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Corso di Dottorato di Ricerca in Oncologia Clinica e Sperimentale e Immunologia

Ciclo XXXVI

Breast cancer biological characterization - Evaluation of the prognostic and/or predictive role of clinical, pathological and molecular biomarkers to dissect breast cancer heterogeneity: focus on estrogen-low breast cancer

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ABSTRACT

Background

Although 1% is the recommended cutoff for defining triple-negative breast cancer (TNBC), growing evidence suggests that 10% cutoff may better recapitulate TNBC. Conversion to TNBC at relapse is associated with poor survival. We primarily aim to assess the prognostic impact of phenotypic conversion to estrogen receptor (ER)-low BC in patients experiencing relapse.

Methods

Relapsing BC patients from two Institutions were included. Patients were categorized in: TNBC (ER=0%, HER2-0/low), ER-low (ER=1-9%, HER2-0/low), Luminal-like (ER=10-100%, HER2-0/low), HER2+. Overall survival (OS) and post-relapse survival (PRS) were adopted as endpoints.

Results

877 patients were included. The proportion of ER-low tumors was 2.6% on primary BC and 2.7% on relapse.

When assessing the prognostic impact of primary BC phenotype, TNBC and ER-low subtypes showed similar and significantly poorer OS and PRS as compared to Luminal-like and HER2+ subtypes. In particular, median OS [mos] was: TNBC 68.6 vs ER-low 47.6 vs Luminal-like 125.4 vs HER2+ 121.4, $p<0.001$. PRS analysis described the same phenomenon ($p<0.001$). Superimposable findings were observed when considering the prognostic impact of tumor phenotype at relapse (OS, $p<0.001$; PRS, $p<0.001$).

At relapse, 6.4% of TNBC (n=6), 2.5% of Luminal-like (n=10) and 1.9% of HER2 BC cases (n=3) converted to ER-low phenotype, with a total conversion rate to ER-low BC of 2.8%. Among Luminal-like primary BC patients, those converting to ER-low phenotype at relapse showed the worst survival outcome as compared to those with stable Luminal-like disease or evolving to either TNBC or HER2+ BC at relapse. In detail, median OS (mos) was: concordant Luminal-like 131.3 vs conversion to HER2+ 129.9 vs conversion to TNBC 75.9 vs conversion to ER-low 50.6, $p<0.001$. Superimposable findings were observed for PRS.

Conclusions

ER-low BC was associated with unfavorable prognosis, similar to TNBC and significantly poorer than Luminal-like and HER2+. Luminal-like BC patients converting to ER-low phenotype experienced the worst survival rates, even worse than those converting to TNBC, possibly due to the limited access to TNBC treatment algorithms. Our study supports the assimilation of ER-low BC to TNBC.

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1 BACKGROUND

Breast cancer (BC) represents the most frequently diagnosed solid tumor in women across the globe. In addition, despite the constant improvements in terms of curability rates, it represents the second-leading cause of cancer related death in women world-wide[1]. In Italy, BC accounts for approximately 55.7000 new cases per year (2022) and 12.500 new deaths per year (2021)[2], thereby making it evident the substantial financial, social and health-related burden of this disease.

1.1 Breast cancer classification

BC represents a highly heterogenous disease, classified, in the clinical practice setting, into 3 major subtypes based on the expressions of hormone receptors (HR, estrogen receptor [ER] and progesterone receptor [PgR]) and HER2 overexpression or gene amplification: HR+/HER2-, HER2+ and triple-negative (TN)[3]. The therapeutic landscape of BC still mostly relies on this stratification, driving access to the following – not completely exhaustive – macro-therapeutic areas: endocrine-based treatments (HR+ BC), anti-HER2-based therapy (HER2+ BC), and chemotherapy (all subtypes, especially TNBC AND her2+) with the possible association of immunotherapy (TNBC)[3]. However, in the last years, a finer classification based on the assessment of these traditional biomarkers has been put forward. In particular, the dichotomic stratification in HER2 +/- has now evolved as a three-tier classification[4], namely HER2+ (immunohistochemistry [IHC] score 3+ or gene amplification by in situ hybridization [ISH]), HER2-low ([IHC score 1+/2+ in the absence of gene amplification by ISH) and HER2-null (IHC score 0) based on the availability of novel anti-HER2 treatment opportunities (anti-HER2 antibody drug conjugate, [ADCs]) in patients traditionally classified as HER2- but showing low levels of HER2 expression (the so-called HER2-low category)[5]. Importantly, this distinction between HER2-null and HER2-low BC is purely operational, since available data do not support HER2-null and HER2-low subtypes as biologically distinct entities. Moreover, the ER +/- dichotomization (≥ or < 1%)[6] is being challenged by the increasing availability of data supporting the ER-low subgroup (1-9%, formally defined as ER+) as more clinically and biologically similar to pure-TN rather than the ER frankly positive subtype in terms of molecular features, prognostic impact and treatment sensitivity[7–11]. These considerations pose the pressing need to generate data on this – so far – neglected entity.

1.2 Focus on ER-low breast cancer

According to available international guidelines[6], tumors with ER expression ranging from 1% to 100% are interpreted as positive and this position is strictly anchored to data suggesting a potential benefit from endocrine therapy also in case of low expression of ER. Indeed, studies addressing this topic have mainly been conducted in the 1990s and subsequently meta-analyzed in a large work involving 20 trials and more than 200.000 patients[12]. In particular, while it has been confirmed that patients with tumors showing very low levels of ER expression by ligand-binding assay (LBA, <10 fmol ER/mg protein) had no benefit from 5-year tamoxifen, patients exhibiting levels of ER ranging from 10 to 20 fmol ER/mg protein derived clinical benefit from the endocrine treatment, quantifiable as a one third reduction in the risk of recurrence. Although no formal conversion from LBA and standard IHC method for ER expression is currently unanimously accepted in terms of predictive power, the cutoff of 10 fmol/mg has been suggested as functionally equivalent to the 1% threshold by standard IHC, thus making 1% the most conservative cutoff for defining benefit and access to endocrine treatment. As a direct consequence, this consideration has been inflexibly interpreted as the mere assimilation of the ER-low phenotype within the ER-positive disease, thus leading to the systematically exclusion of ER-low BC patients from clinical trials designed for the TN subtype with regulatory purposes, and, therefore, from TNBC treatment algorithms.

However, accumulating evidence suggests that ER-low/HER2-neg BC shares clinical behavior and molecular features more with TNBC than frankly ER-positive disease, in terms of long-term prognosis, treatment sensitiveness (chemotherapy and immunotherapy) as well as intrinsic molecular subtype by gene expression.

In detail, from a clinical point of view, available evidence is consistent in suggesting that patients with ER-low BC experience similar survival rates to TNBC's, and worse than patients with ER frankly positive/HER2- tumors. On the same ground lie the growing body of data reporting similar pCR rates after neoadjuvant treatment for TNBC and ER-low BC phenotypes, higher that that achieved by the ER+/HER2- counterpart.
In particular, in a cohort of more than 3000 HER2- stage II-III patients undergoing neoadjuvant treatment, patients with TN or ER-low tumors were associated with significantly higher pCR rates as compared to those with ER≥10%[11]. Interestingly, overlapping rates of time to relapse (TTR) and overall survival (OS) were observed for patients whose tumors exhibited ER <1% or ER 1% to 9%, which were significantly poorer that that observed for patients with ER≥10% tumors. Consistently, a pooled analysis of 3 neoadjuvant trials revealed that TNBC and ER-low patients showed similar pCR rates after neoadjuvant treatment, which were significantly higher than that observed within the ER frankly positive cohort[9]. In addition, TNBC and ER-low patients were associated with similar survival rates in terms of disease-free survival (DFS), distant DFS (DDFS) and OS, which were significantly poorer than that of the ER-positive BC subgroup. Similar findings were observed in a study involving 406 patients with ER<10%/HER2- BC treated with neo/adjuvant chemotherapy, where similar OS and invasive relapse-free survival (iRFS) rates were observed when discriminating between patients with ER<1% vs 1-9%. In addition, in the subgroup treated in the neoadjuvant setting (n=165), similar pCR rates were reported for the two sub-cohorts (TNBC and ER-low)[7].
Moreover, particularly appealing appear the results from the NeoPACT phase II trial[13], where patients with ER<10%/HER2- tumor were treated with neoadjuvant pembrolizumab + taxane-carboplatin-based treatment (de-escalated chemotherapy backbone without anthracyclines). Overall, the study reported encouraging pCR rates (58%) with neoadjuvant immunotherapy + chemotherapy and, importantly, when focusing on the 16% of patients with ER-low phenotype, pCR rates were superimposable to that observed in the TNBC cohort, thus suggesting that TNBC and ER-low BC may also share sensitiveness to immunotherapy-based treatments.
These findings, overall, suggest that ER-low BC shares clinical features with TNBC, and behaves in a clearly more aggressive manner as compared to ER frankly positive subtype.

From a biological point of view, the currently available body of data is consisting in highlighting substantial molecular similarities between TNBC and ER-low BC mostly in terms of depletion of ER-associated gene expression, enrichment for the basal-like intrinsic molecular subtype and immune features. In more detail, gene expression analysis from 465 HER2- tumors revealed that average ESR1 mRNA expression was similar across TN and ER-low tumors, and significantly lower than in ER frankly positive tumors[8]. In addition, both ER-low and TNBC were enriched for basal-like tumors, while an enrichment for tumors classified as Luminal A was observed among cases with ER≥10%. Other studies[9,10] subsequently confirmed these findings in more than 4000 cumulative patients, strengthening the notion that ER-low and TNBC share features also in terms of molecular stratification. Additionally, it has been recently reported that ER-low versus TNBC show similar levels of TILs, CD8/FOXP3 ratio and rate of positive PD-L1 expression as well as similar positive prognostic effect of high TILs[14].

To conclude, data generated so far indirectly – albeit strongly - suggest that ER-low BC patients should be granted access to therapeutic opportunities currently available for TNBC, without neglecting the distinct features in terms of potential endocrine-sensitiveness. Unfortunately, the most important therapeutic breakthroughs that affected TNBC in the last years, formally apply only for patients defined as TNBC based on the ASCO/CAP recommendations, thus making the ER-low category an unmet clinical need.

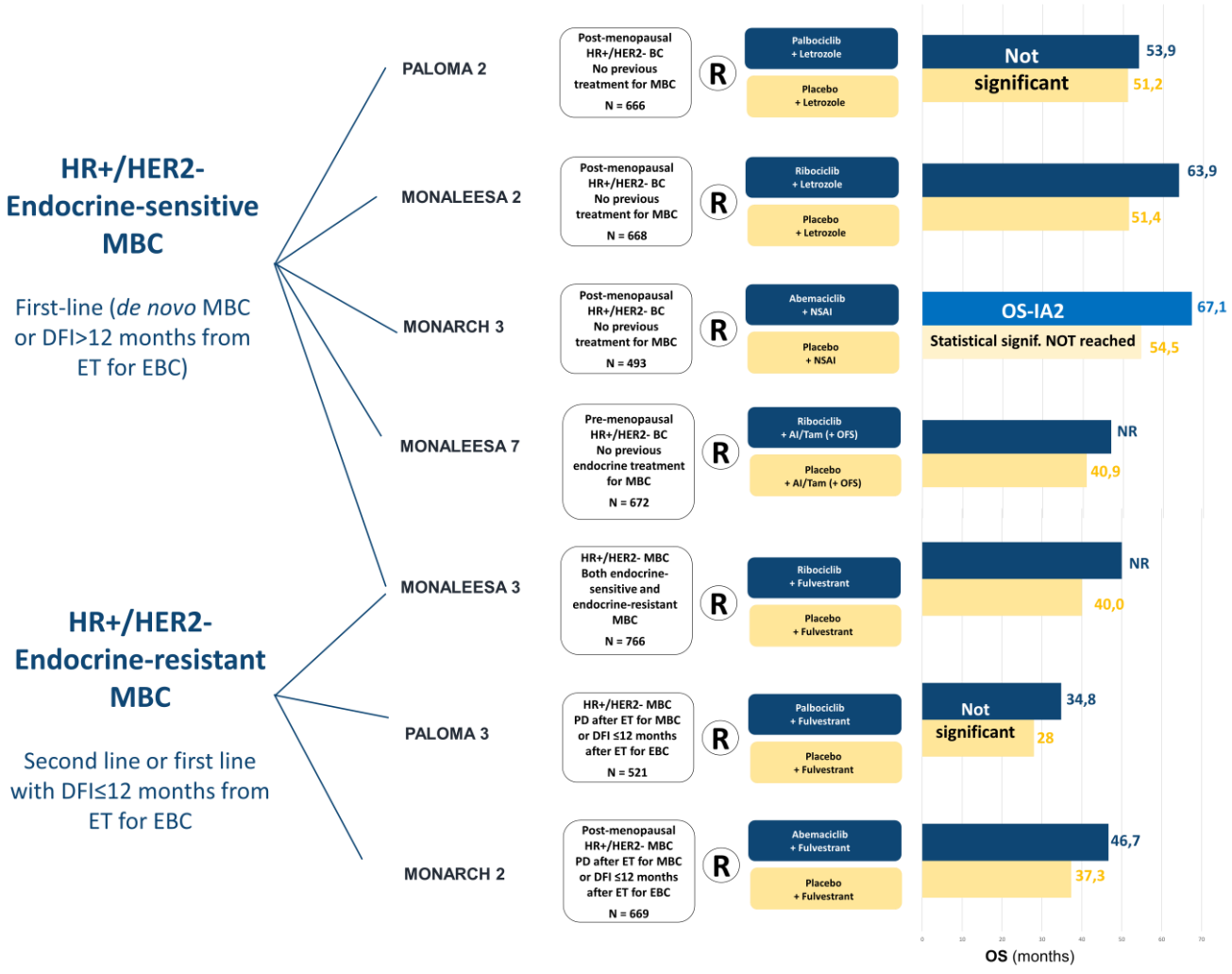
1.3 Advanced breast cancer treatment: focus on HR+/HER2- and TN BC

In the last years we have witnessed unprecedented broadening of the therapeutic armamentarium of advanced BC, leading to a substantial improvement of survival rates[15]. Despite these breakthroughs, once metastatic, BC remains a virtually incurably condition. The complexity of advanced BC management is driven by several factors underpinning the possibility of access to the best available care: most effective drugs, multidisciplinary and specialized care, high-quality imaging, pathology and locoregional treatments. In

addition, metastatic BC patients represent a very heterogenous population and one of the main determinants of patients' outcome is represented by disease biology, with triple-negative subtype surely representing the most evident unmet clinical need within the contemporary landscape of metastatic disease. A summary of treatment algorithm of metastatic disease, with special emphasis for HR+/HER2- and TN BC are summarized below.

1.3.1 HR+/HER2- breast cancer

In the first-line setting, the association of endocrine treatment plus CDK 4/6 inhibitor currently represents the standard option in most cases, based on the observed benefit in terms of progression-free survival (PFS) and, in some cases, also OS, along with the preservation of quality of life[16–26]. In case of visceral crisis (severe organ dysfunction with potentially fatal implications in the short-term period), chemotherapy may represent an option with the aim of achieving a rapid response. Currently, there are 3 CDK 4/6 inhibitors routinely and globally used in the clinic: palbociclib, ribociclib and abemaciclib. The choice of the endocrine backbone to be associated to CDK 4/6 inhibitors ranges from aromatase inhibitors to the selective endocrine degrader fulvestrant, with the main driver for choosing between the two being represented by the state of endocrine-sensitiveness/-resistance, as shown in Figure 1. In patients exhibiting a pre-menopausal status, ovarian function suppression should be achieved.



After progression to first-line therapy, both endocrine-based and chemotherapy-based treatments represent viable options based on several considerations, including patient features, depth and duration of the benefit from first-line treatment, disease burden. The combination of fulvestrant plus PI3K/AKT inhibitors are effective treatments in the aromatase-inhibitor resistant setting. In particular, the combination of fulvestrant + the PI3K inhibitor alpelisib is currently approved for HR+/HER2- BC patients progressing to aromatase inhibitor-based treatment in the light of the results from the phase III randomized SOLAR-1 trial[27]. The clinical value of this combination has been further solidified by the ByLieve phase II trial[48]. Importantly, this combination is currently not available in Europe for patients progressing after CDK 4/6 inhibitors, thus making this treatment a merely virtual option. In addition, the combination of fulvestrant + the AKT inhibitor capivasertib has recently shown, within the CAPItello-291 phase III trial, clinical activity and efficacy in HR+/HER2- BC patients progressing after aromatase inhibitors with or without CDK 4/6 inhibition both in the subgroup of AKT altered and non-altered tumors[28]. Other treatment options for pre-treated HR+/HER2- disease also include endocrine therapy plus the mTOR inhibitor everolimus, based on the BOLERO-2 phase III trial, where the addition of everolimus has been reported to significantly improve PFS, with, however, no impact on OS over endocrine therapy alone[29].

Once viable endocrine-based options have been exhausted, international guidelines recommend the use of chemotherapy. In this context, besides all traditional chemotherapeutic agents, including capecitabine, vinorelbine, eribulin, gemcitabine given as single agents or in combination[3], in the last years novel agents, namely ADCs, have monopolized the scene. ADCs' key structural components are the antibody, the payload and the linker. The first is responsible for the antigenic specificity of the drug, the second represents the effector component of the ADC and the latter, connecting the payload to the antibody, is the main director of the so-called bystander effect, which is capable of substantially enhancing the antitumor activity and efficacy of the drug. Within HR+/HER2- advanced BC, there are 2 ADCs with a current clinical positioning: the anti-HER2 ADC trastuzumab deruxtecan for HER2-low patients and the anti-TROP2 ADC sacituzumab govitecan. In particular, the Destiny-Breast04 trial set trastuzumab deruxtecan (FDA and EMA approved) as an effective treatment option for HER2-low BC patients who had received one or two previous lines of chemotherapy (win the presence of an endocrine-refractory disease)[5] and the TROPIC02 trial set sacituzumab govitecan (FDA and EMA approved) as a viable treatment options for HR+/HER2- metastatic BC patients treated with at least 2 and up to 4 previous lines of chemotherapy and at least one previous line of endocrine therapy including CDK 4/6 inhibitor[30].

Finally, in patients harboring germline BRCA1/2 mutations, the OlympiAd[31–33] and Embraca[34,35] phase III trials set the PARP-inhibitors olaparib and talazoparib, respectively, as valuable treatment options in patients previously treated with chemotherapy and endocrine therapy.

1.3.2 Triple-negative breast cancer

Given the absence of both HR expression and HER2 overexpression, chemotherapy represents the main treatment backbone for TN advanced BC.

The association of immune checkpoint inhibitors (atezolizumab [EMA approved] or pembrolizumab [FDA and EMA approved]) plus chemotherapy represents the standard first-line treatment options for PD-L1-positive patients, based on the results from the Impassion-130[36] and KEYNOTE-355[37,38] randomized phase III trials, respectively.

The anti-TROP2 ADC sacituzumab govitecan has been granted approval for relapsed or refractory metastatic TNBC patients who have received two or more prior systemic therapies, including at least one of them for advanced disease, based on the phase III ASCENT trial results[39]. The anti-HER2 ADC Trastuzumab Deruxtecan also received approval for HER2-low BC patient, including TNBC, who have received prior chemotherapy in the metastatic setting or developed disease recurrence during or within 6 months of completing adjuvant chemotherapy[5].

Similar to HR+/HER2- BRCA-associated advanced BC, the treatment armamentarium of patients with TNBC harboring a BRCA1/2 germline mutation also includes single-agent PARP-inhibitors (olaparib and talazoparib) after a previous treatment with both anthracycline and taxane-based chemotherapy[31–35].

1.4 Breast cancer instability

A substantial and consistent body of evidence suggests the BC biology may evolve during the evolution of the disease itself. This instability may be surrogated by the observation of a relatively high frequency of HR and HER2 status modifications from primary BC to disease relapse. The rate of receptor discordance widely varies across literature data, with generally higher discordance rates for ER, ranging from 10% to 60%, followed by PgR, with discordance rates ranging from 3% to 15% and, HER2, typically showing the more stable phenotype, with discordance rates generally lower than 10%[40–44]. Based on this notion, along with the expanding knowledge regarding intra-tumor heterogeneity and time-dependent clonal selection during tumor progression and under therapeutic pressure[45–48], international guidelines currently endorse tissue characterization of the disease at relapse since this approach may optimize the management of metastatic BC, even in terms of treatment adaptation[3]. Within this framework, there is still disagreement on whether to prioritize the primary tumor phenotype or, conversely, the metastatic lesion phenotype in terms of treatment selection/access. It has been recently outlined[33] that, in case of important differences in ER/PgR and HER2 status between the primary tumor and recurrence, expert opinion and limited clinical trial evidence for HER2-targeted therapies indicate that patients should be managed according to receptor status of the recurrent disease biopsy. However, tumor heterogeneity deserves to be taken into account for each new line of treatment and a re-biopsy may be appropriate in cases of mixed/unexpected response patterns[3].

Interestingly, in recent years, the concept of phenotypic instability has further evolved by envisaging the most contemporary stratification of HER2 status, taking into account the emerging HER2-low category. In particular, in our pivotal study we reported a not negligible 38% rate of HER2 conversion from primary BC to relapse, with cases switching from HER2-0 to HER2-low or from HER2-low to HER2-0 representing the main determinants of such instability[49]. These findings have been further confirmed by subsequent reports[50], thus stressing the importance of relapse biopsy in case of a primary HER2-0 tumor, since this approach may open new therapeutic opportunities in a relevant proportion of patients.

Importantly and additionally, receptor discordance has gained increasing interest in the light of accumulating evidence suggesting a prognostic impact of such phenomenon. In particular, a negative prognostic value of ER single receptor discordance between primary breast cancer and metastatic sites has been consistently reported, with ER loss representing the main determinant of this dismal prognostic impact[41,51,52]. A similar negative prognostic association has been suggested for HER2 conversion, both in terms of general discordance and HER2 loss, however, data in this regard are less solid[41,51,52].
A further step forward is represented by the observation that not only single receptor discordance, but also BC phenotypic conversion retains a solid prognostic role. In particular, a retrospective analysis of 139 patients undergoing histological confirmation of breast cancer recurrence, revealed that those with discordant phenotypes in matched samples of primary tumor and metastatic tissue experienced significantly poorer survival rates as compared to patients exhibiting concordant phenotypes. Interestingly, among discordant cases, patients exhibiting a conversion into triple-negative phenotype experience the poorest outcome[41].
Taken together, these data highlight that the management of metastatic BC should also account for the phenomenon of receptor/phenotypic instability, since it affects a not negligible proportion of advanced BC patients, with important implications also in terms of prognostic stratification.

The aim of the present PhD project was to biologically characterize BC through the investigation of clinical, pathological and molecular biomarkers, ultimately aiming at dissecting BC heterogeneity. Given the

complexity of the topic addressed, the research activity during the PhD period spanned multiple sub-projects.

In the first year of the PhD Course, we focused on the dynamics of HER2-low expression. In particular we conducted two sub-studies:

a. Evaluation of the dynamic evolution of HER2 expression from primary BC to disease relapse in patients undergoing tissue re-sampling, resulting in 1 mini-oral presentation at an international meeting and 1 paper published on an international paper.
b. Evaluation of the dynamic evolution of HER2-low expression from baseline biopsy to residual disease after neoadjuvant treatment in patients failing to achieve pCR, resulting in 1 poster presentation at an international meeting and 1 paper published on an international paper.

In the second year we focused on the development of a prognostic model based on residual cancer burden (RCB) and TILs on residual disease (RCB-TIL composite score) in HER2+ breast cancer patients treated with anti-HER2-based neoadjuvant treatment, resulting in 1 plenary session oral presentation at an international meeting and 1 paper published on an international paper.

In the third year we focused on the biological characterization of HR+/HER2- BC. In particular, we conducted three sub-studies:

c. Development of an immune-genomic model based on the assessment of TILs and Oncotype-Dx Recurrence Score (RS) in a large adjuvant population[53–56], resulting in 1 mini-oral presentation at an upcoming international meeting and 1 paper under review on an international paper.
d. Dynamic tracking of PI3K/PTEN/mTOR pathway and prognostic value in HR+/HER2- BC patients with residual disease after neoadjuvant chemotherapy, resulting in 1 paper published on an international paper.;
e. Evaluation of the dynamic evolution of ER-low expression and prognostic impact of the acquisition of ER-low phenotype at BC relapse, object of the present PhD thesis.

For the purposes of the present thesis, we decided to focus on the evaluation of the dynamic evolution of ER-low expression and prognostic impact of the acquisition of ER-low phenotype at BC relapse since it represents, in our opinion, one of the hottest topics within BC experimental scenario.

2 METHODS

2.1 Population

BC patients undergoing biopsy or surgical resection of either locoregional recurrence or distant metastasis at two different Italian Institutions (Istituto Oncologico Veneto – IRCCS, Padova and Treviso Hospital, Italy) between 1999 and 2022 were identified. Those patients for whom ER and HER2 status evaluation was available on both primary tumor and matched relapse samples were included in this analysis. Patients experiencing contralateral BC in the absence of other sites of recurrence or those with ipsilateral relapse on prior breast conserving surgery were excluded. Similarly, patients with de novo stage IV breast cancer were excluded.

Tumor samples of primary breast cancer and paired locoregional recurrence/distant metastases were retrieved from the Archive of the respective Pathology Departments.

Patients clinicopathologic characteristics including age, stage at diagnosis, HR status and expression, HER2 status and expression, timing and site of locoregional and/or distant relapse, and survival status were recorded.

2.2 Pathology

ER expression was retrieved from the original pathology report and categorized as follows: ER-null in case of IHC staining in 0% of cancer cells, ER-low in case of IHC staining in 1-9% of cancer cells and ER+ in case of IHC staining in at least 10% of cancer cells[6]. Progesteron receptor (PgR) status was retrieved from the original pathology report and classified as positive in case of positive IHC staining in at least 10% of cancer cells.

HER2 expression was categorized as follows: HER2-null in case of IHC score 0, HER2-low in case of IHC score 1+ or 2+ in the absence of gene amplification by in situ hybridization, HER2+ in case of IHC score 3+ and/or HER2 amplification by ISH. HER2 status was retrieved from the original pathology report and was evaluated according to ASCO/CAP recommendations in place at the time of diagnosis[57–59]. However, all cases diagnosed between 2007 and 2013 were reviewed by IHC to comply with the 10% cut-off of cells staining for HER2-positivity.

Based on the matched ER and HER2 expression, patients were categorized according to the following subgroups: TNBC (ER-null/HER2-null or HER2-low), ER-low (ER-low/ HER2-null or HER2-low), Luminal-like (ER+/HER2-null or HER2-low), HER2+ (any ER/HER2+).

For the evaluation of primary BC, surgical samples were preferred over biopsies, with the exception of cases undergoing neoadjuvant treatment, for whom baseline biopsy was instead used.

2.3 Statistical analysis

Statistical analyses were carried out using IBM SPSS Statistics (version 22.0), software (IBM Corp, Armonk, NY, USA).

Descriptive statistics were performed to analyze patient demographics and clinical characteristics. For continuous variables mean, median, range values and quartiles were computed. Student-T test, and The Mann-Whitney and Kolmogorov-Smirnov nonparametric tests were used to study the distribution of continuous variables across groups defined by clinicopathologic characteristics. Chi-squared test ($\chi 2$) was used to compare categorical variables across subgroups. We built Sankey diagrams to graphically show the evolution of BC subtypes from primary to recurrent BC.

Overall survival (OS) was defined as the time from the date of primary BC diagnosis and the date of death or last follow-up. Post-recurrence survival (PRS) was defined as the time from the date of diagnosis of locoregional relapse/distant metastasis and the date of death or last follow-up. Post-distant recurrence survival (PDRS) was defined as the time from the date of diagnosis of distant metastasis and the date of death or last follow-up. The Kaplan-Meier method was used to estimate survival curves and the log-rank test was adopted to test for differences across groups. The Cox-regression model was adopted to calculate hazard ratios and 95% confidence intervals (CI).

All reported p values are two-sided, and significance level was set at $p<0.05$.

3 RESULTS

3.1 Patient cohort and clinicopathologic features

A total of 877 patients with relapsed BC were included. Clinicopathologic features of the entire cohort are shown in Table I.

Features		N (%)		Median (Q1-Q3)
Age (yrs)				50.95 (43.25-61.02)
Primary BC phenotype	TNBC	96 (11)		
	ER-low	23 (2.6)		
	Luminal-like	426 (48.6)		
	HER2+	167 (19)		
	NA	165 (18.8)		
Tumor grading (primary BC)	1	46 (5.2)		
	2	322 (36.7)		
	3	427 (48.7)		
	NA	82 (9.4)		
Histology (primary BC)	NST	683 (77.9)		
	Lobular	130 (14.9)		
	Other	37 (4.2)		
	NA	27 (3)		
Ki67 (primary BC)				25 (11-40)
Early BC treatment	Neoadjuvant CT	216 (24.6)		
	Adjuvant CT	485 (55.3)		
	Adjuvant ET	527 (60.1)		
	Neoadjuvant anti-HER2	45 (5.1)		
	Adjuvant anti-HER2	84 (9.6)		
Relapse site	Local	416 (47.5)		
	Distant	Overall	746 (85.3)	
		Visceral	437 (58.8)	
Relapse BC phenotype	TNBC	163 (18.6)		
	ER-low	24 (2.7)		
	Luminal-like	485 (55.3)		
	HER2+	159 (18.1)		
	NA	46 (5.3)		

Table I *Clinicopathological features of the study cohort*

Abbreviations: yrs, years; BC, breast cancer; TNBC, triple-negative breast cancer; ER, estrogen receptor; NA; not available; BC, NST, no special type; CT, chemotherapy; ET, endocrine therapy.

Primary tumor phenotype was available for 712 cases and the distribution was as follows: TNBC 13.5%, ER-low 3.2%, Luminal-like 59.8%, HER2+ 23.4%. Relapse tumor phenotype was available for 831 cases and the distribution was as follows: TNBC 19.6%, ER-low 2.9%, 58.4%, HER2+ 19.1%.

Clinicopathologic features according to primary tumor phenotype are shown in Table II.

Table II *Clinicopathologic features according to primary tumor phenotype*

Primary BC phenotype			TNBC	ER-low	Luminal-like	HER2+
Age (yrs), median			51.58	51.58	54.21*	47.54
Grading, N (%)		1	0 (0)	0 (0)	31 (7.3)*	3 (1.8)
		2	14 (14.6)	4 (17.4)	200 (46.9)*	38 (22.8)
		3	80 (83.3)*	17 (73.9)*	166 (39)	118 (70.7)*
Histology, N (%)		NST	81 (84.4)	20 (87)	328 (77)	140 (83.8)
		Lobular	3 (3.1)	2 (8.7)	80 (18.8)*	22 (13.2)*
		Other	12 (12.5)*	1 (4.3)	11 (2.6)	4 (2.4)
Ki67, median			50*	38*	20	25*
HER2 status, N (%)		0	58 (60.4)*	15 (65.2)*	207 (48.6)	0 (0)
		Low	38 (39.6)	8 (34.8)	219 (51.4)	0 (0)
		Positive	0 (0)	0 (0)	0 (0)	167 (100)
Relapse site, N (%)	Local		58 (35.6)*	11 (45.8)	174 (35.9)	75 (47.2)
	Distant	Overall	78 (47.9)	19 (79.2)	373 (76.9)	136 (85.5)
		Liver	22 (22.9)	7 (30.4)	163 (38.3)*	0 (0)
		Lung	37 (48.1)*	8 (42.1)	79 (21.3)	0 (0)
		Bone	18 (18.8)	8 (34.8)	200 (46.9)*	0 (0)
		Brain	8 (10.4)*	3 (15.8)*	10 (2.7)	0 (0)
		Lymph-node	24 (25)	2 (8.7)	103 (24.2)	0 (0)
		Soft tissue	7 (7.3)	1 (4.3)	20 (4.7)	0 (0)

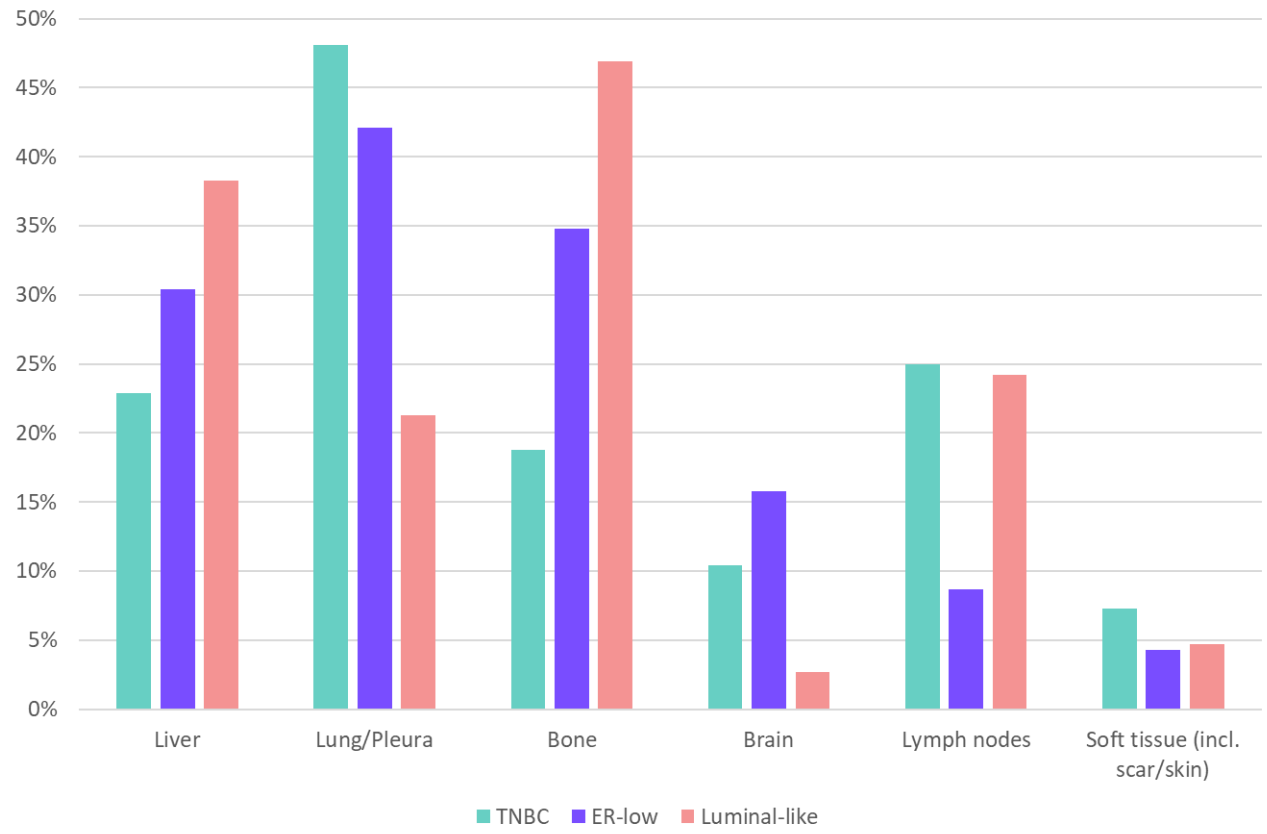
*statistically significant relationships

Abbreviations: yrs, years; BC, breast cancer; TNBC, triple-negative breast cancer; ER, estrogen receptor

Focusing on HER2- cases, as compared to Luminal-like disease, ER-low and TNBC subtypes were significantly associated with higher ki67 expression ($p<0.001$), higher proportion of grade 3 tumors ($p<0.001$), and HER2-null cases ($p=0.044$).

We also examined the patterns of relapse, observing that, compared to Luminal-like BC, TNBC and ER-low shared a higher tendency to brain ($p<0.001$) and lung (not statistically significant numerical trend) involvement as first site of relapse. Conversely, Luminal-like disease showed a significantly higher tendency to bone and liver involvement, as shown in Figure 2.

Figure 2 *Site of first relapse according to tumor phenotype, focusing on HER2- BC*



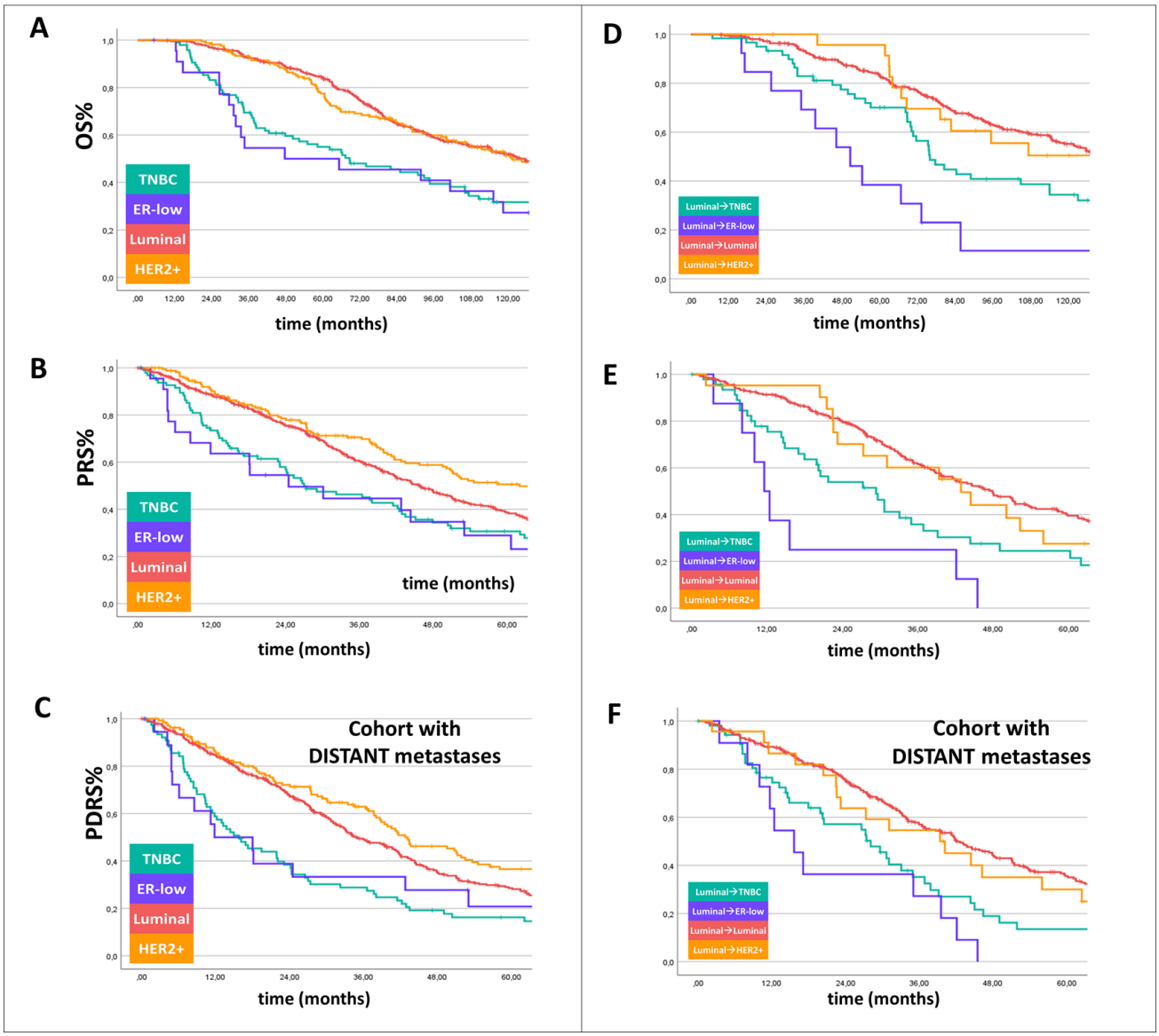
Abbreviations: TNBC, triple-negative breast cancer; ER, estrogen receptor

3.2 Prognostic impact of ER-low BC

Primary BC phenotype had a significant impact on prognosis. In particular, TNBC and ER-low subtypes showed similar and significantly poorer OS and PRS as compared to Luminal-like and HER2+ subtypes (median OS [mos]: TNBC 68.6 vs ER-low 47.6 vs Luminal-like 125.4 vs HER2+ 121.4, p<0.001, Figure 3A; median PRS [mos]: TNBC 27.0 vs ER-low 24.5 vs Luminal-like 45.8 vs HER2+ 61.9, p<0.001, Figure 3B). In addition, when focusing on the subgroup of patients with distant relapse (n=602), TNBC and ER-low patients exhibited poorer PDRS as compared to Luminal-like and HER2+ patients (median PDRS [mos]: TNBC 15.8 vs ER-low 11.8 vs Luminal-like 34.9 vs HER2+ 42.8, p<0.001, Figure 3C).

Superimposable results were observed when considering BC phenotype at relapse (OS, p<0.001; PRS, p<0.001).

Figure 3 *Kaplan Meier curves for overall survival (A,D), post-relapse survival (B,E) and post-distant relapse survival (C,F) according to primary tumor phenotype (A-C) and phenotype conversion in the cohort of Luminal-like primary breast cancer (D-F)*



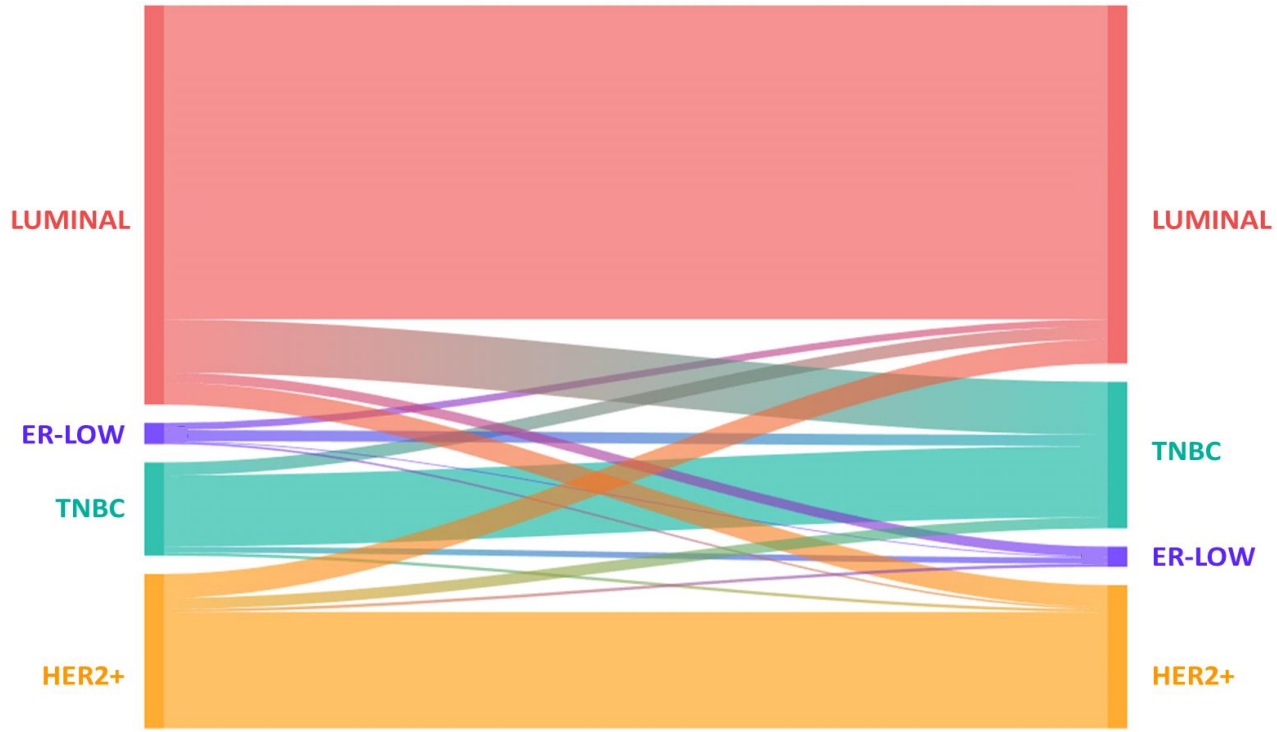
Abbreviations: TNBC, triple-negative breast cancer; ER, estrogen receptor; OS, overall survival; PRS, post-relapse survival; PDRS, post-distant relapse survival.

3.3 Phenotype discordance between primary BC and relapse and prognostic impact

The matched evaluation of primary and relapsed BC was available in 666 cases. The evolution of BC phenotype from primary to recurrent BC is shown in Figure 4.

In detail, 6.4% of TNBC (n=6), 2.5% of Luminal-like (n=10) and 1.9% of HER2 BC cases (n=3) converted to ER-low phenotype at relapse, with a total conversion rate to ER-low BC of 2.8%. In HER2- primary BC cases, HER2 expression (null vs low) did not affect the probability of conversion to ER-low disease at relapse.

Figure 4 *Sankey diagram showing the evolution of tumor phenotype from primary breast cancer to relapse.*



Abbreviations: TNBC, triple-negative breast cancer; ER, estrogen receptor

Patients converting from non-ER-low primary BC to ER-low relapse showed numerically poorer OS and PRS as compared to those not showing an evolution towards ER-low disease. In particular, median OS (mos) was: non-ER-low primary BC/non-ER-low relapse 121.7 vs non-ER-low primary BC/ER-low relapse 66.7, p=0.115 and median PRS (mos) was: non-ER-low primary BC/non-ER-low relapse 48.0 vs non-ER-low primary BC/ER-low relapse 42.1, p=0.227.
Focusing on patients with distant relapse, significantly poorer outcome was observed in case of conversion from non-ER-low primary BC to ER-low relapse with respect to cases without this phenomenon. In detail median OS (mos) was: non-ER-low primary BC/non-ER-low relapse 107.0 vs non-ER-low primary BC/ER-low relapse 50.6, p<0.001, median PRS (mos) was: non-ER-low primary BC/non-ER-low relapse 41.9 vs non-ER-low primary BC/ER-low relapse 15.6, p=0.007.

In the subgroup of patients with Luminal-like primary BC, those converting to ER-low phenotype at relapse showed the worst survival outcome as compared to those with stable Luminal-like disease or evolving to either TNBC or HER2+ BC at relapse. In detail, median OS (mos) was: concordant Luminal-like 131.3 vs conversion to HER2+ 129.9 vs conversion to TNBC 75.9 vs conversion to ER-low 50.6, p<0.001; median PRS (mos) was: concordant Luminal-like 96.7 vs conversion to HER2+ 118.7 vs conversion to TNBC 30.6 vs conversion to ER-low 17.1, p<0.001. Kaplan Meier curves are shown in Figure 3D-E.
Similar results were observed in patients with distant relapse. In particular, median OS (mos) was: concordant Luminal-like 126.7 vs conversion to HER2+ 107.2 vs conversion to TNBC 77.6 vs conversion to ER-low 50.6, p<0.001; median PRS (mos) was: concordant Luminal-like 47.8 vs conversion to HER2+ 42.8 vs conversion to TNBC 29.3 vs conversion to ER-low 11.5, p<0.001; median PDRS (mos) was: concordant Luminal-like 41.2 vs conversion to HER2+ 39.3 vs conversion to TNBC 27.2 vs conversion to ER-low 11.6, p<0.001, Figure 3F.

4 DISCUSSION AND CONCLUSIONS

BC is a very heterogeneous disease in terms of clinical behavior and biology, and ER expression represents one of the main determinants of such heterogeneity. In this context, current ASCO/CAP recommendations endorse the adoption of a 1% cutoff to distinguish between ER+ BC and TNBC, however, this dichotomization is being progressively challenged by a mounting body of evidence supporting the ER-low subgroup as more clinically and biologically similar to pure-TN rather than the ER frankly positive subtype. The present work represents the first study to evaluate, in a large multi-institutional cohort of relapsing BC patients, the clinical and biological landscape of ER-low disease as well as the prevalence and prognostic impact of the acquisition of ER-low phenotype at BC relapse.

We observed a low prevalence of ER-low disease, which accounted for less than 5% of all BC primary and relapse cases, thus confirming in a large cohort of relapsing BC that ER-low BC represents a relatively rare entity[6].

We subsequently observed that ER-low phenotype shared with TNBC more aggressive clinical course and biological features as compared to Luminal-like disease. In particular, both TNBC and ER-low BC patients showed feature of enhanced biological aggressiveness, with also implications in terms of patterns of relapse. Interestingly we observed that, relative to Luminal-like BC, ER-low patients showed a tendency to early relapse with a significantly higher risk of brain involvement as first site of disease recurrence, thus behaving similar to how it is usually described for TNBC[60,61]. It was therefore not surprising to observe that ER-low phenotype was associated with dismal prognosis in terms of OS and PRS in the overall cohort and in the subgroup of patients experiencing distant relapse, with superimposable survival rates of TNBC's. Our findings well fit within the current framework of available evidence supporting the ER-low disease as clinically and biologically comparable to TNBC, and further strengthen the notion that ER-low BC should incorporated into the TNBC definition, without however neglecting its distinct features in terms of potential endocrine-sensitivity.

The mail aim of this study was to investigate the rate of conversion to ER-low phenotype at relapse and to assess the prognostic impact of this phenomenon.
In detail, in our cohort of relapsing BC patients, 3% showed an evolution to ER-low phenotype at recurrence, with the strongest tendency observed for primary TNBC. In particular, 6.4% of TNBC patients evolved towards ER-low phenotype, thus identifying a subset of patients for which tumor resample could have opened the possibility to get access to Luminal-like BC treatment algorithms. This observation further strengthens the importance to retest disease biology at relapse, since it may open new therapeutic opportunities in case of receptor gain. Conversely, 2.5% of patients with Luminal-like primary disease switched towards ER-low disease at relapse. Although, for these patients, endocrine-based treatment formally still represents an option, there is great uncertainty regarding the actual clinical value of this strategy in case of subtotal loss of ER expression. Notably, we observed that patients with non-ER-low primary BC acquiring ER-low phenotype at relapse experienced significantly poorer outcome than patients not exhibiting such phenomenon. Splitting this observation according to primary tumor phenotype and focusing on patients with originally Luminal-like disease, we observed that cases for which the evolution of tumor phenotype coincided with the acquisition of an ER-low phenotype not only experienced significantly poorer prognosis than patients preserving Luminal-like phenotype during the course of disease, but this negative prognostic impact appeared to be even more evident with respect to cases showing a complete loss of ER expression (exhibiting a pure TNBC phenotype at relapse). This observation generates two orders of considerations. Firstly, we confirmed that ER loss (total of subtotal) has a detrimental impact on long-term prognosis, as already well described previously[41]. In this context it is worth mentioning that a previous report from our group similarly described that conversion to TNBC, defined as either complete or subtotal loss of ER expression, was associated with poor outcome[41]. However, the absence of stratified data according to ER loss subcategory (total vs subtotal) precluded the possibility to appraise the actual contribution of the evolution towards ER-low phenotype. Our results suggest, for the first time, that the subtotal loss of ER

expression is - at least - comparable to the acquisition of a pure TNBC phenotype at relapse in terms of prognostic implications. Notably, the negative prognostic impact of this phenomenon was confirmed both in terms of OS and PRS, thus suggesting that the evolution towards ER-low phenotype negatively affected the entire natural history of the disease, both from the time of primary BC diagnosis and of tumor relapse. The second consideration builds on the observation that patients converting to ER-low phenotype did even worse than those converting to TNBC. Although hypothesis generating, this finding may be the direct consequence of the limited access of ER-low BC patients to TNBC treatment algorithms. Indeed, the endorsement of a 1% cutoff for the definition of ER positivity is almost entirely driven by data supporting endocrine-based strategies to be effective also in case of low ER expression. However, within the contemporary experimental scenario, this aspect has been inflexibly interpreted as a mere absorption of ER-low phenotype within the Luminal-like cohort, thus resulting in the systematic exclusion of ER-low BC from TNBC trials with regulatory intents, precluding ER-low BC patients to be considered in the drug registration process of TNBC drugs. We believe that this may represent the most convincing interpretation of our survival analyses.
Of course, although these data deserve to be confirmed in prospective populations, enriched for patients receiving contemporary treatment strategies, we expect them to urge the BC scientific community to increase the awareness level towards this issue and endorse the inclusion of ER-low BC patients in potentially practice-changing clinical trials for TNBC, without however precluding the access to clinical trials intended for the ER+ disease.

The main strength of the present work is that it represents the first study evaluating the clinical impact of ER-low phenotype acquisition at relapse. In addition, the multi-institutional design may have resulted in minimizing the bias related to single-laboratory receptor assessment. Finally, the large sample size allowed this study to be informative regarding the prognostic impact of ER-low disease, which represents a relatively rare entity.

Some limitations must be acknowledged as well. Firstly, the retrospective nature of this work may have resulted in the enrichment of our population for patients exhibiting outlier behaviors. In addition, this study included a patient cohort spanning from 1999 and 2022 and this may have been responsible for a high heterogeneity in terms of treatments administered for either early or advanced disease, with the inevitable enrichment for historical cases receiving treatments following anachronistic algorithms.

In conclusions we demonstrated for the first time that BC evolution to ER-low phenotype at relapse is associated with dismal prognosis, even worse than the complete loss of ER expression, thus imposing the pressing need to grant ER-low patients access to treatment strategies formally developed in a purely TNBC experimental scenario. We also expect our results to solicit industry and academy driven trials to ensure ER-low BC patients the access to clinical trials potentially leading to the clinical positioning of novel drugs for TNBC. However, one must recognize that efforts in this direction are already ongoing, especially within academic clinical trials[13].

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