjaj, Sylvain Brehée, Peter Van Loo, Patrick Maisonneuve, Roberto Salgado, Thomas Van Brussel, Dieter Lambrechts, Ron Bose, Otto Metzger, Christine Galant, François Bertucci, Martine Piccart-Gebhart, Giuseppe Viale, Elia Bignozzi, Peter J. Campbell, and Christos Sotiriou

See accompanying editorial on page 1838

ABSTRACT

Purpose
Invasive lobular breast cancer (ILBC) is the second most common histologic subtype after invasive ductal breast cancer (IDBC). Despite clinical and pathologic differences, ILBC is still treated as IDBC. We aimed to identify genomic alterations in ILBC with potential clinical implications.

Methods
From an initial 630 ILBC primary tumors, we interrogated oncogenic substitutions and insertions and deletions of 360 cancer genes and genome-wide copy number aberrations in 413 and 170 ILBC samples, respectively, and correlated those findings with clinicopathologic and outcome features.

Results
Besides the high mutation frequency of CDH1 in 65% of tumors, alterations in one of the three key genes of the phosphatidylinositol 3-kinase pathway, PIK3CA, PTEN, and AKT1, were present in more than one-half of the cases. HER2 and HER3 were mutated in 5.1% and 3.6% of the tumors, with most of these mutations having a proven role in activating the human epidermal growth factor receptor/ERBB pathway. Mutations in FOXA1 and ESR1 copy number gains were detected in 9% and 25% of the samples. All these alterations were more frequent in ILBC than in IDBC. The histologic diversity of ILBC was associated with specific alterations, such as enrichment for HER2 mutations in the mixed, nonclassic, and ESR1 gains in the solid subtype. Survival analyses revealed that chromosome 1q and 11p gains showed independent prognostic value in ILBC and that HER2 and AKT1 mutations were associated with increased risk of early relapse.

Conclusion
This study demonstrates that we can now begin to individualize the treatment of ILBC, with HER2, HER3, and AKT1 mutations representing high-prevalence therapeutic targets and FOXA1 mutations and ESR1 gains deserving urgent dedicated clinical investigation, especially in the context of endocrine treatment.

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INTRODUCTION

Invasive lobular breast cancer (ILBC) is the most frequent special histologic type of breast cancer (5% to 15%) after invasive ductal breast cancer (IDBC). 1 2 This neoplasia is typically characterized by small, discohesive epithelial cells, which mostly express the estrogen receptor (ER, encoded by the ESR1 gene), lack HER2 (ERBB2) amplification, and lost the cell adhesion molecule E-cadherin (CDH1). Besides the most prevalent classic subtype, several special ILBC subtypes have been described on the basis of their architecture (alveolar, solid, and trabecular) or cytonuclear characteristics (apocrine, histiocytoid, pleomorphic, and signet ring, further grouped into the mixed-nonclassic subtype). 2 3 The solid and mixed nonclassic subtypes have been associated with worse survival. 2 3

From a clinical perspective, ILBCs generally show an indolent clinical behavior and tend to exhibit a peculiar metastatic pattern. 3 Although patients with ILBC tend to have lower response rates to conventional chemotherapeutic agents than patients with IDBC, some results have suggested that they might derive increased benefit...
from treatment with aromatase inhibitors.\textsuperscript{1,5} Despite these histologic and clinical differences, patients with ILBC are treated as those with IDBC because of the relative paucity of research dedicated to this disease.

Several studies have devoted efforts to characterize the mutational landscape of breast cancer (reviewed in Gonçalves et al\textsuperscript{9}), with a clear underrepresentation of ILBC, which limits the understanding of the genomic alterations that define these cancers. In the current study, we assessed genomic alterations that recur in cancer-related genes in, to our knowledge, the largest cohort of primary ILBC tumors collected for this purpose. We aimed to correlate those recurrent alterations with clinical and pathologic features and to identify those associated with breast cancer–free interval (BCFI).

### METHODS

**Patients and Samples**

Formalin-fixed paraffin-embedded primary ILBC samples from 630 patients (Data Supplement) were selected from four institutional biobanks (Institut Jules Bordet and Cliniques Universitaires Saint Luc, Brussels, Belgium; European Institute of Oncology, Milan, Italy; and Institut Paoli-Calmettes, Marseille, France) according to the following criteria: availability of a formalin-fixed paraffin-embedded sample from the surgical specimen of the primary tumor; no distinct invasive neoplastic components other than pure lobular histologic subtype by central revision; minimal tumor cellularity of 10% to have an unbiased representation of ILBC histotypes (Data Supplement); and availability of at least 600 ng of double-stranded DNA. Patients who received neoadjuvant treatment and/or had previous malignancies at the time of ILBC diagnosis were excluded. All patients received a diagnosis between January 1994 and December 2008 and were treated with surgery followed by medical treatment per local guidelines at the time of presentation. For 235 of 630 patients, we also collected material from an uninvolved lymph node as a source of germline DNA. ILBC histologic subtypes were defined in Desmedt et al.\textsuperscript{10} Other patient characteristics are provided in Table 1. The study protocol was approved by the internal ethics committee of each participating center.

**Targeted Sequencing**

The 630 primary ILBC samples underwent targeted sequencing analysis of 360 cancer-related genes (Data Supplement). We discarded samples that did not have the required coverage to detect mutations for any cellularity with a prespecified type I error probability $\alpha = .05$ and power of $1 - \beta = .80$, on the basis of Carter et al.\textsuperscript{11} At a median sequencing coverage over exonic regions of 103X, 413 tumors were retained for final analysis of substitutions and insertions and deletions (Data Supplement). Germline DNA was interrogated for 196 (47.5%) patients. Single base substitutions were called independently in each sample by using CaVEMan (Cancer Variants through Expectation Maximization),\textsuperscript{12} whereas small somatic insertions and deletions were identified by using a modified version of Pindel.\textsuperscript{13} To avoid the inclusion of potential germline variants in samples without germline DNA and to focus on mutations with a likely role in cancer, we further considered only oncogetic mutations as defined in Desmedt et al.,\textsuperscript{10} henceforth referred to as mutations for the sake of simplicity. The Cancer Genome Atlas (TCGA) database was used for comparison of frequencies after applying the same definition for oncogenicity.

**Copy Number and Gene Expression Analysis**

Log\textsubscript{2} ratios were calculated from the sequencing coverage, normalized with single nucleotide polymorphism data derived from sequencing data of nontumor DNA samples, and smoothed by guanine-cytosine correction. One hundred seventy samples passed the quality criteria for copy number aberration (CNA) analysis. GeneChip U133 Plus 2.0 Array (Affymetrix, Santa Clara, CA) gene expression microarray data were available for 117 patients with ILBC with mutation data.\textsuperscript{11}

### Statistical Analysis

For association analysis of genomic alterations with clinical and pathologic parameters, we limited the investigations to oncogenic mutations observed in $\geq 5\%$ of the 413 samples retained for mutational analysis and to CNAs that occurred in $\geq 5\%$ of the 170 samples with adequate quality criteria. These cutoffs were warranted by exploratory power considerations. In survival analyses, we considered BCFI as the primary end point on the basis of STEEP System criteria outlined by Hudis et al.\textsuperscript{12} Follow-up was censored at 12 years in consideration of the declining number of patients after that time point. Details are provided in the Data Supplement.

### Table 1. Patient and Sample Characteristics ($n = 413$)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>No.</th>
<th>%</th>
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<td></td>
</tr>
<tr>
<td>$&lt; 50$</td>
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<tr>
<td>50-64</td>
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<td>$\geq 65$</td>
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<td>pT(m)</td>
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<tr>
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<td>$&lt; 20%$</td>
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<td>$\geq 20%$</td>
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<td>1.5</td>
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<td>ER-negative</td>
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<td>HER2 status*</td>
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<td>0.5</td>
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<td>Classic</td>
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<tr>
<td>Solid</td>
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<td>15.7</td>
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<td>Mixed, nonclassic</td>
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<td>Trabecular</td>
<td>28</td>
<td>6.8</td>
</tr>
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</table>

Abbreviations: ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; Ki-67, protein encoded by the MCM7 gene; PgR, progesterone receptor.

*The ER and HER2 status denote the pathologic assessment by immunohistochemistry and combined immunohistochemistry/fluorescent in situ hybridization status of these markers.
Fig 1. Genomic landscape of ILBC. (A) Histogram of the frequency and type of mutations of the genes recurrently mutated in more than 2% of the primary ILBC tumors. (B) Lollipop plots of protein coding sequences and major domains of CDH1, TBX3, and FOXA1. Gold and red circles represent missense and truncating mutations (nonsense, nonstop, frameshift insertion and deletion, and splice site substitution), respectively. The length of the vertical lines corresponds to the frequency of the respective mutation. (C) Genome-wide view of copy number alterations present in ILBC. Gains are indicated in blue and losses in gold. (D and E) Scatter plots of the prevalence of the mutated genes in estrogen receptor (ER)-positive/HER2-negative IDBC (n = 338) from The Cancer Genome Atlas on the x-axis and the prevalence of mutated genes in ER-positive/HER2-negative ILBC (n = 371) from the present cohort on the y-axis. The color of the dots represents the significance of the comparison, with gold and orange representing FDRs < 10%. Cad, Cadherin prodomain like; Cadherin_C, Cadherin cytoplasmic; FDR, false discovery rate; HER2, human epidermal growth factor receptor 2; Fork Head_N, forkhead N-terminal region; HNF_C, HNF3 C-terminal domain; IDBC, invasive ductal breast cancer; ILBC, invasive lobular breast cancer.
RESULTS

Recurrent Mutations in Protein-Coding Genes

We observed mutations in 103 of the 360 genes interrogated, of which 15 were recurrently mutated in at least 2% of the tumors (Figs 1A and 1B; Data Supplement). Some genes were mainly affected by truncating mutations (eg, CDH1, GATA3), whereas for others, missense substitutions prevailed (eg, PIK3CA, FOXA1). Besides the expected high mutation frequency of CDH1, present in 65% of cases, we observed that 50% of the tumors were mutated in at least one of the three key genes of the phosphatidylinositol 3-kinase (PI3K) pathway, namely PIK3CA, the negative regulator PTEN, and the downstream effector kinase AKT1. In agreement with the literature, these mutations were mutually exclusive in all but five of 207 tumors that harbored a mutation in one of these genes. Of note, the activating AKT1 substitution E17K was found in all but two patients who carried AKT1 mutant tumors.

Several transcriptional regulators were recurrently altered with high prevalence, such as TBX3, FOXA1, KMT2C, GATA3, and ARID1A. Mutations in TBX3, a member of the T-box transcription factors family, were observed in 13% of cases, principally in the protein T-box domain. FOXA1 and GATA3, mutated in 9% and 7% of the tumors, respectively, are key players in the recruitment of the ER transcription complex. Mutations in the chromatin regulatory factors KMT2C and ARID1A were present in 8% and 6%, respectively. With the exception of KMT2C mutations, which have been demonstrated to facilitate cell cycle progression, the transcriptional consequences of the described alterations are currently unknown.

Finally, two members of the human epidermal growth factor receptor (HER)/ERBB family, HER2 and HER3, were mutated in 5.1% and 3.6% of the tumors, respectively (Fig 2; Data Supplement), with three of 35 mutated cases being simultaneously HER2 amplified, as revealed by routine fluorescent in situ hybridization analysis. We observed 16 different substitutions in these genes. The majority of those were concentrated in the tyrosine kinase domain of the respective genes, each associated with a highly recurrent mutation (L755S for HER2 and E928G for HER3; Fig 2A). Each of these mutations, together with the S310F, D769Y, and V777L HER2 substitutions, and the S846L and Q809R HER3 mutations are known to activate the HER/ERBB pathway16–18 (Figs 2C–2F). Although the recurrent HER2 L869R substitution has never been characterized in breast cancer, it is homologous to substitutions observed in the activation loops of the EGFR, FLT3, and BRAF kinases20,21 (Data Supplement). Hence, the functional relevance of at least eight of the 16 substitutions, present in 27 of 35 of the HER2 and HER3 mutated tumors (77%), is supported by structural and/or in vitro evidence. In the subset of tumors with available gene expression data, we further observed that the scores of an established microarray signature of HER2 pathway activation22 were above the median for the majority of these HER2 and HER3 mutated tumors (Fig 2B).

Recurrent CNAs

We identified 25 arm-level changes and 20 focal gains/deletions, recurrent in at least 5% of the 170 tumors with CNA data (Fig 1B; Data Supplement). Of note, the HER2 amplification status identified by routine assessment was confirmed by the present analysis in all tumors but one.

CDH1 deletion was present in 94% of the patients (160 of 170, 152 as arm-level loss and eight as a focal event). In the subset of tumors stained for CDH1 by immunohistochemistry (156 of 170), absence of expression was observed in 133 (85%) cases (Fig 2G), with protein loss being statistically more frequent in tumors that harbored both a deletion and a mutation compared with those that retained at least one intact copy of the gene (P < .001).

We also observed recurrent, focal copy number gains at 6q25.1, which encompasses ESR1, in 25.3% (43 of 170) of the cases. This focal gain was associated with a higher ESR1 mRNA signature score12 (P = .03; Fig 2H) as well as with increased mRNA expression of TFF1,23 a canonical ESR1 transcriptional target (P = .04), which altogether point to a functional role for this aberration in ILBC. Among recurrent focal alterations, noteworthy gains were found in 1q13.3 (CCND1, 38.2%), 8q24.21 (MYC, 31.2%), 15q26.3 (IGFIR, 31.2%), 8p11.23 (FGFR1, 25.3%), and 12q24.21 (TBX3, 18.8%), and losses were found in 1p36.22 (ARID1A, 22.9%) and 8q24.23 (PTK2, 18.2%), often associated with significant in–cis transcriptional modulation (Data Supplement).

Comparison With Publicly Available Data

We used the publicly available data from TCGA to compare the mutation frequencies in the present ILBC cohort with the ILBC cases analyzed by TCGA (n = 159) and to investigate the differences between ER-positive/HER2-negative ILBCs from the present series (n = 371; Data Supplement) and ER-positive/HER2-negative IDBCs from TCGA (n = 338). Sequencing results generated independently on these two ILBC cohorts were comparable, with significant differences observed only for CDH1 and TBX3, which had a higher prevalence in the present cohort compared with TCGA ILBC cases (Data Supplement). A possible higher prevalence was also observed for AKTI, whereas there was a lower prevalence for NFI and RUNX1. These differences in mutation prevalence might be explained by clinicopathologic differences between the two ILBC cohorts (TCGA series characterized by larger, less differentiated tumors). In contrast, losses were significantly more prevalent in ILBC than IDBC, such as ESR1 and ETVI gains (Fig 1C; Data Supplement).
Association of Genomic Alterations With Clinical and Pathologic Features

We first investigated the association of recurrent mutations and CNAs with clinicopathologic features (Table 2; Data Supplement). TP53 mutations were more frequent in older patients and in high-grade, highly proliferative tumors, as assessed by Ki-67 (protein encoded by the MKI67 gene). On the contrary, PIK3CA mutations were found to be associated with low proliferative tumors. HER2 mutations were further associated with high grade and progesterone receptor negativity and MAP3K1 mutations with node-negative...
tumors. At the copy number level, among others, 6q25.1 (ESR1) and 8p11.23 (FGFR1) gains were more frequent in older and younger patients, respectively, whereas 1p36.22 (MTOR/ARID1A) deletions were associated with high proliferative and HER2-amplified tumors (Table 2). Of note, PTEN and TBX3 mutations as well as 11q14.1 gains (PAK1) were associated with the presence of multifocal tumors, which corroborates the clinical perspective that those genes are involved in cell motility and tissue invasion.\textsuperscript{5-22}

We then sought to identify genomic alterations associated with the various histologic subtypes of ILBC. In addition to the already-described clinical associations with such subtypes,\textsuperscript{2} we found that the mixed nonclassic and solid subtypes were enriched in ERBB2 and ARID1A mutations, respectively, whereas both were associated with TP53 mutations (Data Supplement). By copy number analysis, the solid subtype was more often characterized by 11p36.22 (ARID1A) deletions and by 1p36.22 (ARID1A) deletions, the mixed nonclassic subtype frequently presented with 1p36.22 (ARID1A) deletions, and the alveolar subtype was enriched in 11q13.3 (CCND1) and 11q14 (PAK1) gains. Altogether, these results show that distinctive genomic alterations likely underlie the phenotypic appearance of ILBC histologic subtypes (Fig 3).

**Breast Cancer–Free Survival Analyses**

We took advantage of the long-term follow-up data available for this cohort (median follow-up, 9.5 years) to explore whether specific genomic aberrations could be associated with prognosis (BCFI; Data Supplement). Univariable assessment of mutated genes and CNAs identified TP53 mutations and several CNAs as associated with the investigated end point (Figs 4A and 4C). Only three CNAs were, however, retained by the stability selection with the lasso-penalized Cox proportional hazard regression model, which also included classic clinicopathologic variables. Specifically, 1q gains were associated with a better outcome, whereas 17q12 and 11p gains were associated with a worse outcome (Figs 4D-4F). Hazard proportionality assessment suggested that AKT1 and HER2 mutations may have time-dependent effects associated with short-term risk, even after adjusting for standard clinicopathologic variables (Fig 4B; Data Supplement).

**DISCUSSION**

ILBC represents a significant proportion of the breast cancer population and is associated with distinct clinical and pathologic features compared with IDBC. However, no specific treatment recommendation exists for this disease. We therefore aimed to identify and characterize the recurrent genomic alterations associated with ILBC in the largest cohort investigated, to our knowledge, at the genomic level to identify biologically and clinically meaningful determinants for such disease.

We show that ILBC presents a different mutational landscape from that of IDBC. In addition to the expected increased frequency of genomic alterations that involve CDH1, ILBC is characterized by a peculiarly high prevalence of mutations that affect PI3K and HER/ERBB family members, ESR1-specific and other transcriptional regulators as well as CNAs that involve, among others, ESR1 gains in one-fourth of the cases. Of note, no novel ILBC-specific mutated genes, not included in our analysis, were discovered.

### Table 2. Association of the Recurrent Genomic Alterations Retained by the Stability Selection With Clinical and Pathologic Characteristics: Genes Recurrently Mutated in > 2% of the Patient Sample

<table>
<thead>
<tr>
<th>Variable</th>
<th>Mutated Genes</th>
<th>Mutated Tumors Per Category (%)</th>
<th>Odds Ratio (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>ER (positive v negative)</td>
<td>HER2</td>
<td>4.6 v 12.0</td>
<td>0.37 (0.11 to 1.61)</td>
<td>.167</td>
</tr>
<tr>
<td></td>
<td>FOXA1</td>
<td>8.2 v 20.0</td>
<td>0.44 (0.16 to 1.43)</td>
<td>.161</td>
</tr>
<tr>
<td></td>
<td>RUNX1</td>
<td>2.8 v 12.0</td>
<td>0.19 (0.06 to 0.85)</td>
<td>.032*</td>
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<tr>
<td></td>
<td>TP53</td>
<td>6.7 v 16</td>
<td>0.34 (0.12 to 1.19)</td>
<td>.0871</td>
</tr>
<tr>
<td>PgR (positive v negative)</td>
<td>BRCA2</td>
<td>1.6 v 3.9</td>
<td>0.37 (0.10 to 1.41)</td>
<td>.140</td>
</tr>
<tr>
<td></td>
<td>HER2</td>
<td>3.9 v 8.8</td>
<td>0.40 (0.17 to 0.99)</td>
<td>.047*</td>
</tr>
<tr>
<td>HER2 (positive v negative)</td>
<td>HER3</td>
<td>10 v 3.3</td>
<td>4.12 (0.74 to 15.91)</td>
<td>.0951</td>
</tr>
<tr>
<td></td>
<td>KMT2C</td>
<td>25 v 7.2</td>
<td>5.85 (1.83 to 16.89)</td>
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</tr>
<tr>
<td></td>
<td>PIK3CA</td>
<td>20 v 44.8</td>
<td>0.31 (0.09 to 0.84)</td>
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<td>Grade (3 v 1 and 2)</td>
<td>HER2</td>
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<td>2.89 (1.02 to 7.85)</td>
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<td>PIK3CA</td>
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<td>TP53</td>
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</tr>
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<td>Ki-67 (≥ 20 v &lt; 20)</td>
<td>PIK3CA</td>
<td>35.5 v 46.6</td>
<td>0.64 (0.41 to 0.99)</td>
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<td>15.3 v 3.9</td>
<td>4.30 (2.03 to 9.51)</td>
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<td>Age (50-64 v &lt; 50 years)</td>
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<td>1.53 (0.94 to 2.56)</td>
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<td>3.28 (1.21 to 8.94)</td>
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<td>TBX2</td>
<td>19.2 v 11.5</td>
<td>1.90 (1.02 to 3.45)</td>
<td>.045*</td>
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</table>

Abbreviations: CI, confidence interval; ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; Ki-67, protein encoded by the MXI1 gene; PgR, progesterone receptor.

*P < .05.
†P < .1.
‡P < .01.
§P < .001.
through the exome sequencing approach adopted by TCGA, which supports the maturity of biology-driven next-generation sequencing–targeted panels, such as the one we deployed.

The clinically relevant findings of the present work can be unfolded into three main categories. First, we identified alterations in one of three key genes of the PI3K pathway, PIK3CA, PTEN, and AKT1, in 50% of ILBC cases, each more frequently mutated in ER-positive/HER2-negative ILBC than in ER-positive/HER2-negative IDBC tumors. Whereas PIK3CA mutant cancers were associated with low proliferation rates, as defined by Ki-67, patients affected by AKT1 mutated tumors (4.1%) were associated with a short-term risk of relapse.

Second, in a sufficiently large cohort of well-annotated primary ILBC, this study points out that the prevalence of HER2 and HER3 mutations is significantly higher than in primary IDBC, with 8.5% (35 of 413) of the investigated ILBC cases carrying an HER2 and/or HER3 mutation. The functional evidence of oncogenicity for the majority of mutations (71%) together with the demonstration of transcriptional activation of the pathway in most HER2 and HER3 mutated tumors emphasize the actionable nature of these alterations. Overall, 60 (14.5%) tumors either were HER2 amplified (as determined by fluorescent in situ hybridization) or carried an HER2/HER3 mutation. This proportion of HER/ERBB pathway alterations was even higher (23.1%) in the mixed, non-classic subtype, which suggests that the treatment of nearly one-fourth of these aggressive tumors could be individualized. With the large number of ILBC cases in this study, we confirmed that HER2 mutations occur at a higher frequency in ILBC than in invasive breast cancer overall.

Finally, we identified mutations in genes involved in the genomic action of ER, such as GATA3 (7.3%) and FOXA1 (9.0%),
which were present at significantly lower and higher frequencies than ER-positive/HER2-negative IDBC, respectively. Both FOXA1 and GATA3 are important for the expression of ESR1-target genes because of their key role in the transcription factor complex, which assembles at the estrogen response element of genes transcriptionally regulated by ESR1. Therefore, we can expect these alterations to play a role in modulating response to hormone treatment. However, this will need to be investigated in the context of a clinical trial; the institutional and retrospective nature of the present cohort precluded such analysis. In addition to these mutations, copy number gains of FOXA1 and 6q25.1 (ESR1) were further observed in 5.9% and 25.3% of the cases, respectively, which could also affect the ER-related transcriptional program. We have shown that ESR1 copy number gains are associated with increased ESR1 mRNA together with an increased expression of the ESR1-target gene TFF1. Of note, this alteration had a higher prevalence in the aggressive solid histotype, with 44% of the cases presenting 6q25.1 gains compared with 17% of classic ILBC cases. Such a finding is especially relevant as a result of experimental data that suggested that tumors with increased ESR1 copy number levels could show increased sensitivity to estradiol therapy.

In conclusion, we provide evidence that the clinical and pathologic features that distinguish ILBC from IDBC are mirrored by a peculiar genomic landscape. Given the higher prevalence of HER2, HER3, and AKT1 mutations in ILBC than in IDBC and the existence of drugs that target these alterations, we recommend the characterization of ILBC tumors for these genes. In addition, FOXA1 mutations and ESR1 gains urgently deserve dedicated clinical investigation, especially in the context of endocrine treatment. As our understanding of the molecular features that
characterize ILBC increases, we can begin to individualize the treatment of this disease.

### Conception and design: Christine Desmedt, Giancarlo Pruneri, Giuseppe Viale, Christos Sotiriou

### Financial support: Christine Desmedt, Christos Sotiriou

### Administrative support: Christine Desmedt

### Provision of study materials or patients: Denis Larisomt, Otto Metzger, Françoise Bertucci, Martine Piccart-Gebhart, Giuseppe Viale

### Collection and assembly of data: Christine Desmedt, Gabriele Zopppoli, Gunes Gundem, Giancarlo Pruneri, Denis Larisomt, Debora Fumagalli, Françoise Rothé, Delphine Vincent, Naima Kheddoumi, Ghizlane Rouas, Samira Majjabi, Sylvain Brohée, Thomas Van Brussel, Diether Lambrechts, Otto Metzger, Christine Galant, Françoise Bertucci, Giuseppe Viale, Peter J. Campbell

### Data analysis and interpretation: Christine Desmedt, Gabriele Zopppoli, Gunes Gundem, Giancarlo Pruneri, Marco Fornili, David Brown, Sylvain Brohée, Peter Van Loo, Ron Bose, Martine Piccart-Gebhart, Giuseppe Viale, Elia Biganzoli, Peter J. Campbell, Christos Sotiriou

### Manuscript writing: All authors

### Final approval of manuscript: All authors

### REFERENCES


### Affiliations

Christine Desmedt, Gabriele Zopppoli, Denis Larisomt, Debora Fumagalli, David Brown, Françoise Rothé, Delphine Vincent, Naima Kheddoumi, Ghizlane Rouas, Samira Majaji, Sylvain Brohée, Roberto Salgado, Martine Piccart-Gebhart, and Christos Sotiriou, Institut Jules Bordet; Christine Galant, Cliniques Universitaires Saint Luc, Brussels; Peter Van Loo, University of Leuven; Thomas Van Brussel and Diether Lambrechts, VIB Vesalius Research Center, Leuven, Belgium; Gabriele Zopppoli, University of Genoa and Istituto di Ricerca a Carattere Clinico-Scientifico San Martino-National Cancer Institute, Genoa; Giancarlo Pruneri, Patrick Maisonneuve, and Giuseppe Viale,
Genomics of Lobular Breast Cancer

European Institute of Oncology; Marco Fornili and Elia Biganzoli, University of Milan, Fondazione Istituto di Ricovero e Cura a Carattere Scientifico Istituto Nazionale Tumori, Milan, Italy; Gunes Gundem and Peter J. Campbell, Wellcome Trust Sanger Institute, Cambridgeshire; Peter Van Loo, The Francis Crick Institute, London, United Kingdom; Ron Bose, Washington University School of Medicine, St Louis, MO; Otto Metzger, Dana-Farber Cancer Institute, Boston, MA; and François Bertucci, Institut Paoli-Calmettes, Marseille, France.

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Christine Desmedt
No relationship to disclose

Gabriele Zoppoli
Travel, Accommodations, Expenses: Life Technologies

Gunes Gundem
No relationship to disclose

Giancarlo Pruneri
No relationship to disclose

Denis Larsimont
Travel, Accommodations, Expenses: Roche

Marco Fornili
No relationship to disclose

Debora Fumagalli
No relationship to disclose

David Brown
No relationship to disclose

Françoise Rothé
No relationship to disclose

Delphine Vincent
No relationship to disclose

Naima Khedoumi
No relationship to disclose

Ghizlane Rouas
No relationship to disclose

Samira Majjaj
No relationship to disclose

Sylvain Brohéé
No relationship to disclose

Peter Van Loo
No relationship to disclose

Patrick Maisonneuve
No relationship to disclose

Roberto Salgado
Travel, Accommodations, Expenses: Roche

Thomas Van Brussel
No relationship to disclose

Diether Lambrechts
Consulting or Advisory Role: Genentech, Sanofi, Bayer AG, Boehringer

Ingeleim, Multiplicom

Speakers' Bureau: Eli Lilly, Roche

Research Funding: Genentech (Inst), Sanofi (Inst), Bayer AG (Inst)

Patents, Royalties, Other Intellectual Property: Named as an inventor on several patents related to response to antiangiogenesis

Ron Bose
Honoraria: Genentech

Consulting or Advisory Role: Genentech

Otto Metzger
Honoraria: Novartis, Eisai, AbbVie, Roche

Research Funding: Pfizer, Genentech, Eisai

Christine Galant
No relationship to disclose

François Bertucci
No relationship to disclose

Martine Piccart-Gebhart
Consulting or Advisory Role: AstraZeneca, Eli Lilly, Inivivis

Pharmaceutical, MSD, Novartis, Pfizer, Genentech, Synthon, Radius

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Giuseppe Viale
Consulting or Advisory Role: DakoCytomation, Genentech, AstraZeneca

Speakers' Bureau: Novartis

Travel, Accommodations, Expenses: Roche, Celgene

Elia Bignazoli
No relationship to disclose

Peter J. Campbell
Stock or Other Ownership: 14M Genomics

Consulting or Advisory Role: 14M Genomics

Christos Sotiriou
Consulting or Advisory Role: Nanostring Technologies, Astellas Pharma, Vertex

Travel, Accommodations, Expenses: Nanostring Technologies, Astellas Pharma, Vertex, Roche
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