



## Original article

# Targeted next-generation sequencing identifies genomic abnormalities potentially driving the prognosis of early-stage invasive lobular breast carcinoma patients stratified according to a validated clinico-pathological model



Luisa Carbognin <sup>a, b</sup>, Michele Simbolo <sup>c</sup>, Anna Calì <sup>c</sup>, Caterina Vicentini <sup>d</sup>, Pietro Delfino <sup>c</sup>, Isabella Sperduti <sup>e</sup>, Matteo Fassan <sup>f</sup>, Francesco Schettini <sup>g</sup>, Maria Vittoria Dieci <sup>h, i</sup>, Gaia Griguolo <sup>h, i</sup>, Sara Pilotto <sup>a, j</sup>, Elena Fiorio <sup>j</sup>, Grazia Arpino <sup>g</sup>, Valentina Guarneri <sup>h, i</sup>, Sabino De Placido <sup>g</sup>, Pierfranco Conte <sup>h, i</sup>, Erminia Manfrin <sup>c</sup>, Matteo Brunelli <sup>c</sup>, Giovanni Scambia <sup>b</sup>, Aldo Scarpa <sup>c, d</sup>, Giampaolo Tortora <sup>k, 1</sup>, Emilio Bria <sup>k, 1, \*</sup>

<sup>a</sup> University of Verona, Verona, Italy

<sup>b</sup> Division of Gynecologic Oncology, Department of Woman and Child Health and Public Health, Fondazione Policlinico Universitario A. Gemelli IRCCS, Roma, Italy

<sup>c</sup> Department of Diagnostics and Public Health, University of Verona, Azienda Ospedaliera Universitaria Integrata, Verona, Italy

<sup>d</sup> ARC-Net Research Centre and Department of Pathology, University of Verona, Verona, Italy

<sup>e</sup> Biostatistics, 'Regina Elena' National Cancer Institute, Roma, Italy

<sup>f</sup> Department of Medicine (DIMED), Surgical Pathology Unit, University of Padova, Padova, Italy

<sup>g</sup> Medical Oncology, Federico II University, Napoli, Italy

<sup>h</sup> Division of Medical Oncology 2, Istituto Oncologico Veneto, IRCCS, Padova, Italy

<sup>i</sup> Department of Surgery, Oncology and Gastroenterology, University of Padova, Padova, Italy

<sup>j</sup> U.O.C. Medical Oncology, Azienda Ospedaliera Universitaria Integrata, Verona, Italy

<sup>k</sup> Oncologia Medica, Fondazione Policlinico Universitario A. Gemelli IRCCS, Università Cattolica Del Sacro Cuore, Roma, Italy

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## ABSTRACT

**Introduction:** The clinico-pathological and molecular factors that drive the prognosis of invasive lobular breast carcinoma (ILC) are not entirely explored. In this regard, the development and validation of a prognostic model for ILC and the investigation of the distribution of molecular abnormalities (focusing on CDK4/6 alterations) according to prognosis were the aims of this study.

**Patients and methods:** Two clinico-pathological multi-center data-sets of early-stage ILC patients (Training/Validation Set, TS/VS) were gathered. A 3-class model was developed according to the multivariate analysis for disease-free-survival (DFS) and externally validated. Mutational, copy number variation and transcriptomic analyses by targeted next generation sequencing (NGS) were performed (and validated with quantitative PCR) in an explorative cohort of patients with poor and good prognosis.

**Results:** Data from overall 773 patients (TS/VS: 491/282) were gathered. The developed model significantly discriminated low/intermediate/high risk in the TS (10-years DFS: 76.3%/67.6%/39.8%, respectively,  $p < 0.0001$ ) and in the VS ( $p < 0.0001$ ). In the explorative cohort for molecular analysis (34 patients), CDK4 gain was present exclusively in the poor prognosis group (35.0%,  $p = 0.03$ ; OR 7.98, 95%CI 1.51–42.1,  $p = 0.014$ ). Moreover, CDK4 and 6 overexpression showed a trend toward an association with poor prognosis (OR 2.7, 95%CI 0.4–18.1,  $p = 0.3$ ; OR 3.29, 95%CI 0.56–19.25,  $p = 0.18$ ).

**Conclusions:** A risk stratification model, able to accurately separate early-stage ILC patients' prognosis into different risk classes according to clinico-pathological variables, allowed to investigate potential biomarkers of prognosis with targeted NGS. CDK4 gain is suggested for future validation as a prognostic biomarker and a potential therapeutic opportunity in ILC patients.

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\* Corresponding author. Fondazione Policlinico Universitario A. Gemelli IRCCS, Università Cattolica Del Sacro Cuore, Largo Agostino Gemelli 8, 00168, Roma, Italy.

E-mail address: [emilio.bria@unicatt.it](mailto:emilio.bria@unicatt.it) (E. Bria).

<sup>1</sup> G.T. and E.B. share last co-authorship.

## 1. Introduction

Invasive lobular carcinoma (ILC), accounting for 5%–15% of all invasive breast tumours, represents the second most common histologic type of breast cancer (BC), after invasive carcinoma of no special type (NST) [1]. The lobular histotype is generally characterized by luminal phenotype and lower mitotic index and grading in comparison with NST [2]. Although these aspects are typically associated with a favorable prognosis, the overall long-term survival of ILC appears worse than that of invasive carcinoma of NST [3]. Even the recent genomic characterization of ILC underlined that this histotype represents a peculiar entity of BC [4–6].

However, despite the several dissimilarities between lobular and ductal histology, the prognostic and therapeutic aspects of ILC are currently borrowed from the ductal histotype. Thus, an issue for clinical practice is represented by the identification of predictors of prognosis for ILC to assist in the choice of a pertinent therapeutic decision [7]. Recently, the correlation between genomic alterations and the risk of recurrence suggested that chromosome 1q gain was predictor of a better outcome, whereas 11p gain was predictor of worse outcome [5]. However, the prognostic role of genomic alterations varies considerably and does not emerge equally from studies [8], providing limited insight into the prognosis of this histotype.

A possible strategy to identify potential prognostic factors consists in the early identification of patients characterized by ‘best’ and ‘worst’ prognosis. These patients, defined as ‘outliers’, may potentially be featured by peculiar molecular alterations accountable for their specific outcome. Moreover, this strategy may help to combine the clinical aspects with the recent technologies [9].

In this regard, the aim of this study was to develop and validate a prognostic nomogram for early stage ILC patients, combining clinico-pathological factors, to finally identify those prognostic ‘outliers’ candidate to undergo genomic analysis with targeted next generation sequencing (NGS).

## 2. Materials and methods

### 2.1. Patients’ population

Consecutive patients with diagnosis of early stage ILC underwent surgery at 3 Italian institutions (Verona and Padova providing data for the training set (TS) and Napoli for the validation set (VS) between 1990 and 2013 were considered eligible. Inclusion criteria were ‘pure’ ILC diagnosis (stage I–III), curative surgery and availability of clinico-pathological parameters.

### 2.2. End-points

The aims of this study were 1) to develop a clinico-pathological prognostic model in a multi-center population of ILC (TS), to finally recognize ‘outlier’ patients, in terms of disease-free survival (DFS) and overall survival (OS); 2) to validate the nomogram in an external patients’ cohort (VS); 3) to explore potential molecular drivers of prognosis with NGS in a subset of prognostic ‘outliers’.

### 2.3. Statistical analysis

Descriptive statistics were adopted to summarize appropriate study data. The Hazard Ratios (HR) and the 95% Confidence Interval (CI) were assessed for each variable applying the Cox univariate model [10]. A multivariate hazard model was calculated adopting the stepwise regression (forward selection, enter limit and remove limit,  $p = 0.10$  and  $p = 0.15$ , respectively), to detect independent prognostic factors [11]. The outcomes were DFS and OS. To

accomplish the multivariate model overfit, an internal cross-validation technique was performed [12–15]. Further details are reported in Supplementary Material.

### 2.4. Prognostic score assessment

A step-by-step protocol was pursued consistent with the methodological approach for developing a nomogram for cancer prognosis proposed by Iasonos et al. [13]. The recommendations for tumor marker prognostic studies (REMARK) criteria were followed [16,17]. To develop the prognostic model [18], the log-hazard rates obtained from the Cox model were adopted to derive weighting factors of a prognostic index, aimed to recognize differential risks of death. Coefficients estimates were ‘normalised’ dividing by the smallest one and rounding the resulting ratios to the nearest integer value [19]. A continuous score, assigning to patients an ‘individualized’ risk, was generated. Patients’ outcomes, according to the prognostic score, were exhibited by distributing patients into three risk classes. The cut-offs were selected at nearly equal distance along the range of values [20]. Finally, an external validation of the DFS model was explored in the VS. The receiver operating characteristic analysis allowed to estimate the accuracy of the prognostic model, by the area under the curve (AUC) determination with standard error (SE).

### 2.5. Samples and molecular analysis

Formalin-fixed and paraffin-embedded (FFPE) ILC samples from patients at poor prognosis (defined with the prognostic model) were selected from the University of Verona according to the availability of a tissue sample from the surgical specimen of the primary tumor. In order to compare the molecular pattern of these samples at poor prognosis with those at good, we selected a subgroup of cases at good prognosis according to the developed prognostic model. The collected material has been subjected to targeted NGS analysis for somatic mutation (SM), copy number variation (CNV) and transcriptomic analysis. In addition, quantitative-PCR, immunohistochemistry (IHC) and fluorescent in situ hybridization (FISH) for the validation of gene alterations of interest were performed. Finally, stromal tumor-infiltrating lymphocytes (sTIL) were also evaluated. Fisher’s exact test and *Peto Odds Ratio (OR)* for estimating the risk of association of a given biomarker with each prognostic class were used as appropriate. Finally, in order to identify potential biological profiles associated with the prognosis, the multiple correspondence analysis (MCA) was accomplished [21].

### 2.6. Mutational, CNV and transcriptomic analyses

Matched tumor/normal DNA and RNA from all FFPE samples was subjected to NGS. To analyse DNA, an Ion Ampliseq custom panel was used to investigate SM and CNV status of all exons of 26 selected genes upon the results of published whole genome sequencing and exome breast cancer data, as described in the supplementary material [4,22]. Mutational load and chromosome integrity number (CIN) were also evaluated as illustrated in previous studies [23]. Available RNA was converted in cDNA and subjected to analysis using Ion Ampliseq Transcriptome Human Gene Expression Kit [24].

### 2.7. Immunohistochemistry, FISH and sTIL assessment

Immunohistochemistry for PD1, PD-L1, phtTOR, CDK4 and CDK6 was performed on surgical specimens using 4  $\mu$ m FFPE tissue. Fluorescent in situ hybridization for PD-L1, CDK4, ESR1 and mTOR

was performed as well. Stromal TIL were assessed on hematoxylin and eosin-stained sections according to the International TILs Working Group 2014 recommendations [25].

### 3. Results

#### 3.1. Patients' characteristics

Clinico-pathological information from 773 patients (491 for TS and 282 for VS) with ILC who underwent surgery was gathered [26]. Patients' characteristics of the TS are reported in Table 1. At a median follow-up of 77 months (range 1–396), median DFS was 175 months (95%CI 153–197), with a 5-/10-year rate of 82.4%/70.5%, respectively. Median OS was 213 months (95%CI 190–236), with a 5-/10-year rate of 91.8%/82.2%, respectively.

#### 3.2. Survival analysis

The univariate analysis was reported in Table S1. At the multivariate analysis, T-category (1–2) and negative nodal status were independent predictors for longer DFS; age at diagnosis <60 years, negative nodal status and Ki67 < 5% were independent factors for longer OS. A Ki67 threshold of 5% was adopted as optimal prognostic cut-off considering the previously reported and validated results in a large series of ILC [2]. At the internal cross-validation analysis, all variables were confirmed as independent factors (Table 2).

#### 3.3. Prognostic score assessment

All the covariates showing independent prognostic role in the Cox model (T-category, nodal status, age at diagnosis and Ki67) were included in the prognostic index. According to the HR achieved at the multivariate analysis, a prognostic scoring index was allocated to each patient in order to categorize the individual risk of recurrence and death (Table S2). Based on the prognosis, patients were separated into three risk groups for DFS and OS: 1) low risk of recurrence and death: score < 2 (ie, the best outcome estimate); 2) intermediate risk of recurrence and death: score = 2; 3) high risk of recurrence and death: score > 2 (ie, worst outcome estimate). According to the prognostic model, a significant prognostic difference between patients at low, intermediate, and high risk was obtained for DFS (10-year: 76.3%/67.6%/39.8%,  $p < 0.0001$ ; AUC 0.60 (SE, 0.03)); Fig. 1, Panel A) and OS (10-year: 92.7%/82.7%/67.1%,  $p < 0.0001$ ; AUC 0.66 (SE, 0.03)).

#### 3.4. External validation analysis

The VS consisted of 282 patients (Table S3); median follow-up 86 months (range 1–348). The model, developed in the TS, has been proven to be likewise able to discriminate the DFS in the VS (Fig. 1, Panel B). Indeed, a significant prognostic difference between patients at low, intermediate, and high risk was obtained (10-year: 81.5%/53.4%/44.0%,  $p < 0.0001$ ; AUC 0.70 (SE, 0.03)). Considering the overall population (TS plus VS; Table S4 and Additional File 1), the performance of the model adjusting for the set was confirmed ( $p < 0.001$ ), without difference in terms of DFS between the TS and VS (HR 1.02, 95%CI 0.77–1.37,  $p = 0.88$ ).

#### 3.5. Patients' sample

Patient's samples for molecular analysis were selected based on the inclusion criteria: 1) selection according to the prognostic model (patients with the 'best' and 'worst' outcome, excluding patients with intermediate outcome), and 2) tissue availability at

**Table 1**

Clinico-pathological and therapeutic characteristics in patients with invasive lobular carcinoma (Training Set, N = 491).

Characteristics	Subcategories	ILC [TS] Patients N (%)
Menopausal status	Premenopausal	142 (28.9)
	Postmenopausal	349 (71.1)
Grading	1	124 (25.3)
	2	155 (31.6)
	3	75 (15.3)
	Unknown	137 (27.9)
Oestrogen Receptor status	Positive	460 (93.7)
	Negative	17 (3.5)
	Unknown	14 (2.8)
Progesterone Receptor status	Positive	412 (83.9)
	Negative	52 (10.6)
	Unknown	27 (5.5)
Ki67	<5%	136 (27.7)
	≥5%	313 (63.7)
	Unknown	42 (8.6)
HER2 status	Positive	27 (5.5)
	Negative	353 (71.9)
	Unknown	111 (22.6)
T category according to TNM [7 <sup>o</sup> Edition]	1	276 (56.2)
	2	148 (30.1)
	3	40 (8.1)
	4	23 (4.7)
	Unknown	4 (0.9)
Lymph-nodal status	Positive	180 (36.7)
	Negative	297 (60.5)
	Unknown	14 (2.8)
Vascular Invasion	Present	87 (17.7)
	Absent	290 (59.1)
	Unknown	114 (23.2)
Multifocality	Present	93 (18.9)
	Absent	375 (76.4)
	Unknown	23 (4.7)
Type of surgery	Tumorectomy	130 (26.5)
	Quadrantectomy	165 (33.6)
	Mastectomy	196 (39.9)
Adjuvant hormonal therapy	Yes	432 (88.0)
	No	534 (11.0)
	Unknown	5 (1.0)
Adjuvant chemotherapy	Yes	199 (40.5)
	No	292 (59.5)
Adjuvant Trastuzumab	Yes	11 (2.2)
	No	480 (97.8)
Adjuvant radiotherapy	Yes	301 (61.3)
	No	177 (36.0)
	Unknown	13 (2.6)

Legend-Table 1. ILC, invasive lobular carcinoma; TS, training set; N, number.

the coordinating center (University of Verona). These tissue samples were available for 20 and 14 patients scored at poor and good prognosis class according to the DFS model, respectively (Table S5, Fig. S1, Fig. S2).

#### 3.6. Molecular features according to prognosis

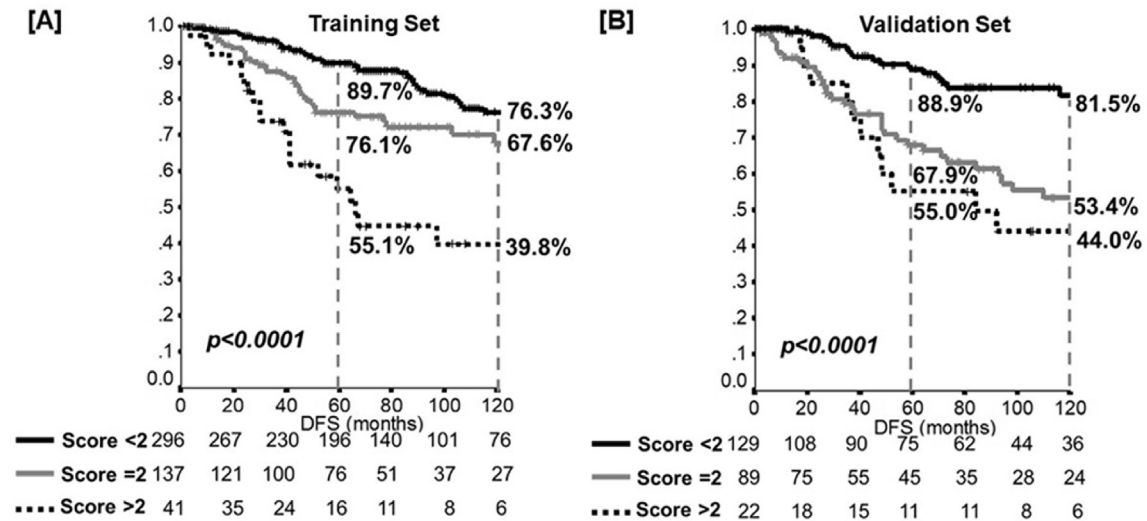
Mutations were observed in 29 cases of all series for 26 genes analysed. In detail: one mutation was observed in 10/34 cases (29.4%), more than one in 19/34 (55.9%) cases while no alteration in 5/34 cases (14.7%). A mean of 12.4 mutations/Mb was achieved. The most commonly mutated gene in the whole cohort was *CDH1* (38.2%), followed by *PIK3CA* (29.4%) and *TP53* (20.6%) (Fig. 2 (Panel A), Table 3, Table S6). Loss of heterozygosity of *CDH1* (44.1%) and *ARID1A* (38.2%) were the most frequent CNV events, followed by gain in *FGFR1* and *ESR1* (each 12/34; 35.3%).

The prevalence of gene SM and CNV according to prognostic groups was reported in Table 3 and Supplementary Material. Interestingly, gain of *CDK4* (7/34; 21.2%) was exclusively present in this poor prognosis group, whereas no good prognosis case showed

**Table 2**  
Multivariate analysis in patients with invasive lobular carcinoma (Training Set, N = 491).

Predictors	DFS HR (95% CI) [p-value]	Replication Rate [Internal Validation]	OS HR (95% CI) [p-value]	Replication Rate [Internal Validation]
T-category according to TNM (7 <sup>o</sup> Edition) [3–4 vs. 1–2]	1.78 (0.97–3.25) [0.062]	60%	–	–
Nodal Status [Positive vs. Negative]	2.46 (1.50–4.05) [<0.0001]	95%	3.32 (1.71–6.45) [<0.0001]	100%
Age [>60 years vs. ≤60 years]	–	–	2.19 (1.14–4.21) [0.019]	90%
Ki67 [≥5% vs. <5%]	–	–	2.47 (1.02–5.94) [0.044]	80%

Legend-Table 2. DFS, disease-free survival; HR, hazard ratio; CI, confidence interval; OS, overall survival, T, tumor.



**Fig. 1.** Disease-free survival (DFS) according to the risk-class model in the Training set [Panel A] and in the Validation set [Panel B]. p-value: log-rank analysis.

this alteration ( $p = 0.03$ ). Moreover, *CDK4* gain resulted in a statistically significant higher chance to be associated with poor prognosis (OR 7.98, 95%CI 1.51–42.1,  $p = 0.014$ ) (Fig. 2, Panel B). Regarding the association of *CDK4* gain and clinico-pathological characteristics, this alteration was more frequent in node positive ( $p = 0.03$ ) and high proliferative tumours, as defined by Ki67, despite a non-significant statistically difference ( $p = 0.14$ ).

When grouping the molecular alterations involved in the regulation of G1/S phase cell cycle progression herein evaluated (*CDK4* and *CCND1* gain or *CDKN2A* mutation), this signature was significantly more associated with poor prognosis in the whole patients' sample (OR 6.24, 95%CI 1.59–24.5,  $p = 0.009$ ), and in the RB1 wild type population (32 patients, OR 5.33, 95%CI 1.33–21.28,  $p = 0.018$ ).

RNA was available for 20 samples (12 poor and 8 good). Ninety genes (as reported in Fig. 3) were differentially expressed between the two prognostic groups (Table S7, Supplementary Results, Table S8).

According to the FISH analysis, *CDK4* gain (Fig. S3, Panel A) was detected in the 10% of patients at poor prognosis (those presenting also *CDK4* gain at NGS), while no *CDK4* gain was detected in patients at good, without a significant difference between the two groups. According to the IHC analysis, nuclear *CDK4* overexpression (score 3+) (Fig. S3, Panel B) was detected in the 20% and 7.1% of patients at poor and good prognosis, respectively ( $p = 0.62$ ). No association between *CDK4* gain at NGS and nuclear *CDK4* overexpression at IHC was observed. *CDK4* gain at FISH and *CDK4* overexpression were more frequently associated with poor prognosis, despite a non-statistically significant difference, with a similar trend for *CDK6* overexpression as well (Fig. S3, Panel C;

Fig. S4). Patients with poor prognosis resulted to have a significantly higher chance to be cumulatively associated with abnormalities in *CDK4/CDK6* overall expression or CNV (Fig. S5, Supplementary Material).

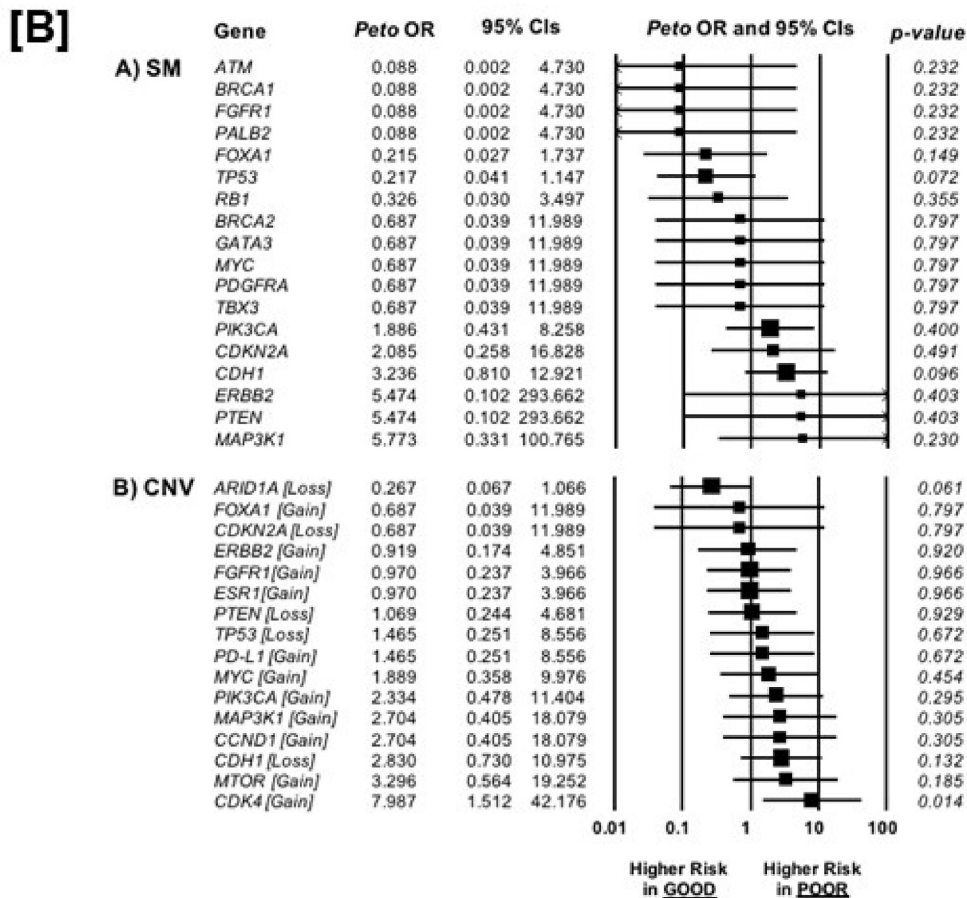
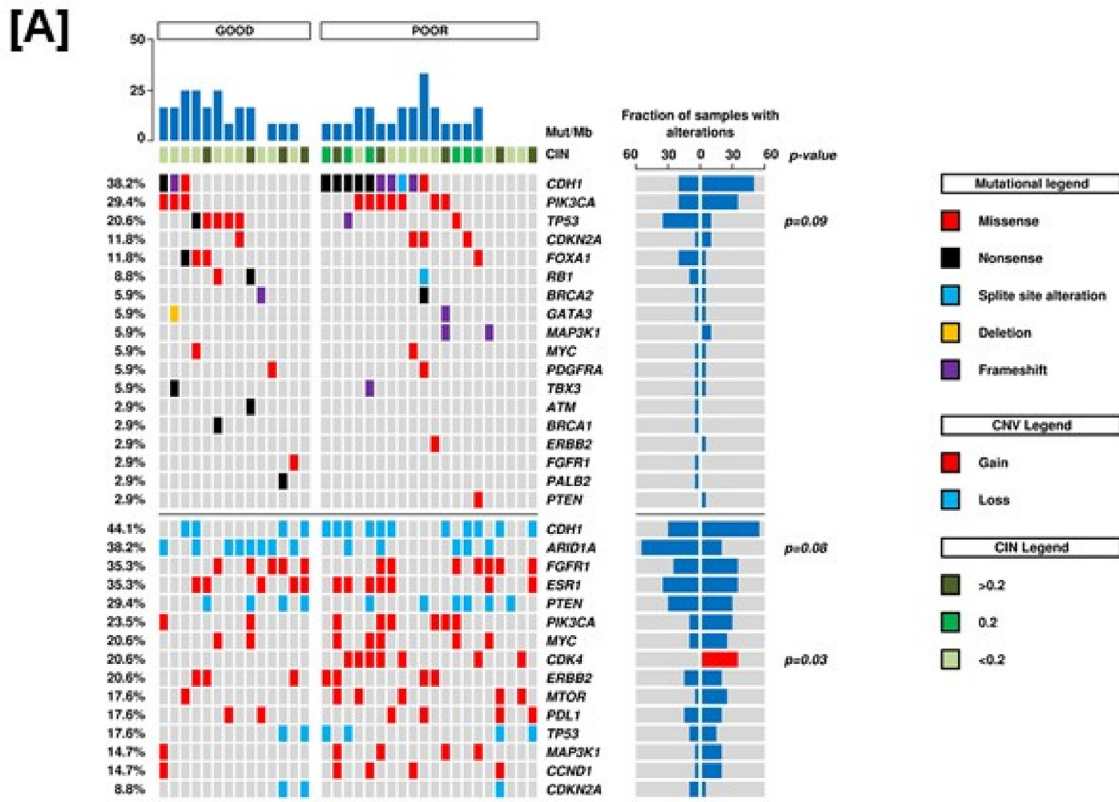
Regarding the sTIL assessment, the median value was 1% (range 0–30%), with only 6% of cases presenting a TILs percentage  $\geq 10\%$ . No significant association between TILs level (considered as a categorical variable,  $<1\%$  and  $\geq 1\%$ ) and prognosis was found (OR 1.75, 95%CI 0.45–6.8,  $p = 0.41$ ). In addition, no association between TILs level and *CDK4* gains was identified.

Finally, the MCA suggests that patients with good prognosis (upper left quadrant) and poor prognosis (lower right quadrant) seem to be correlated with distinct molecular alterations as defined by the distribution of some biomarkers, that appear far from the origin, and diagonally opposite (Fig. S6).

#### 4. Discussion

The current multicenter study suggests that the combination of clinico-pathological parameters seems able to stratify the prognosis of patients affected by early-stage ILC. Moreover, the external validation supported the discrimination performance of the prognostic model, highlighting the potential ability of this model to accurately separate ILC patients into three risk classes according to their individual risk of recurrence.

The risk classification system for DFS comprises the two traditional clinico-pathological parameters adopted in daily clinical practice: T-category and nodal status. These factors represent the milestone variables driving the prognosis and the choice of adjuvant treatment in the context of early-stage BC, together with the



**Fig. 2.** Comparison of mutational load, chromosome integrity number, somatic mutations and copy number variation between patients with poor prognosis and patients with good prognosis [Panel A]. Odds Ratio analysis [Panel B] of somatic mutations (Panel A) and copy-number variation (Panel B) according to prognosis: an OR<1 indicates a higher chance to be associated with good prognosis; an OR>1 indicates a higher chance to be associated with poor prognosis. CIN, chromosome integrity number; CNV, copy number variation; OR, odds Ratio; CI, confidence interval; SM, somatic mutation; CNV, copy number variation.

hormone receptor and HER2 status [7]. Regarding OS, age and Ki67 represent the variables included in the prognostic model, besides the nodal status. Previous retrospective series reported other potential prognostic factors for ILC, such as the tumor grade; however, they did not consider the role of Ki67, a proliferation marker usually involved in the treatment decision of early BC, and they also included metastatic tumours [27]. Conversely (and similarly to our data) tumor histological grade did not result to be an independent prognostic factor for early-stage ILC in a different retrospective series [28]. With regard to the Ki67 role in the lobular histotype, we previously suggested that a very low cut-off of Ki67 (4%–5%) may be able to significantly discriminate the prognosis of patients with 'pure' ILC [2,29].

Our analysis does not represent a simple investigation of prognostic factors for ILC (as previously reported by different retrospective studies); the novelty of this study consists in the development and validation of a prognostic tool (consisting of the combination of reliable clinico-pathological factors with different prognostic weight according to the model's score assessment) easy to adopt in the clinical practice. These results may help the clinicians to estimate the DFS and OS of ILC, a histotype where several aspects (including the prognosis) represent a matter of research for the personalized medicine. Indeed, although the common prognostic factors are indiscriminately applied for both lobular and ductal histology, determining that ILC patients are substantially treated in the same way as those affected by invasive carcinoma of NST, the overall prognosis of the two histotypes appears different [3,27]. Thus, this peculiar prognostic aspect supports the hypothesis that specific molecular features might drive the ILC prognosis. In this regard, the selection of 'exceptional' responders may increase the likelihood of finding a molecular characteristic that could account for the outcome [9].

Based on this approach, we performed the molecular analysis in an explorative subset of 'worst' prognostic performers, comparing the results with those of a subset of 'best' performers. The most interesting finding of our analysis is represented by the potential negative prognostic role of *CDK4* gain, detected by NGS analysis. Moreover, the *CDK4* amplification, detected by FISH analysis, and the nuclear *CDK4* overexpression displayed a trend toward an association with poor prognosis.

The involvement of *CDK4* amplification/gain in tumorigenesis has been found in various tumours including malignant gliomas and sarcomas [30,31]. In the context of BC, a previous analysis, conducted in 95 patients undergone surgery (80 carcinoma of NST and 15 ILC), suggested that *CDK4* amplification (detected in 15.8% of patients) was associated with high tumor cell proliferation [32]. In another study, included 102 cases of carcinoma of NST, the immunohistochemical nuclear expression of *CDK4*, detected in about 70% of tumor samples, did not show any significant correlations with the main clinical prognostic factors nor with survival [33].

However, no previous studies reported the prognostic role of *CDK4* in the specific context of ILC. According to the TCGA data, no *CDK4* alterations was detected in 127 ILC. On the contrary, the genomic characterization of ILC conducted by Desmedt et al. identified *CDK4* gain in 1.7% of the 170 lobular tumours with CNA data. The discrepancy in *CDK4* gain prevalence between our series and the others may be explained by differences in term of prognostic characteristics, considering that we detected *CDK4* gain only in patients selected for their extremely poor prognosis. Thus, this potential prognostic role deserves to be further explored in the featured context of ILC with worse outcome.

The combined subset analysis of the molecular alterations involved in the G1/S phase regulation strengthens a potential role of this cell cycle pathway in the prognosis of ILC. However, the low

**Table 3**

Prevalence of somatic mutations and copy number variations analysis of the 26 genes in the 34 invasive lobular carcinoma patients according to prognostic groups.

Gene	Alteration	Poor Group Patients N (%)	Good Group Patients N (%)	Total N (%)	<i>p</i> -value*
<i>AKT1</i>	–	–	–	–	–
<i>ARID1A</i>	Loss	5 (25.0)	8 (57.1)	13 (38.2)	0.08
<i>ATM</i>	SM	0	1 (7.1)	1 (2.9)	–
<i>BRCA1</i>	SM	0	1 (7.1)	1 (2.9)	–
<i>BRCA2</i>	SM	1 (5.0)	1 (7.1)	2 (5.9)	–
<i>CCND1</i>	Gain	4 (20.0)	1 (7.1)	5 (14.7)	–
<i>CDH1</i>	SM	10 (50.0)	3 (21.4)	13 (38.2)	–
	Loss	11 (55.0)	4 (28.6)	15 (44.1)	–
<i>CDK4</i>	Gain	7 (35.0)	0	7 (20.6)	0.03
<i>CDKN2A</i>	SM	3 (15.0)	1 (7.1)	4 (11.8)	–
	Loss	1 (5.0)	2 (14.3)	3 (8.8)	–
<i>ERBB2</i>	SM	1 (5.0)	0	1 (2.9)	–
	Gain	4 (20.0)	3 (21.4)	7 (20.6)	–
<i>ESR1</i>	Gain	7 (35.0)	5 (35.7)	12 (35.3)	–
<i>FGFR1</i>	SM	0	1 (7.1)	1 (2.9)	–
	Gain	7 (35.0)	5 (35.7)	12 (35.3)	–
<i>FOXA1</i>	SM	1 (5.0)	3 (21.4)	4 (11.8)	–
	Gain	1 (5.0)	1 (7.1)	2 (5.9)	–
<i>GATA3</i>	SM	1 (5.0)	1 (7.1)	2 (5.9)	–
<i>MAP3K1</i>	SM	2 (10.0)	0	2 (5.9)	–
	Gain	4 (20.0)	1 (7.1)	5 (14.7)	–
<i>MTOR</i>	Gain	5 (25.0)	1 (7.1)	6 (17.6)	–
<i>MYC</i>	SM	1 (5.0)	1 (7.1)	2 (5.9)	–
	Gain	5 (25.0)	2 (14.3)	7 (20.6)	–
<i>PALB2</i>	SM	0	1 (7.1)	1 (2.9)	–
<i>PDGFRA</i>	SM	1 (5.0)	1 (7.1)	2 (5.9)	–
<i>PDL1</i>	Gain	4 (20.0)	2 (14.3)	6 (17.6)	–
<i>PGR</i>	–	–	–	–	–
<i>PIK3CA</i>	SM	7 (35.0)	3 (21.4)	10 (29.4)	–
	Gain	6 (30.0)	2 (14.3)	8 (23.5)	–
<i>PTEN</i>	SM	1 (5.0)	0	1 (2.9)	–
	Loss	6 (30.0)	4 (28.6)	10 (29.4)	–
<i>RB1</i>	SM	1 (5.0)	2 (14.3)	3 (8.8)	–
<i>TBX3</i>	SM	1 (5.0)	1 (7.1)	2 (5.9)	–
<i>TP53</i>	SM	1 (5.0)	5 (35.7)	7 (20.6)	0.09
	Loss	4 (20.0)	2 (14.3)	6 (17.6)	–

Legend-Table 3. N, Number; SM, somatic mutation; *p*-value\* according to Fisher's exact test (only *p*<0.10 are reported).

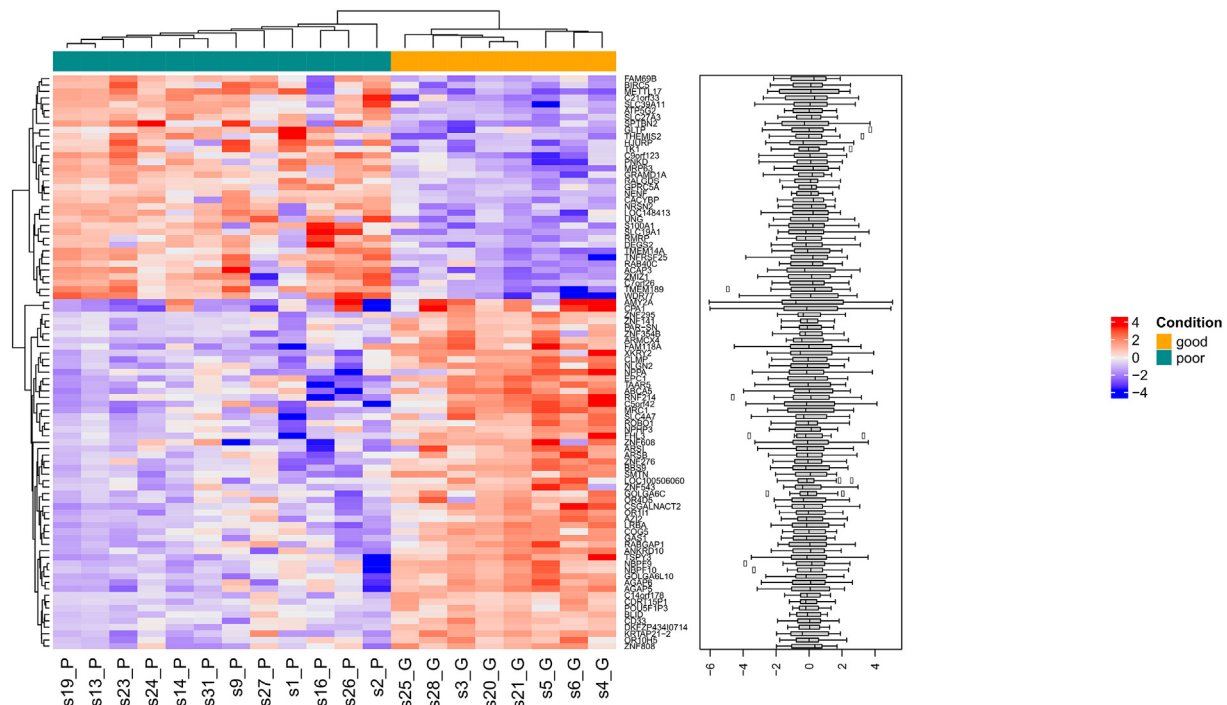
number of analysed samples and the criteria in the selection of patients, limit the possible implication of such data, given the risk of a false positive result.

As expected in the lobular histotype, the most frequent alteration is represented by the *CDH1* mutation and loss of heterozygosity, without a different distribution in the two prognostic groups. Similarly, mutations in *PIK3CA*, *TP53*, *CDKN2A* and *FOXA1* does not present a different distribution according to prognosis.

The incidence of *FOXA1* mutation and *ESR1* gain are similar to that reported by Desmedt et al. (11.8% vs. 9% and 35.3% vs. 25.3%, respectively) [5]. Considering that *FOXA1* plays a key role in the endocrine signaling, these gene alterations need to be further explored in the context of ILC, where the endocrine therapy represents the main therapeutic strategy [34].

The unsupervised clustering analysis of the transcriptome evidenced differences between the two prognostic groups in terms of gene expression level. Unlike a previous integrative study [4], our analysis showed that a series of genes, overexpressed in the poor prognosis group, may play a relevant role in the oncogenesis. For example, the *METTL17* interacts with both the AF1 and AF2 domains of ER $\alpha$ / $\beta$ . In a recent study, the observation that knockdown of *METTL17* reduces BC cell growth suggests that *METTL17* regulates the cancer cell growth possibly through modulation of ER $\alpha$  function as well as the expression of ER $\alpha$ / $\beta$  target genes [35].

Concerning the presence of sTIL, our analysis showed that most of ILCs are characterized by low sTIL levels, without differences



**Fig. 3.** Heatmap displaying normalised expression values of the 90 differentially expressed genes between the two prognostic groups at a p-value cutoff of 0.05. Hierarchical clustering correctly separates the 'good' and the 'poor' samples.

between the two prognostic cohorts. These results are consistent with a recent analysis reporting that the percentage of sTIL in ILC was lower compared to that in invasive carcinoma of NST and that the sTIL level did not represent an independent prognostically variable [36].

The most notable limit of this study, beyond the retrospective design and the absence of a central pathology review, is represented by the small sample size for the molecular analysis. Therefore, the estimation in the frequency of genomic alterations is imprecise. A larger cohort is needed to validate the results in independent datasets. Despite this, the stratification according to prognosis, based on a large cohort of ILC patients (more than 750), offers the possibility to better identify potential molecular differences associated with outcome.

## 5. Conclusions

A risk stratification model, able to potential separate early-stage ILC patients' prognosis into different risk classes according to clinico-pathological variables, allowed to investigate potential biomarkers of prognosis with targeted NGS in an explorative cohort. The reported molecular analysis needs to be interpreted as hypothesis generating. *CDK4* gain is suggested for future validation as a prognostic biomarkers and potential therapeutic opportunity.

## Ethical approval

The study was approved by the local Ethics Committees (Coordinating Center [University of Verona]: Prot. CESC n° 24,163, May 20th, 2014).

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.breast.2020.01.034>.

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