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Molecular subtyping and target identification in triple negative breast cancer through immunohistochemistry biomarkers

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Abstract

Background The Triple-Negative Breast Cancer (TNBC) molecular subtyping and target identification based on Immunohistochemistry (IHC) is of considerable worth for routine use. Yet, literature on this topic is limited worldwide and needs to be enriched with data from different populations.

Methods We assessed the IHC expression of subtyping biomarkers (Cytokeratins 5, 14 and 17, Epidermal Growth Factor Receptor, Claudins 3 and 7, E-cadherin, Vimentin and Androgen receptor) and predictive biomarkers (Tumor-infiltrating lymphocytes (TILs) density, Breast Cancer Antigen 1 (BRCA1) and P53) in a cohort of TNBC patients. Clinico-pathologic parameters and overall survival (OS) were investigated as well.

Results The patients were aged 50.11 ± 12.13y (more than 40y in 76.56% of patients), and 23.44% had a BC family history. They were in a non-advanced stage: 51.6% T2 stage, 56.2% negative lymph node involvement, 76.6% without metastasis and 64.1% grade II Scarff-Bloom-Richardson classification (SBR).

The IHC subtypes were: 53.1% Basal-like1 (BL1), 6.3% Basal-like2 (BL2), 17.2% Mesenchymal (MES), 9.4% Luminal Androgen Receptor (LAR), 4.7% Mixed subtype and 9.4% "Unclassified" type. The LAR subtype involved the youngest patients (40.17 ± 8.68y, $p = 0.02$). The "Unclassified" subtype expressed the p53 mutated-type pattern more frequently (100%, $p = 0.07$). The BRCA1 mutated pattern and TILs infiltration were present in (23.44% and 37.5% of patients, respectively).

The OS of the subtypes differed significantly ($p = 0.007$, log-rank test). The subtypes median OS were, respectively, 15.47 mo. (Unclassified), 18.94 mo. (BL2), 27.23 mo. (MES), 27.28 mo. (Mixed), 30.88 mo. (BL1), and 45.07 mo. (LAR). There was no difference in the OS following age, BRCA1 expression, p53 pattern and TILs density. Though, the OS following the TNM stage was different ($p = 0.001$). A multivariable Cox proportional hazards regression analysis showed that TNM staging and TNBC subtypes, independently influence the OS ($p < 0.001$ and $p = 0.017$, respectively).

Hence, IHC is useful in TNBC subtyping for prognostic purposes and in the identification of therapeutic biomarkers. Further investigation is required to confirm our results and to implement IHC as a routine tool to improve patient's care.

Keywords Immunohistochemistry, Molecular classification, Triple-negative breast cancer, BRCA1, P53, Androgen receptor, TILs, EGFR

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Background

Breast cancer (BC) is the most prevalent cancer worldwide [1]. The Triple Negative Breast Cancer (TNBC) is a heterogenous group of BC with a high aggressivity. The American Society of Clinical Oncology (ASCO) and the College of American Pathologists (CAP) define the TNBC as a BC that shows less 1% expression of progesterone receptor (PR) and estrogen receptor (ER) when examined through immunohistochemistry (IHC). Furthermore, TNBC is characterized by either 0 to 1 + expression of HER2 (Human Epidermal Growth Factor Receptor-2) by IHC with negative results in fluorescent in situ hybridization (FISH) testing [2, 3]. Hence, this rapidly progressive BC type typically lacks molecular targets for therapy. Consequently, chemotherapy and surgery remain the primary modalities in the clinical management of TNBC patients, as well as emerging therapies such as antibody–drug conjugates (ADCs) that offer a targeted approach maximizing therapeutic benefit. Statistically, TNBC accounts for 10 to 25% of incident BC depending on patient ethnic background [4, 5].

In-depth gene expression profiling was carried out by Lehmann and his colleagues who identified six different subgroups of TNBC: Luminal Androgen Receptor (LAR), Mesenchymal (MES), mesenchymal stem-like (MSL), Basal-like1 (BL1), Basal-like1 (BL2) and immunomodulatory (IM) [6]. Further research revealed that the stroma-associated tumor cells and infiltrating lymphocytes were the source of the IM and MSL subtype transcripts. Consequently, they revised their classification and grouped the subtypes into four main categories: BL1, BL2, MES and LAR subtypes [7]. In another work, [8] used mRNA profiling to identify four subgroup of TNBC; LAR, MES, BL immune-suppressed (BLIS), and BL immune-activated (BLIA) subtypes. Actually, the MES described by Burstein almost completely corresponded to the IM and MSL subtypes of Lehmann et al. [9].

Subtyping the TNBC can help provide valuable insights into its underlying biology, and identify potential therapeutic targets to personalize treatment. Lehmann et al., have shown, retrospectively and by gene expression profiling, that TNBC molecular subtypes differ in the rate of OS and pathological complete response to neoadjuvant chemotherapy, with BL1 being the most favourable subtype [7]. Similarly, [8] showed that the disease-free survival of every molecular TNBC subtypes is decreasing in the following order BLIA > MES > LAR > BLIS. More recently, using surrogate IHC, Leeha et al. showed different overall survival rates between TNBC subgroups, with the BLIS subtype having the worst outcome [10].

It is important to know that there are differences within and between ethnic groups regarding both the frequency of TNBC and its outcome [11–13]. These outcome differences are not fully explained by insurance status disparities and potentially disparate access to care [14, 15]. One of the possible explanations may be the heterogeneity of tumour biology between ethnicities. Interestingly, Ding et al. found that BLIS subtype is more frequent in Hispanic women (53%) while Asian women accounted for a lower proportion of BLIS (19%) but higher proportion of LAR (38%) compared to other ethnic groups [16]. As well, Jiang et al. compared the frequency of their mRNA-based TNBC subtypes in Chinese patients to Caucasian and African-American using the Cancer Genome Atlas (TCGA) database. They found BLIS, IM, and MES subtypes had comparable distribution between these ethnicities, while LAR subtype in the Chinese cohort was the most frequent [17]. Additionally, the TNBC frequency across Africa is highest in West Africa (45.7%) and lowest in Central Africa (14.9%) [18].

Most of our knowledge on TNBC biology and outcome was obtained using gene expression profiling as the preferred technique for molecular subtyping. However, this technique has a limited utility as a conventional diagnostic tool because it is time-consuming and the associated expenses are prohibitive. Hence, IHC surrogate panels would offer a practical solution to these problems [10, 19–21]. Additionally, there have been no previous studies on the molecular subtypes of TNBC in North African patients. Hence, the aim of this study was to characterize the distribution and prognostic relevance of IHC-defined molecular subtypes in a cohort of North African TNBC patients.

Patients, materials and methods

Patients and clinicopathological data

This was a prospective cohort study. Among the 573 patients diagnosed with breast carcinoma at both pathology departments of the Cancer Control Center (CLCC) and of the university hospital of Batna city, during the time period between 2018 and 2023, eighty-nine cases were diagnosed as TNBC. After removing tissue blocks with poor quality and those with a scarcity of material, 64 tissue blocks were available for further investigation. The inclusion criteria were patients with primary TNBC whose tissues, before chemotherapy or radiotherapy, were available. The TNBC was diagnosed when ER, PR and HER2 staining was negative. The negativity threshold set for hormone receptors is (< 1%) according to the Allred score. HER2 negativity was defined as IHC scores of 0 or 1 + . Cases with an IHC score of 2 + were classified as equivocal and FISH was performed to determine HER2 amplification status. They were considered HER2-negative if no amplification was observed by FISH [3].

Clinicopathological data were obtained from the medical records at the Oncology department of the CLCC.

According to the recommendations in the 7th edition of the American Joint Committee on Cancer (AJCC) guidelines, Tumor-Node-Metastasis (TNM) staging was carried out [22]. We calculated the Nottingham Prognostic Index (NPI) according to Todd et al. [23]. Then patients were separated into three prognostic groups: Good prognostic group GPG (NPI ≤ 3.4), Moderate prognostic group MPG (3.4 < NPI ≤ 5.4) and Poor prognostic group PPG (NPI > 5.4).

The treatment decisions were primarily based on the TNM staging of the disease. Patients received taxane-based chemotherapy, administered either as adjuvant or neoadjuvant therapy to surgery. Radiotherapy was utilized in cases classified as Stage III. Targeted therapies (like anti-PD1 monoclonal antibodies or ADCs) were not employed in our cohort.

The study protocol was approved by the Thematic Research Agency in Health and Life Sciences. Necessary precautions to protect participant's information were taken according to the principles of the World Medical Association Declaration of Helsinki and its later amendments. An informed written consent was obtained from all the patients.

Histological analysis and Immunohistochemistry

Morphological classification and tumor grading were based on the original pathology reports from the time of diagnosis. Representative tissue blocks for IHC were selected based on the presence of viable tumor tissue, as indicated in the histopathological records. These blocks corresponded to primary tumors obtained prior to the initiation of treatment, ensuring that the specimens represented the initial tumor state before any therapeutic intervention. Hematoxylin and eosin (H&E)-stained slides from each patient were carefully examined and the classification of the World Health Organization (WHO) was used to group the histologic subtypes [24]. The Scarff-Bloom-Richardson (SBR) grading system was used to determine the histologic grade.

To determine the tumor-infiltrating lymphocytes (TILs) density, the stromal TILs percentage was measured. The proportion of mononuclear inflammatory cells within the tumor's overall stromal region was measured to establish this percentage following the International TIL Working Group 2014 [25].

For the IHC assay, we started with an optimization on positive and negative control tissues. These conditions were then applied to the samples. Two types of controls were performed in this study: a negative control of the reagent was conducted by omitting the specific reagent in our sample, and positive and negative control tissue, following the recommendations provided in the datasheets (provided by the manufacturer) for each marker studied (for example: for AR, the positive control tissue is: Prostate, and the negative control tissue is: Cerebellum). Tumor sections of 4–5 µm were deposited on silane-coated adhesive slides, then dried overnight in an oven at 30°C. The samples were then deparaffinized by immersing the slides in three changes of xylene for 10 min each, and rehydrated in alcohol at decreasing concentrations (100%, 95%, 70%) for 5 min each, and then in distilled water (two washes during 5 min). For the restoration of the antigen, two unmasking buffers were used for 50 min at 90C° then they were cooled at room temperature for 30 min.

The detection step was carried out by the use of the (Novolink kit, Leica, as per the guidelines provided by the manufacturer). Briefly, the slides were neutralized by peroxidase block and protein block, for 30 min in the dark, followed by the incubation, for 30 min, with the corresponding primary antibody for each marker supplied by Leica Biosystem (the antibodies, their clones, dilutions, and positivity threshold are detailed in Table 1). Then, the secondary antibody conjugated to horseradish peroxidase (HRP), and the Novolink polymer were added. Finally, the DAB chromogen (3.3' -diaminobenzidine) which reacts with the HRP was added for 5 min. The

Table 1 Immunohistochemistry antibody panel for TNBC subtyping and target identification

Antibody	Clone	Dilution	Positivity threshold	Threshold reference	Location
ER	6F11	/	1%	[26]	Nuclear
RP	16	/	1%	[26]	Nuclear
Ki67	K2	/	20%	[27]	Nuclear
HER2	CB11	1/40	10%	[28]	Membrane
AR	AR27	1/25	1%	[28, 29]	Nuclear
p53	DO-7	/	5%	[28]	Nuclear
EGFR	113	1/20	10%	[28]	Membrane
CK5	XM26	/	10%	[28]	Cytoplasm Membrane
CK17	E3	/	10%	[28]	Cytoplasm Membrane
CK14	LL002	/	10%	[28]	Cytoplasm Membrane
BRCA1	MS110	1/100	5%	[28]	Nuclear
VIM	SRL33	1/400	50%	[30]	Cytoplasm Membrane
CLD3	4B12	1/200	80%	[31]	Membrane
CLD7	4B11	1/200	80%	[31]	Membrane
E-Cad	36B5	1/25	10%	[32]	Membrane

ER Estrogen Receptor *RP* Progesterone Receptor *HER2* Human Epidermal Growth Factor Receptor-2 *AR* Androgen Receptor *P53* Protein 53 *EGFR* Epidermal Growth Factor Receptor *CK5/CK17/CK14* CK for cytokeratin *BRCA1* Breast Cancer Antigen 1 *VIM* Vimentin *CLD3/CLD7* Claudins 3 and 7 *E-Cad* E-Cadherin

All antibodies were obtained from Leica Biosystems

sections were counterstained with hematoxylin for 4 min and dried at 80°C for 4 min. Following this, the slides were mounted with Biomount Eukitt® and covered with coverslips.

Immunohistochemical staining interpretation and scoring

In this study, two pathologists examined the slides, independently. All readings of IHC scoring were in total concordance among the pathologists, except for slight differences in some TILs percentages that did not change their classification as (< 10% or ≥ 10%). Hence, no adjudication process was required. In the event of a disagreement, a consensus approach would have been adopted, involving a joint review of the case. If consensus could not be reached, a third senior pathologist would have been consulted to provide a final adjudication. The positivity thresholds for IHC markers were determined based on established literature and are detailed in (Table 1) with a comprehensive assessment of staining intensity (0–3), cellular localization, and proportion of positive cells. The expression pattern was evaluated across membrane, nuclear, cytoplasmic, or combined compartments, depending on the studied biomarker (Table 1), utilizing a semi-quantitative scoring methodology that considered both staining intensity and percentage of positive cells. Staining intensity was graded on a scale from 0 (negative) to 3 (strong positive), with percentage of positive cells quantified in 10% increments, resulting in a final score calculated by multiplying intensity and percentage scores. All the markers, were scored based on the whole slide assessment. The average of pathologists' readings was considered the final score, ensuring a comprehensive and systematic evaluation of protein expression. Protein expression profiles were used to classify TNBC subtypes as described in [19]:

- BL1 subtype: EGFR expression is negative and CK5 and/or CK14 expression is positive.
- BL2 subtype: EGFR expression that is positive regardless of CK5 or CK14 positivity.
- Mesenchymal subtype (MES): Vimentin expression is positive and E-cadherin, Claudins 3 and 7 have a diminished expression.
- LAR subtype is associated with the positive expression of AR.
- Mixed subtype: situations where more than one subtype's phenotype is displayed.
- Unclassifiable subtype (or Unclassified): scenarios that did not fit into any of the previously listed subtype categories.

Regarding p53 IHC staining pattern, we considered it as "mutation-type" (totally negative or diffuse strong nuclear staining in more than 50% of tumor cells) or as "wild-type" (nuclear staining in less than or equal to 50% of tumor cells with heterogeneous intensity) following the pathology report [33].

Survival analysis

Patients were followed from the date of diagnosis until the end of follow-up period (15 December 2023), death, or loss to follow-up. The survival endpoint was Overall Survival (OS) defined as the time from diagnosis to death from any cause. Patients alive at the end of the follow-up or lost to follow-up were censored. The cumulative probability of survival over time was estimated by Kaplan–Meier method, and the Log-rank test was used to compare survival distributions between groups. Survival time was counted in months.

Statistical analysis

To meet the objectives of the study, a descriptive statistical analysis of the clinicopathological and histological characteristics of the studied population is carried out. The chi-2 test (or Fisher test where needed) is applied to compare the categorical variables. We used the Kruskal–Wallis test to assess whether the distribution of categorical variables is the same between NPI prognosis groups. One-way analysis of variance was used to study quantitative response variables according to the categorical variable "molecular sub-types". Some ordinal variables (stages) where regrouped when necessary. The overall survival was assessed using the Kaplan–Meier test. The p value is set at 0.05.

Results

Clinical features of the cohort

Among the included 573 breast cancer patients, 89 cases (15.53%) were diagnosed with TNBC, of which 64 cases were available for analysis in this study. The average age of the latter group is 50.11 ± 12.13 years, ranging from 27 to 84 years. The population's age distribution revealed that the majority (76.56%) is over 40 years old. We found a family history of breast cancer in 23.44% of patients.

As shown in Table 2, the histological type was predominantly represented by infiltrating ductal carcinoma (89.1%). The average tumor size was 3.7 cm, and 51,6% of the patients were classified in T2 stage. According to the SBR classification, all the tumors were either in the grade II or III, with a predominance of grade II (64.1%). The percentage of Ki-67 expression was above 20% in 79.7% of the cases. Lymph node involvement was observed in 43.8% of all cases, and no metastasis was found in more than 76.6% of cases. Overall, this information show that the studied population doesn't seem in an advanced stage of the disease.

Table 2 Clinicopathologic characteristics of TNBC patients

Characteristics	Patients N (%)
Age	
≤ 40 y	15 (23.44)
> 40 y	49 (76.56)
Menopausal status	
Premenopausal	25 (39.1)
Postmenopausal	39 (60.9)
Family history of breast cancer	
Presence	15 (23.44)
Absence	49 (76.56)
Histological type	
Infiltrating ductal carcinoma	57 (89.1)
Metaplastic	3 (4.7)
Medullary carcinoma	4 (6.3)
SBR grade	
II	41 (64.1)
III	23 (35.9)
Ki-67 expression	
< 20%	13 (20.3)
≥ 20%	51 (79.7)
Tumor size (cm)	
≤ 5	50 (78,13)
> 5	14 (21,88)
Tumor stage (T)	
T1	8 (12.5)
T2	33 (51.6)
T3	9 (14.1)
T4	14 (21.9)
Lymph node status	
Positive	28 (43.8)
Negative	36 (56.3)
Metastasis status	
M0	49 (76.6)
M1	15 (23.4)
TNM stage	
I	8 (12.5)
II	24 (37.5)
III	18 (28.1)
IV	14 (21.9)
NPI	
GPG	22 (34.37)
MPG	21 (32.81)
PPG	21 (32.81)

SBR (Scarff-Bloom and Richardson), TILs (Tumor Infiltrating Lymphocytes) TNM (Tumor-Node-Metastasis), NPG (Nottingham Prognostic Index), GPG (Good Prognostic Group), MPG (Moderate Prognostic Group), PPG (Poor Prognostic Group)

Molecular classification of TNBC according to IHC markers

Following the expression pattern of the different IHC markers (Table 3), six molecular sub-groups of TNBC tumors were observed. We found 53.1% of the cases in the BL1 subtype, 6.3% in the BL2, 17.2% in the MES and 9.4% un the LAR subtype. Approximately 9.4% of the cases were placed in a category called "Unclassified type" because they did not show definite expression of the used markers. Additionally, 4.7% of the cases showed an expression of both MES and BL markers, which placed them in the category of "Mixed subtype" (Table 4).

Clinicopathologic features in TNBC subtypes

The Table 4 lists the clinicopathological characteristics of each subtype of TNBC. Patients who are in the Mixed subtype were elder than patients from the other groups (67.67 ± 5.50 y), followed by patients from the Unclassified group (55.33 ± 11.32 y). Whereas, the youngest patients were in the LAR group (40.17 ± 8.68 y) ($p = 0.02$). Additionally, the comparison of the SBR grade between the subtypes showed a significant difference; notably, the MES subtype was observed more frequently in an advanced grade (SBR grade III) (54.54%, $p = 0.03$). However, the positive lymph node involvement was more frequent in the Unclassified subtype, although the difference did not reach statistical significance (66.67%, $p = 0.06$).

The p53 mutated-type staining pattern was observed in 79.68% of all the TNBC patients and there is no significant difference between the subtypes ($p = 0.07$).

BRCA1 loss of expression (mutant type) was present in 23.44% of our population. But this negative expression was more frequent in patient with a family history of BC (80%).

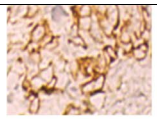
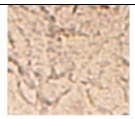
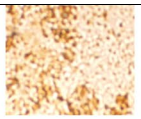
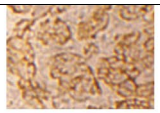
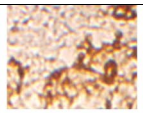
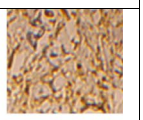
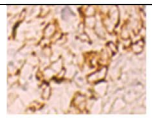
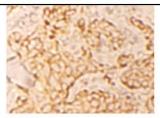
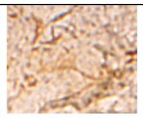
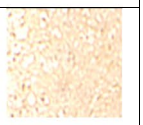
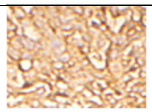
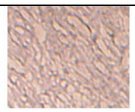
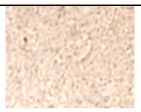
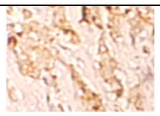
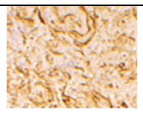
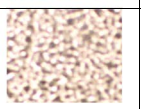
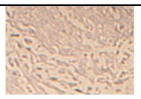
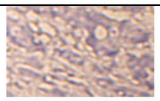
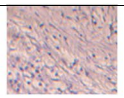
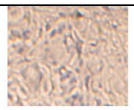
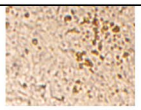
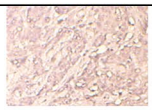
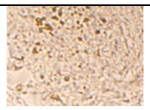
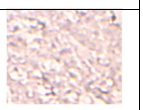
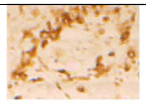
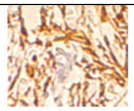
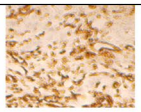
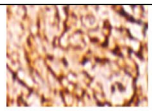
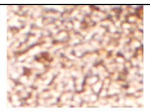
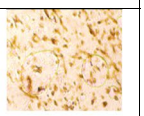
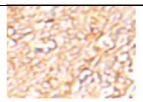
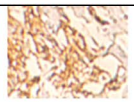
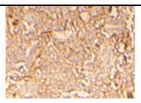
The comparison between the TNBC subtypes regarding the other parameters: Ki-67 expression, tumor size, TILs percentage, lymph node involvement, TNM stage and NPI, did not show any significant difference (Table 4).

TNBC subtypes and overall survival

The OS of all the patients was estimated using Kaplan–Meier survival analysis. At the end date of follow-up, 36 patients were alive, 27 patients had died and one person was lost to follow-up. The median follow-up for all patients was 27.23 months, ranging from 0.62 to 70.63 months.

Comparison of the survival curves between TNBC subtypes showed a significant difference ($p = 0.007$) (Fig. 1a). Median OS were varying: the subtypes BL2 and 'Unclassified' showed the shortest median OS; 18.94 and 15.47 months, respectively. On the other hand, the LAR and the BL1 subtypes exhibited relatively favorable OS, with medians of 45.07 and 30.88 months, respectively. The MES subtype and the Mixed subtype had median OS of 27.23 and 27.28 months, respectively. In order to determine whether the OS of young patients with TNBC (age ≤ 40 y) and older patients (age > 40 y) are different,

Table 3 TNBC classification following expression pattern of different IHC markers

	BL1	BL2	LAR	MES	MIXED	UnCLASSIFIED
CK5						
CK14						
CK17						
EGFR						
AR						
VIM						
E-CAD						
CLD3						
CLD7						
BRCA1						
p53						
TILs						

Subtypes of TNBC were identified by immunohistochemical characteristics. LAR (luminal androgen receptor); MES (mesenchymal), BL1 or 2 (Basal like 1 or 2), CK (cytokeratin), CLD (Claudin); VIM (Vimentin), E-CAD (E-Cadherin); AR (Androgen receptor); EGFR (epidermal growth factor receptor); BRCA1 (Breast Cancer Antigen 1); TILs (Tumor-infiltrating lymphocytes) density, Microscopic magnification x40.

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Table 4 Clinicopathologic features according to the TNBC subtype

Characteristics	*BL1*	*BL2*	*MES*	*LAR*	*MIXED*	*Unclassified*	*p value*
Subtype size, n (%)	34 (53.1)	4 (6.2)	11 (17.1)	6 (9.4)	3 (4.7)	6 (9.4)	
Average age (y)	48.82 ± 11.50	50.75 ± 8.15	51.64 ± 13.67	40.17 ± 8.68	67.67 ± 5.50	55.33 ± 11.32	0.02*
Age categories n (%)#							
≤ 40	9 (26.47)	1 (25)	1 (9.09)	4 (66.67)	0 (0)	0 (0)	0.08
> 40	25 (73.52)	3 (75)	10 (90.91)	2 (33.33)	3 (100)	6 (100)	
Menopausal status, n (%)#							0.38
Premenopausal	15 (44)	2 (50)	4 (36)	4 (66.67)	0 (0)	0 (0)	
Postmenopausal	19 (56)	2 (50)	7 (64)	2 (33.33)	3 (100)	6 (100)	
Histological type, n (%)#							
IDC	31 (91.17)	4 (100)	9 (81.81)	5 (83.33)	3 (100)	5 (83.33)	0.896
Metaplastic	2 (5.88)	0 (0)	1 (9.09)	0 (0)	0 (0)	0 (0)	
Modulatory	1 (2.94)	0 (0)	1 (9.09)	1 (16.67)	0 (0)	1 (16.67)	
SBR grade, n (%)#							0.03*
II	18 (52.94)	4 (100)	5 (45.45)	6 (100)	3 (100)	5 (83.33)	
III	16 (47.05)	0 (0)	6 (54.54)	0 (0)	0 (0)	1 (16.6)	
Ki67, n (%)#							
< 20%	8 (23.52)	1 (25)	0 (0)	1 (16.6)	1 (33.33)	2 (33.33)	0.33
≥ 20%	26 (76.47)	3 (75)	11 (100)	5 (83.33)	2 (66.67)	4 (66.67)	
P53, n (%)#							0.07
Wild Type	6 (17.64)	1 (25)	1 (9.09)	3 (50)	2 (66.67)	0 (0)	
Mutated Type	28 (82.35)	3 (75)	10 (90.91)	3 (50)	1 (33.33)	6 (100)	
BRCA-1, n (%)#							0.58
Positive	25 (73.52)	4 (100)	9 (81.81)	5 (83.4)	3 (100)	3 (50)	
Negative	9 (26.47)	0 (0)	2 (18.18)	1 (16.6)	0 (0)	3 (50)	
TILs, n (%)#							0.30
< 10%	25 (73.52)	2 (50)	5 (45.45)	4 (66.67)	2 (66.67)	2 (33.33)	
≥ 10%	9 (26.47)	2 (50)	6 (54.54)	2 (33.33)	1 (33.33)	4 (66.67)	
Tumor size, n (%)#							
≤ 5 cm	25 (73.52)	3 (75)	8 (72.72)	5 (83.33)	3 (100)	6 (100	0.80
> 5 cm	9 (26.46)	1 (25)	3 (27.27)	1 (16.67)	0 (0)	0 (0)	
Tumor stage, n (%)#							0.52
T1 + T2	18 (53)	3 (75)	8 (73)	4 (67)	3 (100)	5 (83)	
T3 + T4	16 (47)	1 (25)	3 (27)	2 (33)	0 (0)	1 (17)	
TNM stage, n (%)#							0.19
I + II	14 (41)	1 (25)	8 (73)	3 (50)	3 (100)	3 (50)	
III + IV	20 (59)	3 (75)	3 (27)	3 (50)	0 (0)	3 (50)	
Lymph node involvement, n (%)#							0.06
Positive	17 (50)	2 (50)	4 (36.36)	1 (16.6)	0 (0)	4 (66.67)	
Negative	17 (50)	2 (50)	7 (63.63)	5 (83.33)	3 (100)	2 (33.33)	
NPI groups, n (%)#							0.18
GPG	9 (26.47)	1 (25)	4 (36.36)	3 (50)	3 (100)	2 (33.33)	
MPG + PPG	25 (73.53)	3 (75)	7 (63.64)	3 (50)	0 (0)	4 (66.67)	

* Statistically significant. #Percentages are within subtype. BL1 or 2 (Basal like 1 or 2), MES (mesenchymal), LAR (luminal androgen receptor). SBR (Scarff-Bloom and Richardson), TILs (Tumor Infiltrating Lymphocytes) TNM (Tumor-Node-Metastasis), NPG (Nottingham Prognostic Index), GPG (Good Prognostic Group), MPG (Moderate Prognostic Group), PPG (Poor Prognostic Group), IDC (Infiltrating ductal carcinoma)

we compared between them but we did not find any significant difference ($p = 0.75$).

We performed also other comparisons of the OS regarding additional biomarkers. The comparison of the OS following the expression of BRCA1, p53 pattern and the TILs density did not show any differences ($p > 0.05$).

However, the comparison between the OS following the TNM stage showed a significant difference ($p < 0.01$)

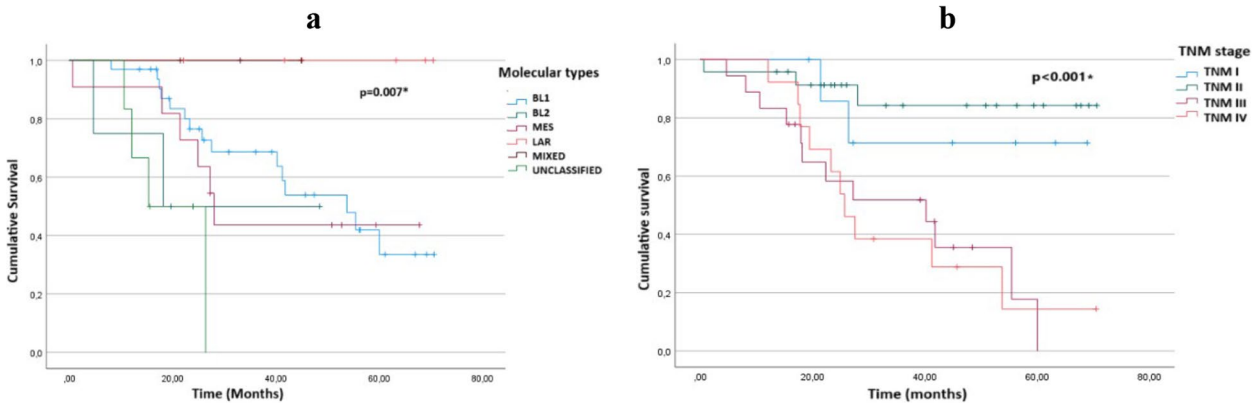

Fig. 1 Survival analysis. The OS of the different TNBC subtypes (**a**) and TNM stages (**b**) using Kaplan–Meier analysis. BL1 or 2 (Basal like 1 or 2), MES (mesenchymal), LAR (luminal androgen receptor), *$p < 0.05$

(Fig. 1b). This prompted us to perform a multivariable Cox proportional hazards regression analysis to evaluate the simultaneous influence of TNM staging and TNBC subtypes on OS. The result showed the persistence of a significant influence of each of them ($p < 0.001$ and $p = 0.017$, respectively).

Discussion

In this study, we used an IHC profiling to categorize a TNBC North African population into six subtypes (BL1, BL2, MES, LAR, Mixed and unclassified), and we compared between them in terms of clinicopathological characteristics and OS rates. We found that the BL1 is the most frequent subtype. A univariate analysis showed that there are significant differences in OS among TNBC subtypes ($p = 0.007$), with median OS ranging from 15.47 months in the Unclassified subtype to 45.07 months in the LAR subtype. The OS differed also significantly by TNM stage ($p = 0.001$). In a multivariable Cox proportional hazards regression analysis, we found that TNBC subtypes with TNM staging influenced OS, independently ($p < 0.001$ and $p = 0.017$, respectively).

Cytokeratins are the main IHC markers of BL BC [34], especially the CK14 which has proven to be the most accurate one [35]. This is why we have included CK14 in our panel. Despite using the same IHC markers as Kumar et al. [19], we found different subtype proportions. Our TNBC cohort had a higher percentage of patients with BL1 & BL2 (53.1% & 6.2%) but a lower percentage of patients with LAR (9.4%) than those reported by Kumar et al. (13.1% & 1.6% for BL1 & BL2, respectively, and 16.7% for LAR) (Table 5). At the other hand, we found that the proportion of BL subtype (the sum of BL1 & BL2 that is 59.3%) is more akin to the results of Liu et al. (53.9%). This is also valid regarding the percentage of LAR subtype (9.4% in our study vs 11.7% in the study of Liu al.) [36]. The existence of discrepancies in the distribution of TNBC subtypes between different geographic groups was clearly observed with genomic based subtyping [16, 17]. These findings suggest that the proportion variability may stem from differing genetic backgrounds observed among various racial or ethnic groups. In case of IHC based subtyping, the observations tend to be the same. In Table 5, we have grouped the main attempts to subclassify TNBC using IHC markers. We included studies that were conducted since the first description of the basal-like BC by Perou et al. [4] until 2023. In the first group of these studies, IHC markers are inferred either from Lehmann et al. or from Burstein et al. genomic classifications. Whilst the second group includes studies that are inferred from the multi-omics classification established by [17] and known as "Fudan typing". The third group of studies includes those that tried to characterize BL TNBC using surrogate IHC markers along with the genomic gold standard method. The studies in this group only made a distinction between the BL subtype and the non-BL subtype. In this third group, and in order to be able to make comparisons with the other studies, we have manually calculated the percentage of the BL subtype (defined by the expression of CKs and/or EGFR) in the TNBC group (defined by the lack of expression of hormone receptors and HER2) (Table 5). We can notice that even when the same IHC markers were used, the percentage of BL in the TNBC is varying [37–41]. Taken together with our observations and those of Kumar et al. [19], we can suggest that ethnicity may contribute to the variation of IHC-based TNBC subtypes distribution. However, it is not clear what is the trend of this variation since there are differences in the same ethnic group [38, 39], and even in the same population study but at different periods [37, 40]. It is also not clear, why did we found the same proportions of BL and LAR as in the work of

Table 5 A comparative table of IHC based TNBC subtyping studies

Study	Geographic origin	Mean age	BL1	BL2	MES	LAR	MIXE	Unclassified	Survival (months)
Studies inferred from Lehmann or Burstein subtypings									
Our study	North West Arica ($n=64$)	50.11 y	53.1% EGFR- and CK5 and/or CK14+	6.2% EGFR+regardless of CK5 or CK14 positivity	17.1% Vimentin+, E-cadherin, Claudins 3 & 7 diminished expression	9.4% AR+ Lowest mean age: 40.17±8.68 y	4.7% more than one subtype's phenotype is displayed	9.4% Other scenarios	OS ($P=0.01$) LAR: 45.07 BL1: 30.88 MES: 27.23 Mix: 27.28 BL2: 18.94 Unclassified: 15.47
Kumar 2021 [19]	Chandigarh India ($n=245$)	50 y	13.1% EGFR- and CK5 and/or CK14+	1.6% EGFR+regardless of CK5 or CK14 positivity	28.6% Vimentin+, E-cadherin, Claudins 3 & 7 diminished expression	16.7% AR+ Highest mean age: 55 y	15.1% more than one subtype's phenotype is displayed	24.9% Other scenarios	OS: The shorter OS are: MES: 68.2 and Unclassified: 69.2 (But, $P=0.97$) DFS: BL2 median of 35.4 months ($P=0.011$)
Choi 2012 [38]	South Korea ($n=122$)	47.5 y	22.1% CK5/6+and/or EGFR+		23.0% claudin 3, 4 & 7 negative and/or E-cadherin negative	9.8% AR+ Highest mean age: 56.9±13.7	18.9% characteristics of 2 different subtypes	26.2% null tumors not belong to any types described	BL & Unc subtypes: less favorable prognosis AR subtype: better prognosis than others MES & Mix subtypes: intermediate prognosis (not statistically significant)
Liu 2016 [36]	Jilin, China ($n=140$, table II)	54 years	53.9% CK5/6+or CK14		25.3% vimentin+ E-cadherin-; CD44+CD24-/low phenotype; All claudins low	11.7% AR+ Mean age NA but it is higher than BL (> 50y)			OS: AR 96.3 m Basal 75.8 m MES 84.7 m
Yoo 2022 [20]	Seoul Korea ($n=145$)	55 years	18.6% p16, EGFR, CK5/6, and p53		MES (30.3%), MUC1, SMAD4 IM (14.5%), (PDL1, TIL, CD8)	17.9% AR Highest mean age: 61.7±10.4)		12.4%	No difference in OS was noted
Kim 2018 [39]	Seoul, South Korea ($n=200$)	NA	42.5% (CK5/6+and/or EGFR+, BL was further classified as BLIA (IDO1+and FOXC1-) or BLIS type (IDO1- and FOXC1+)		11.5% claudin 3– and/or E-cadherin–,	11% AR+ LAR type was associated with older patient age (> 50 y)	30%	5%	RFS: BLIS & unclassified: shortest RFS LAR, BLIA, & BL: relatively favourable RFS

Table 5 (continued)

Study	Geographic origin	Mean age	BL1	BL2	MES	LAR	MIXE	Unclassified	Survival (months)
Inferred from "Fudan typing"									
Zhao 2020 [21]	Shanghai China ($n=212$)	53.1 y	38.1% BL immune-suppressed (BLIS): AR−, CD8−, FOXC1+		IM: AR−, CD8+), M: AR−, CD8−, FOXC1−, DCLK1+	28.6% AR+ The most associated subtype to old age (> 50 y)		Unclassifiable (AR−, CD8−, FOXC1−, DCLK1−)	IM ($p=0.002$), LAR ($p=0.004$), BLIS ($p=0.014$) subtypes were associated with better RFS than the MES subtype
Leeha 2023 [10]	Thailand ($n=195$)	52.3 y	52.8%; BL immune-suppressed (BLIS): AR−, CD8−, FOXC1+		17.4%; IM: AR−, CD8+), 0.5%; M: AR−, CD8−, FOXC1−, DCLK1+	19% AR+ Highest mean age: 57.7		(AR−, CD8−, FOXC1−, DCLK1−)0.10.3%	LAR and BLIS subtypes were significantly associated with poorer OS compared to the IM subtype in univariate analysis, however, only BLIS was significant in multivariate analysis
Studies that characterized only the Basal-like TNBC									
Nielsen 2004 [40]	NA. British Columbia Cancer Agency trials (between the late 1970s and 1990) ($n=930$)	NA	76% ER-, HER2- CK5/6+and/or EGFR+						
Cheang 2008 [37]	NA. British Columbia Cancer Agency (1986–1992) ($n=636$)	85.5% aged > 40 y	52.58% 5 markers: ER-, PR-, HER2-, CK5/6+and/or EGFR+						
Sutton 2010 [41]	Dallas (USA) ($n=105$)	49 y	65.71% ER-, PR-, HER2-, CK5/6+and/or EGFR+						

LAR (luminal androgen receptor); MES (mesenchymal), BL1 or 2 (Basal like 1 or 2), CK (cytokeratin), CLD (Claudin); VIM (Vimentin), E-CAD (E-Cadherin); AR (Androgen receptor); EGFR (epidermal growth factor receptor); OS (Overall survival), RFS (Relapse free survival), DFS (disease free survival)

Liu et al. [36], while the populations are ethnically different. Acknowledging the contribution of differences in clinical and pathological parameters, as well as methodological approaches that may act as confounders in TNBC subtypes distribution, we also speculate that the environment and the epigenetic variations may act as other possible factors.

In our study, we assessed that it will be useful to categorize the BL group into BL1 and BL2 subtypes, the latter being positive for the expression of EGFR. EGFR is a member of the HER family and has an important role in cell proliferation, migration and apoptosis. It has been shown that EGFR expression in TNBC reduces significantly the OS [40, 42]. Consistently, our results showed that BL2 subtype has a shortest OS (18.94 months, $p=0.007$), and expresses a p53 mutation staining pattern in 75% of BL2 tumors. EGFR represents a promising therapeutic target in TNBC, with potential strategies including monoclonal antibodies and tyrosine kinase inhibitors, with or without mTOR inhibitors [43–46]. Emerging immunotherapeutic techniques, such as Chimeric Antigen Receptor (CAR)-NK and CAR-T cells targeting EGFR, demonstrate promising efficacy in EGFR-positive TNBCs [47, 48].

In our research, the LAR subgroup represented 9.4% of the TNBCs. There is an unanimity in using the AR as unique IHC marker of this subgroup. But when comparing our findings with other studies (Table 5), we can notice that the percentage is varying between them. This is consistent with what is found in the literature because the proportion of AR + tumors within IHC characterized TNBCs ranged from 6.6% to 75% [49]. As well, the LAR is considered the most differential among TNBC subtypes by genomic characterization [6]. In our series, patients in the LAR subtype are the youngest (mean age 40.17 ± 8.68 y, $p=0.02$) and the this subtype tends to be the most associated with age ≤ 40 y but this was not significative ($p=0.08$). When comparing these finding with the other series (Table 5), we can find an association of the LAR subtype with elderly age. The reason of this discrepancy may be due to the fact that the majority of the patients in our LAR subtype (66.67%) are in a premenopausal status. Indeed, Wang et al. described a correlation between AR expression in TNBC and the postmenopausal status [50]. Interestingly, targeting the AR has yielded promising clinical benefits [51, 52]. A phase II trial is ongoing also to test the effect of endocrine therapy ("Therapeutic Targeting of ER Beta in Triple Negative Breast Cancer" (ClinicalTrials.gov Identifier: NCT03941730)), as well as many phase I/II clinical trials testing the effect of CDK4/6 inhibitors, alone or in combination with AR inhibitors, on patients within the LAR subtype [53].

In addition to the LAR and BL subtypes which have drawn significant scientific attention and have undergone comparative examinations, we examined other TNBC subtypes as well. The MES subgroup, referred also to as the claudin-low subtype, was described by [6, 54]. It is characterized by a particular expression pattern of proteins regulating cellular motility (like claudins and E-cadherin witch are implicated in cell–cell adhesion), invasion and epithelial-to-mesenchymal transition (like Vimentin). In our study, we found that the frequency of the MES subtype is 17.1%, which is different from the studies of Kumar and Liu (28.6% & 25.3%, respectively) [19, 36], although they used the same IHC markers as ours (Vimentin +, E-cadherin- and Claudins low). This may be due to ethnical differences since both of these studies were conducted on Asian populations. However, in other studies that used only E-cadherin and Claudins as IHC markers and that were conducted on the same population, different percentages were observed (23% and 11.5%) [38, 39] (Table 5). Hence, other alternative or complementary factors, yet unidentified, may explain proportions discrepancy.

Furthermore, we reported a Mixed subtype that exhibits more than one phenotype of the different subtypes. It represents 4.7% of the TNBC. This is very low compared to what did Kumar et al. found (15.1%) [19]. Curiously, Liu et al. did not yield any information pertaining to this subtype [36] although using the same markers as Kumar et al. and our study. We can observe that, even in other studies identical IHC markers, and in the same population, different percentages of the Mixed subtype were observed (18.9% and 30%) [38, 39] (Table 5). We found also that the Mixed subtype is characterized by some indicators of good prognostic: the inclusion of the eldest patients (67.67 y, $p=0.02$), the prevalence of the p53 wild type pattern and the negative lymph node status, although these associations did not reach significance ($p=0.07$ and $p=0.06$, respectively). In the other similar studies, the clinicopathological characteristics are either different [19] or not reported [36]. The main raison of these inconsistencies should be the definition of the Mixed subtype itself which is given as the existence of more than one phenotype in the subtype (our study and [19]), of two different subtypes [38] or the existence of two or three phenotypes [39]. Alternatively, this may be explained by the different percentages of each phenotype within the mixed subtype itself.

The "Unclassified type" refers to a distinct group of tumours that don't fall under any of the aforementioned classifications. It's worthwhile to note that the gene expression profiling research on TNBC did not find any Mixed or Unclassified subtypes. This may suggest either a heterogeneous molecular profile or the possibility of

additional molecular subgroups not captured by IHC classification.

In parallel to the subtype specific biomarkers, we identified other potential therapeutic targets in our study population. Thus, we found the expression of the BRCA1 protein, encoded by the homonym gene, which is known to be one of the pillars of the DNA repair mechanism by homologous recombination (HR). The mutations of *BRCA1 (*and *BRCA2)* genes account for approximately 25% of hereditary BC [55]. In sporadic BC, somatic *BRCA1* is often inactivated through promoter hypermethylation [56], but the germline *BRCA1* is rarely mutated [57]. In our study, we used the mouse anti-human BRCA1 monoclonal clone MS110 antibody because it is the most effective antibody, currently available, to detect BRCA1 expression [58]. We found that BRCA1 expression is lacking in 23,44% of the TNBCs and doesn't differ between subtypes. The percentage of BRCA1 loss of expression decreases down to 6% if we consider only sporadic TNBCs (no family history of BC), and rises up to 80% if we consider only TNBCs with a family history of BC. The percentage of IHC BRCA1 loss of expression in sporadic TNBCs varies between the different studies (more than 50% in Europeans, 35–40% in Japanese people [59], and 72% in Indians [60]). Since BRCA1 loss of expression in sporadic tumors is mainly due to promotor gene hypermethylation, we can speculate that environment changes between the different populations may be the cause of these discrepancies. Elsewhere, it is important to note that there is a significant association between *BRCA1* mutation and BRCA1 protein expression [61], and this expression can predict *BRCA1* mutation carriers with 80% sensitivity, 100% specificity, 100% positive predictive value and 93% negative predictive value [62]. Hence, it would be possible to use IHC BRCA1 negative expression, together with the family history of BC, in order to guide patients to screen for this gene as a priority. In addition to the importance of this strategy for genetic counselling, it may allow the use of many potential therapies in these patients, such as PARP (Poly (ADP-ribose) polymerase) inhibitors [63, 64] which are approved in patients with germline BRCA mutations, or are being tested in patients with somatic BRCA1/2 mutations [65]. It is noteworthy that increasing evidence indicates PARP inhibitors may even treat patients without BRCA or other HR repair genes mutations [66]. Moreover, PI3K inhibitors can impair BRCA1 expression in TNBCs rendering them sensitive to PARP inhibition [67]. And similar to the LAR subtype, endocrine therapy of TNBCs lacking BRCA1 may be possible because a large part of this subgroup expresses high levels of ERβ [68]. Finaly, as the expression of BRCA1 correlates negatively with those of AR and PARP, it would be possible to use both PARP inhibitors and AR inhibitors in order to treat AR-positive and BRCA1-inactivated TNBC patients [69].

We studied also the p53 expression pattern, the protein of the tumor suppressor gene *TP53.* We found that 79.68% of the TNBCs have a p53 mutated-type pattern. In the study by Kim et al. this percentage was equal to approximately 75% [39], whereas it represents 52.5% in the study by Hashmi et [70]. We found also that the mutated-type pattern was observed in 100% the Unclassified subtype. This observation is akin to the one of Kim et al. [39]. The persistent predominance of the p53 mutated-type pattern in the Unclassified subtype may explain its particular phenotype because *TP53* mutations induce a genomic instability that favours the expression of numerous proteins thus making the classification of the tumors hard. Mutations of *TP53* can be responsible of chemoresistance also [71] which may affect patient prognosis. However, we didn't find a difference between the OS of patients with a p53 mutated-type pattern over those with a wild-type pattern. It is noteworthy that the inhibition of other DNA repair check points in case of TP53 mutation, enhances the vulnerability of DNA to damages induced by chemotherapy [72]. Advanced clinical trials will further clarify this therapeutic potential in p53-deficient TNBC.

It is interesting to know that mutations of the tumor suppressor genes *BRCA1* and *TP53* contribute to the accumulations of genetic alterations that constitute the Tumor Mutational Burden (TMB). The TMB enhances the tumor immunogenicity by creating neo-antigens that stimulate the host immune response. It is a new biomarker to assess the potential response to immunotherapy [73]. Besides TMB, which is difficult to evaluate in the routine clinic, TILs seem a more practical tool to evaluate the responsiveness of the tumor to immunotherapy. Hence, we further characterized our TNBC tumors regarding the density of TILs. We found that the average of TILs density was 37.5% in our sample. This density varies between the different subtypes: 26,47% in the BL1, 50% in the BL2, 54,55% in the 33,33% in the LAR. Similar studies using IHC subtyping either did not find a significant difference in TILs density between TNBC subtypes [20, 38], like what have observed, or did find a significant difference with a predominance in BL (24,3%) and the unclassified subtype (26.4%) [39]. At the other hand, we compared the OS of two groups of TNBCs following the high or low TILs density in order to assess the implication of TILs density in patient prognosis. Our results failed to show any difference between the two groups. A similar result was observed in some studies [20, 38] but not in others [39]. Besides the possibility that these discrepancies may arise from variable genetic backgrounds,

it is also plausible that they are a result of the loose definition of the term "TILs". Indeed, TILs are mononuclear inflammatory cells identified following the International TIL Working Group 2014 [25]. Hence, the composition of TILs is heterogenous giving them an immunosuppressive or an immunostimulant profile. In addition, TILs spatial organization has been shown to influence their behavior in the tumor as well [74]. Finaly, the identification of TILs in TNBC, non-solely participates in defining the prognostic of these tumors but may be a useful tool to predict their responsiveness to immunotherapy. Although no definitive conclusion is made about this predictive role of TILs in cancer immunotherapy, there are promising research about its use as a predictor for Programmed death-ligand 1 (PD-L1) inhibitor and PD-1 inhibitor in clinical trials for metastatic TNBCs [75, 76].

Finally, we found that the OS is significantly different between the subgroups ($p=0.007$). The median OS of BL2 and the Unclassifiable subtypes exhibited the shortest OS (18.94 and 15.47 months, respectively) while the LAR and BL1 subtypes had a prolonged OS (45.07 and 30.88 months, respectively). The MES and the Mixed subtypes had an intermediate OS (27,23 and 27,28 months, respectively). By comparing these results with those from other IHC based TNBC subtyping studies (Table 5), we can find a variability between these studies in the survival rate of each subtype. As the AR subtype is the only subtype that is identified with the same marker in all the studies, we can establish a comparison of survival rates specifically for this subtype. The LAR subtype consistently shows the best survival rate across studies, although not always statistically significant. There are two plausible explanations to this AR expression beneficial effect. The first one would be the possible competitive binding of ARs on the Estrogen Response Elements, which are specific DNA sequences found in the promoter regions of oestrogen-responsive genes, thus leading to an antiproliferative effect [77]. The second one is the possibility that AR induces the up-regulation of let-7a, a ncRNA, that targets the oncogenes c-Myc and KRAS thus reducing proliferation [78]. This observation was strengthened by the positive association of AR and let-7a expression with an improved disease prognosis [79].

Despite having highlighted many aspects of the TNBC subtyping, our study has some limitations like the small size of subgroups. Although we have employed appropriate methods tailored for small sample sizes (Fisher's exact test, Kruskal–Wallis test and log-rank test), and despite having only one person who was lost to follow-up during the study period, it remains crucial to acknowledge that it is necessary to conduct further research with a larger sample size to validate these findings and achieve a more comprehensive understanding of survival outcomes for this particular group. It would be interesting also to have a long-term follow-up data, which allows assessment of the prognostic implications of TNBC subtypes identified in this study. We also recognize that different antibody clones and thresholds for positivity can influence the results and interpretations of IHC-based subtyping. However, there is no universally accepted consensus on IHC-based subgrouping of TNBC. This lack of standardization can lead to discrepancies when comparing data across different research groups. While we acknowledge these challenges, we are confident that the methodology we used provides a reliable basis for subtyping TNBC. We have ensured that the antibody clones and thresholds for positivity used in our study are clearly specified, as these details are critical for ensuring the reproducibility and reliability of our findings.

Conclusion and orientations

Our findings highlight that the TNBC group is a diverse condition consisting in four primary subtypes based on IHC (BL1, BL2, MES and LAR), with cases with overlapping immunophenotypes (Mixed subtype) and cases that couldn't be classified (Unclassified subtype). These subtypes showed significant differences in age and in the OS rates, with the AR subtype including the youngest patients and the longest OS. This research underscores the importance of the IHC subtyping in providing additional prognostic and survival estimation.

The variability in subtype percentages and survival rates between populations highlights the need for larger studies and meta-analyses to establish consensus recommendations for IHC-based TNBC subtyping. It is interesting to note that a similar transition occurred with IHC for breast cancer typing, which eventually became a standard practice.

Additionally, TNBC subtyping offers the additional benefit of guiding therapeutic approaches by identifying actionable biomarkers used in classification, such as AR and EGFR. Therapies targeting these molecules are currently inaccessible due to the lack of biomarker testing. To deal with this limitation, future clinical trials could broaden profiling efforts by incorporating additional molecular targets (DNA repair pathways and immune pathways) alongside subtyping to provide comprehensive information on potential therapies for TNBC.

Abbreviations

AR	Androgen Receptor
BL1	Basal-like1
BL2	Basal-like2
BLIA	Basal-Like immune-activated
BLIS	Basal-Like immune-suppressed
BRCA1	Breast Cancer Antigen 1
CAR	Chimeric antigen receptor
CK5/CK17/CK14	Cytokeratins 5, 17, and 14
CLD3/CLD7	Claudins 3 and 7

DAB	The chromogen (3.3' -diaminobenzidine)
EGFR	Epidermal Growth Factor Receptor
E-Cad	E-Cadherin
FISH	Fluorescent in situ hybridization
GPG	Good prognostic group
HER2	Human Epidermal Growth Factor Receptor-2
HRP	Horseradish Peroxidase
IHC	Immunohistochemistry
IM	Immunomodulatory
LAR	Luminal Androgen Receptor
MES	Mesenchymal stem-like
MPG	Moderate prognostic group
NPI	Nottingham Prognostic Index
OS	Overall Survival
PARP	Poly (ADP-ribose) polymerase
P53	Protein 53
PD-L1	Programmed death-ligand 1
PPG	Poor Prognostic Group
PR	Progesterone Receptor
SBR	Scarff-Bloom-Richardson classification
TILs	Tumor-infiltrating lymphocytes
TNBC	Triple-negative breast cancer
TNM	Tumor-Node-Metastasis
TMB	Tumor Mutational Burden
VIM	Vimentin

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Authors' contributions
RSB carried out sample analysis, retrieved the clinical data, carried out statistical analysis, and wrote the manuscript draft. RB, HL, IS, DH, LO and IB carried out sample analysis. MI and HK coordinated data collection and investigated the study. GB conceived of the study, obtained financial support for the project, carried out the results analyses, and wrote the final version of the manuscript. All authors read and approved the final manuscript.

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Data availability
All data generated or analyzed during this study are included in this published article.

Declarations

Ethics approval and consent to participate
This study, conducted in accordance with the principles of the World Medical Association Declaration of Helsinki and its later amendments, received approval from the Thematic Research Agency in Health and Life Sciences (ATRSSV, June 18, 2019). Informed consent was obtained from all participants involved in the study.

Consent for publication
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Competing interests
The authors declare no competing interests.

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References

1. Ferlay J, Colombet M, Soerjomataram I, Parkin DM, Piñeros M, Znaor A, et al. Cancer statistics for the year 2020: An overview. Int J Cancer. 2021;149:778–89.
2. Hammond MEH, Hayes DF, Dowsett M, Allred DC, Hagerty KL, Badve S, et al. American Society of Clinical Oncology/College of American Pathologists Guideline Recommendations for Immunohistochemical Testing of Estrogen and Progesterone Receptors in Breast Cancer (Unabridged Version). Arch Pathol Lab Med. 2010;134:25.
3. Wolff AC, Hammond MEH, Hicks DG, Dowsett M, McShane LM, Allison KH, et al. Recommendations for Human Epidermal Growth Factor Receptor 2 Testing in Breast Cancer: American Society of Clinical Oncology/College of American Pathologists Clinical Practice Guideline Update. JCO. 2013;31:3997–4013.
4. Perou CM, Sørlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, et al. Molecular portraits of human breast tumours. Nature. 2000;406:747–52.
5. Hines LM, Risendal B, Byers T, Mengshol S, Lowery J, Singh M. Ethnic disparities in breast tumor phenotypic subtypes in Hispanic and non-Hispanic white women. J Womens Health (Larchmt). 2011;20:1543–50.
6. Lehmann BD, Bauer JA, Chen X, Sanders ME, Chakravarthy AB, Shyr Y, et al. Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies. J Clin Invest. 2011;121:2750–67.
7. Lehmann BD, Jovanović B, Chen X, Estrada MV, Johnson KN, Shyr Y, et al. Refinement of Triple-Negative Breast Cancer Molecular Subtypes: Implications for Neoadjuvant Chemotherapy Selection. PLoS ONE. 2016;11: e0157368.
8. Burstein MD, Tsimelzon A, Poage GM, Covington KR, Contreras A, Fuqua SAW, et al. Comprehensive Genomic Analysis Identifies Novel Subtypes and Targets of Triple-Negative Breast Cancer. Clin Cancer Res. 2015;21:1688–98.
9. Pareja F, Geyer FC, Marchiò C, Burke KA, Weigelt B, Reis-Filho JS. Triple-negative breast cancer: the importance of molecular and histologic subtyping, and recognition of low-grade variants. npj Breast Cancer. 2016;2:16036.
10. Leeha M, Kanokwiroon K, Laohawiriyakamol S, Thongsuksai P. Immunohistochemistry-based molecular subtyping of triple-negative breast cancer and its prognostic significance. Pathol Oncol Res. 2023;29:1611162.
11. Giaquinto AN, Sung H, Miller KD, Kramer JL, Newman LA, Minihan A, et al. Breast Cancer Statistics, 2022. CA A Cancer J Clinicians. 2022;72:524–41.
12. Kulkarni A, Kelkar DA, Parikh N, Shashidhara LS, Koppiker CB, Kulkarni M. Meta-Analysis of Prevalence of Triple-Negative Breast Cancer and Its Clinical Features at Incidence in Indian Patients With Breast Cancer. JCO Glob Oncol. 2020;6:1052–62.
13. Lin C-H, Yap YS, Lee K-H, Im S-A, Naito Y, Yeo W, et al. Contrasting Epidemiology and Clinicopathology of Female Breast Cancer in Asians vs the US Population. JNCI: Journal of the National Cancer Institute. 2019;111:1298–306.
14. Ubbaonu CD, Chang J, Ziogas A, Mehta RS, Kansal KJ, Zell JA. Disparities in Receipt of National Comprehensive Cancer Network Guideline-Adherent Care and Outcomes among Women with Triple-Negative Breast Cancer by Race/Ethnicity, Socioeconomic Status, and Insurance Type. Cancers. 2023;15:5586.
15. Wang F, Zheng W, Bailey CE, Mayer IA, Pietenpol JA, Shu X-O. Racial/Ethnic Disparities in All-Cause Mortality among Patients Diagnosed with Triple-Negative Breast Cancer. Can Res. 2021;81:1163–70.
16. Ding YC, Steele L, Warden C, Wilczynski S, Mortimer J, Yuan Y, et al. Molecular subtypes of triple-negative breast cancer in women of different race and ethnicity. Oncotarget. 2019;10:198–208.
17. Jiang Y-Z, Ma D, Suo C, Shi J, Xue M, Hu X, et al. Genomic and Transcriptomic Landscape of Triple-Negative Breast Cancers: Subtypes and Treatment Strategies. Cancer Cell. 2019;35:428-440.e5.
18. Hercules SM, Alnajar M, Chen C, Mladjenovic SM, Shipeolu BA, Perkovic O, et al. Triple-negative breast cancer prevalence in Africa: a systematic review and meta-analysis. BMJ Open. 2022;12: e055735.

19. Kumar S, Bal A, Das A, Bhattacharyya S, Laroiya I, Khare S, et al. Molecular Subtyping of Triple Negative Breast Cancer by Surrogate Immunohistochemistry Markers. Appl Immunohistochem Mol Morphol. 2021;29:251–7.
20. Yoo T-K, Kang J, Lee A, Chae BJ. A triple-negative breast cancer surrogate subtype classification that correlates with gene expression subtypes. Breast Cancer Res Treat. 2022;191:599–610.
21. Zhao S, Ma D, Xiao Y, Li X-M, Ma J-L, Zhang H, et al. Molecular Subtyping of Triple-Negative Breast Cancers by Immunohistochemistry: Molecular Basis and Clinical Relevance. Oncologist. 2020;25:e1481–91.
22. Edge SB, AJCC, editors. AJCC cancer staging manual. 7th ed. New York: Springer; 2010.
23. Todd J, Dowle C, Williams M, Elston C, Ellis I, Hinton C, et al. Confirmation of a prognostic index in primary breast cancer. Br J Cancer. 1987;56:489–92.
24. Tan PH, Ellis I, Allison K, Brogi E, Fox SB, Lakhani S, et al. The 2019 World Health Organization classification of tumours of the breast. Histopathology. 2020;77:181–5.
25. Salgado R, Denkert C, Demaria S, Sirtaine N, Klauschen F, Pruneri G, et al. The evaluation of tumor-infiltrating lymphocytes (TILs) in breast cancer: recommendations by an International TILs Working Group 2014. Ann Oncol. 2015;26:259–71.
26. Allred DC, Harvey JM, Berardo M, Clark GM. Prognostic and predictive factors in breast cancer by immunohistochemical analysis. Mod Pathol. 1998;11:155–68.
27. Goldhirsch A, Winer EP, Coates AS, Gelber RD, Piccart-Gebhart M, Thürlimann B, et al. Personalizing the treatment of women with early breast cancer: highlights of the St Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2013. Ann Oncol. 2013;24:2206–23.
28. Rakha EA, El-Sayed ME, Green AR, Lee AHS, Robertson JF, Ellis IO. Prognostic markers in triple-negative breast cancer. Cancer. 2007;109:25–32.
29. Sunar V, T Dogan H, Sarici F, Ates O, Akin S, Baspinar B, et al. Association between androgen receptor status and prognosis in triple negative breast cancer. J BUON. 2018;23:1325–30.
30. Yamashita N, Tokunaga E, Iimori M, Inoue Y, Tanaka K, Kitao H, et al. Epithelial Paradox: Clinical Significance of Coexpression of E-cadherin and Vimentin With Regard to Invasion and Metastasis of Breast Cancer. Clin Breast Cancer. 2018;18:e1003–9.
31. Katayama A, Handa T, Komatsu K, Togo M, Horiguchi J, Nishiyama M, et al. Expression patterns of claudins in patients with triple-negative breast cancer are associated with nodal metastasis and worse outcome. Pathol Int. 2017;67:404–13.
32. Karsten N, Kolben T, Mahner S, Beyer S, Meister S, Kuhn C, et al. The role of E-Cadherin expression in primary site of breast cancer. Arch Gynecol Obstet. 2022;305:913–20.
33. Alsner J, Jensen V, Kyndi M, Vrou Offersen B, Vu P, Børresen-Dale A-L, et al. A comparison between p53 accumulation determined by immunohistochemistry and *TP53* mutations as prognostic variables in tumours from breast cancer patients. Acta Oncol. 2008;47:600–7.
34. Rakha EA, El-Sayed ME, Green AR, Paish EC, Lee AHS, Ellis IO. Breast carcinoma with basal differentiation: a proposal for pathology definition based on basal cytokeratin expression. Histopathology. 2007;50:434–8.
35. Won JR, Gao D, Chow C, Cheng J, Lau SY, Ellis MJ, et al. A survey of immunohistochemical biomarkers for basal-like breast cancer against a gene expression profile gold standard. Mod Pathol. 2013;26:1438–50.
36. Liu Y-X, Wang K-R, Xing H, Zhai X-J, Wang L-P, Wang W. Attempt towards a novel classification of triple-negative breast cancer using immunohistochemical markers. Oncol Lett. 2016;12:1240–56.
37. Cheang MCU, Voduc D, Bajdik C, Leung S, McKinney S, Chia SK, et al. Basal-Like Breast Cancer Defined by Five Biomarkers Has Superior Prognostic Value than Triple-Negative Phenotype. Clin Cancer Res. 2008;14:1368–76.
38. Choi J, Jung W-H, Koo JS. Clinicopathologic features of molecular subtypes of triple negative breast cancer based on immunohistochemical markers. Histology and Histopathology. 2012;:1481–93.
39. Kim S, Moon B-I, Lim W, Park S, Cho MS, Sung SH. Feasibility of Classification of Triple Negative Breast Cancer by Immunohistochemical Surrogate Markers. Clin Breast Cancer. 2018;18:e1123–32.
40. Nielsen TO, Hsu FD, Jensen K, Cheang M, Karaca G, Hu Z, et al. Immunohistochemical and Clinical Characterization of the Basal-Like Subtype of Invasive Breast Carcinoma. Clin Cancer Res. 2004;10:5367–74.
41. Sutton LM, Han JS, Molberg KH, Sarode VR, Cao D, Rakheja D, et al. Intratumoral Expression Level of Epidermal Growth Factor Receptor and Cytokeratin 5/6 Is Significantly Associated With Nodal and Distant Metastases in Patients With Basal-like Triple-Negative Breast Carcinoma. Am J Clin Pathol. 2010;134:782–7.
42. Viale G, Rotmensz N, Maisonneuve P, Bottiglieri L, Montagna E, Luini A, et al. Invasive ductal carcinoma of the breast with the "triple-negative" phenotype: prognostic implications of EGFR immunoreactivity. Breast Cancer Res Treat. 2009;116:317–28.
43. El Guerrab A, Bamdad M, Bignon Y-J, Penault-Llorca F, Aubel C. Co-targeting EGFR and mTOR with gefitinib and everolimus in triple-negative breast cancer cells. Sci Rep. 2020;10:6367.
44. Liu T, Yacoub R, Taliaferro-Smith LD, Sun S-Y, Graham TR, Dolan R, et al. Combinatorial Effects of Lapatinib and Rapamycin in Triple-Negative Breast Cancer Cells. Mol Cancer Ther. 2011;10:1460–9.
45. Ma J, Dong C, Cao Y-Z, Ma B-L. Dual Target of EGFR and mTOR Suppresses Triple-Negative Breast Cancer Cell Growth by Regulating the Phosphorylation of mTOR Downstream Proteins. BCTT. 2023;15:11–24.
46. You KS, Yi YW, Cho J, Park J-S, Seong Y-S. Potentiating Therapeutic Effects of Epidermal Growth Factor Receptor Inhibition in Triple-Negative Breast Cancer. Pharmaceuticals. 2021;14:589.
47. Xia L, Zheng Z, Liu J, Chen Y, Ding J, Xia N, et al. EGFR-targeted CAR-T cells are potent and specific in suppressing triple-negative breast cancer both *in vitro* and *in vivo*. Clin & Trans Imm. 2020;9: e1135.
48. Hu Z. Tissue factor as a new target for CAR-NK cell immunotherapy of triple-negative breast cancer. Sci Rep. 2020;10:2815.
49. Safarpour D, Pakneshan S, Tavassoli FA. Androgen receptor (AR) expression in 400 breast carcinomas: is routine AR assessment justified? Am J Cancer Res. 2014;4:353–68.
50. Wang C, Pan B, Zhu H, Zhou Y, Mao F, Lin Y, et al. Prognostic value of androgen receptor in triple negative breast cancer: A meta-analysis. Oncotarget. 2016;7:46482–91.
51. Gucalp A, Tolaney S, Isakoff SJ, Ingle JN, Liu MC, Carey LA, et al. Phase II Trial of Bicalutamide in Patients with Androgen Receptor-Positive, Estrogen Receptor-Negative Metastatic Breast Cancer. Clin Cancer Res. 2013;19:5505–12.
52. Traina TA, Miller K, Yardley DA, Eakle J, Schwartzberg LS, O'Shaughnessy J, et al. Enzalutamide for the Treatment of Androgen Receptor-Expressing Triple-Negative Breast Cancer. JCO. 2018;36:884–90.
53. Zhu S, Wu Y, Song B, Yi M, Yan Y, Mei Q, et al. Recent advances in targeted strategies for triple-negative breast cancer. J Hematol Oncol. 2023;16:100.
54. Prat A, Parker JS, Karginova O, Fan C, Livasy C, Herschkowitz JI, et al. Phenotypic and molecular characterization of the claudin-low intrinsic subtype of breast cancer. Breast Cancer Res. 2010;12:R68.
55. Melchor L, Benítez J. The complex genetic landscape of familial breast cancer. Hum Genet. 2013;132:845–63.
56. Ruscito I, Gasparri ML, De Marco MP, Costanzi F, Besharat AR, Papadia A, et al. The Clinical and Pathological Profile of BRCA1 Gene Methylated Breast Cancer Women: A Meta-Analysis. Cancers. 2021;13:1391.
57. Khoo U-S, Ozcelik H, Cheung AN, Chow LW, Ngan HY, Done SJ, et al. Somatic mutations in the BRCA1 gene in Chinese sporadic breast and ovarian cancer. Oncogene. 1999;18:4643–6.
58. Teixeira LA, Candido Dos Reis FJ. Immunohistochemistry for the detection of BRCA1 and BRCA2 proteins in patients with ovarian cancer: a systematic review. J Clin Pathol. 2020;73:191–6.
59. Nomura H, Kataoka F, Aoki D, Jinno H, Kitagawa Y, Sato Y, et al. Expression of potential biomarkers associated with homologous recombination repair in patients with ovarian or triple-negative breast cancer. CBM. 2016;16:145–52.
60. Khan F, Agarwal P, Gupta S, Maurya M, Singh P, Agarwal A, et al. BRCA1 promoter methylation & its immunohistochemical correlation in sporadic breast cancer. Indian J Med Res. 2023;158:47.
61. Bu R, Siraj AK, Al-Obaisi KAS, Beg S, Al Hazmi M, Ajarim D, et al. Identification of novel *BRCA* founder mutations in Middle Eastern breast cancer patients using capture and Sanger sequencing analysis. Intl Journal of Cancer. 2016;139:1091–7.
62. Vaz FH, Machado PM, Brandão RD, Laranjeira CT, Eugénio JS, Fernandes AH, et al. Familial Breast/Ovarian Cancer and *BRCA1/2* Genetic Screening: The Role of Immunohistochemistry as an Additional Method in the Selection of Patients. J Histochem Cytochem. 2007;55:1105–13.

63. Litton JK, Rugo HS, Ettl J, Hurvitz SA, Gonçalves A, Lee K-H, et al. Talazoparib in Patients with Advanced Breast Cancer and a Germline *BRCA* Mutation. N Engl J Med. 2018;379:753–63.
64. Robson M, Im S-A, Senkus E, Xu B, Domchek SM, Masuda N, et al. Olaparib for Metastatic Breast Cancer in Patients with a Germline *BRCA* Mutation. N Engl J Med. 2017;377:523–33.
65. Vidula N, Damodaran S, Bhave MA, Rugo HS, Blouch E, Ruffle-Deignan NR, et al. Phase II study of a PARP inhibitor in somatic *BRCA1/2* mutant metastatic breast cancer (MBC). JCO. 2023;41 16_suppl:TPS1134–TPS1134.
66. Cortesi L, Rugo HS, Jackisch C. An Overview of PARP Inhibitors for the Treatment of Breast Cancer. Targ Oncol. 2021;16:255–82.
67. Ibrahim YH, García-García C, Serra V, He L, Torres-Lockhart K, Prat A, et al. PI3K Inhibition Impairs BRCA1/2 Expression and Sensitizes BRCA-Proficient Triple-Negative Breast Cancer to PARP Inhibition. Cancer Discov. 2012;2:1036–47.
68. Treeck O, Schüler-Toprak S, Ortmann O. Estrogen Actions in Triple-Negative Breast Cancer. Cells. 2020;9:2358.
69. Sang M, Meng L, Ma C, Liu S, Sang M, Chang S, et al. Effect of AR antagonist combined with PARP1 inhibitor on sporadic triple-negative breast cancer bearing AR expression and methylation-mediated BRCA1 dysfunction. Biomed Pharmacother. 2019;111:169–77.
70. Hashmi AA, Sajid A, Hussain M, Zia S, Islam S, Diwan MA, et al. Mutant Phenotype p53 Immunohistochemical Expression Is Associated With Poor Prognostic Parameters and Disease-Free Survival in Triple-Negative Metaplastic Breast Carcinoma. Cureus. 2021. https://doi.org/10.7759/cureus.15244.
71. Muller PAJ, Vousden KH. p53 mutations in cancer. Nat Cell Biol. 2013;15:2–8.
72. Patterson-Fortin J, Bose A, Tsai W-C, Grochala C, Nguyen H, Zhou J, et al. Targeting DNA Repair with Combined Inhibition of NHEJ and MMEJ Induces Synthetic Lethality in *TP53* -Mutant Cancers. Can Res. 2022;82:3815–29.
73. Efremova M, Finotello F, Rieder D, Trajanoski Z. Neoantigens Generated by Individual Mutations and Their Role in Cancer Immunity and Immunotherapy. Front Immunol. 2017;8:1679.
74. Brummel K, Eerkens AL, De Bruyn M, Nijman HW. Tumour-infiltrating lymphocytes: from prognosis to treatment selection. Br J Cancer. 2023;128:451–8.
75. Gonzalez-Ericsson PI, Stovgaard ES, Sua LF, Reisenbichler E, Kos Z, Carter JM, et al. The path to a better biomarker: application of a risk management framework for the implementation of PD-L1 and TILs as immuno-oncology biomarkers in breast cancer clinical trials and daily practice. J Pathol. 2020;250:667–84.
76. Loi S, Adams S, Schmid P, Cortés J, Cescon DW, Winer EP, et al. Relationship between tumor infiltrating lymphocyte (TIL) levels and response to pembrolizumab (pembro) in metastatic triple-negative breast cancer (mTNBC): Results from KEYNOTE-086. Ann Oncol. 2017;28: v608.
77. Peters AA, Buchanan G, Ricciardelli C, Bianco-Miotto T, Centenera MM, Harris JM, et al. Androgen Receptor Inhibits Estrogen Receptor-α Activity and Is Prognostic in Breast Cancer. Can Res. 2009;69:6131–40.
78. Lyu S, Yu Q, Ying G, Wang S, Wang Y, Zhang J, et al. Androgen receptor decreases CMYC and KRAS expression by upregulating let-7a expression in ER−, PR−, AR+ breast cancer. Int J Oncol. 2014;44:229–37.
79. Zhang W, Liu X, Liu S, Qin Y, Tian X, Niu F, et al. Androgen receptor/let-7a signaling regulates breast tumor-initiating cells. Oncotarget. 2018;9:3690–703.

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