Università degli Studi di Ferrara

DOTTORATO DI RICERCA IN
"SCIENZE BIOMEDICHE E BIOTECNOLOGICHE"

CICLO XXXII

COORDINATORE Prof. Pinton Paolo

Role of Androgen Receptor biomarker in breast cancer: a translational study from *in situ* to invasive carcinoma

Settore Scientifico Disciplinare MED/07

Dottoranda
Dott. Ravaioli Sara

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INTRODUCTION

1. BREAST CANCER

1.1 Epidemiology

In 2018, the predicted number of new breast cancers in 28 European Union (EU) countries was 404920, with estimated age-adjusted annual incidence of breast cancer (BC) of 144.9/100000 and mortality of 32.9/100 000, with 98755 predicted deaths.[1] Worldwide, there was about 2.1 million newly diagnosed female breast cancer cases in 2018, accounting for almost one in four cancer cases among women, and about 630000 people died of it.[2] Breast cancer incidence has increased since the introduction of mammography screening and continues to grow with the ageing of the population. The most important risk factors include: genetic predisposition, exposure to estrogens (endogenous and exogenous, including long-term hormone replacement therapy), ionizing radiation, low parity, high breast density and a history of atypical hyperplasia. The Western-style diet, obesity and the consumption of alcohol also contribute to the rising incidence of breast cancer.[3] There is a steep age gradient, with about a quarter of BCs occurring before age 50, and <5% before age 35. The estimated 5-year prevalence of breast cancer (people with a diagnosis within the last 5 years and still alive, with or without disease) in Europe in 2012 was about 1.8 millions cases[1] and a staggering about 7 millions cases worldwide.[2] Prevalence is increasing, due to increased incidence and improvements in treatment outcomes. In most Western countries, the mortality rate has decreased in recent years, especially in younger age groups, because of improved treatment and earlier detection.[4,5] However, BC is still the leading cause of cancer-related deaths for women in Europe and worldwide, although the mortality of lung cancer in women is overcoming BC mortality in some countries.

Breast cancer in males is rare, (about 1% of cases). The major risk factors include clinical disorders carrying hormonal imbalances (especially gynecomastia and cirrhosis), radiation exposure, a positive family history and genetic predisposition.[6]

1.2 Diagnosis and pathology

The diagnosis of BC is based on clinical examination in combination with imaging and confirmed by pathological assessment. Clinical examination includes bimanual palpation of the breasts and regional lymph nodes and assessment for distant metastases (bones, liver and lungs; a neurological examination is only required when symptoms are present). Imaging includes bilateral mammography and ultrasonography (US) of the breast and regional lymph nodes.[7] A

magnetic resonance imaging (MRI) of the breast is not routinely recommended, but should be considered in specific cases, *i.e.* familial BC associated with BRCA mutations, lobular cancers, dense breasts, before neoadjuvant systemic therapy, and to evaluate the response to this therapy. Several new techniques are being tested for screening and diagnostic imaging, such as three-dimensional (3D) mammography (digital breast tomosynthesis), 3D US, shear wave elastography and contrast-enhanced mammography/spectral mammography. None of these are yet routinely implemented but they have the potential to increase diagnostic accuracy, especially in women with dense breasts.

Apart from imaging, pretreatment disease evaluation includes pathological examination of the primary tumor and cytology/ histology of the axillary nodes, if involvement is suspected. Pathological diagnosis should be based on a core needle biopsy, preferably obtained by US or stereotactic guidance. A core needle biopsy (if this is not possible, at least a fine-needle aspiration indicating carcinoma) must be obtained before any type of treatment is initiated. If preoperative systemic therapy is planned, a core needle biopsy is mandatory to ensure a diagnosis of invasive disease and assess biomarkers. In case of multifocal and multicentric tumors, all lesions should be biopsied. A marker (e.g. surgical clip, carbon) should be placed into the tumor at biopsy, to ensure resection of the correct site and to enable pathological assessment of the surgical specimen.

Final pathological diagnosis should be made according to the World Health Organization (WHO) classification[8] and the eighth edition of the American Joint Committee on Cancer (AJCC) tumor, node, metastasis (TNM) staging system.[9] This staging system, apart from purely anatomical information, includes also prognostic information related to tumor biology, such as tumor grade, estrogen receptor (ER), progesterone receptor (PgR), human epidermal growth factor receptor 2 (HER2) and gene expression data, if available. The two most frequent subtypes are invasive carcinoma of the breast, not otherwise specified (NOS, previously named ductal carcinoma) (70%–75%) and lobular carcinoma (12%–15%). The other 18 subtypes exhibit specific morphological traits and are rare (from 0.5% to 5%). Each of these specific subtypes shows a particular prognosis. Of note, a neuroendocrine differentiation can be observed in some cases, without any prognostic or therapeutic consequences for the patient.[8] The pathological report should include presence/absence of ductal carcinoma *in situ* (DCIS), the histological type, grade, immunohistochemistry (IHC) evaluation of ER status (using a standardized assessment methodology) and, for invasive cancer, IHC evaluation of PgR and HER2 expression or HER2 gene amplification. HER2 gene amplification status may be determined directly from all invasive tumors using *in situ* hybridization (ISH) (fluorescent or chromogenic),

replacing IHC or only for tumors with an ambiguous IHC score (2+ of staining intensity).[10] HER2 testing should be carried out according to the American Society of Clinical Oncology–College of American Pathologists (ASCO-CAP) guidelines. HER2 is defined as positive by IHC (3+) when more than 10% of the cells harbor a complete membrane staining, and by ISH if the number of HER2 gene copies is ≥6, or the HER2/chromosome 17 (CEP17) ratio is ≥2 and HER2 copies ≥4, or HER2/CEP17 <2 and HER2 copies ≥6.[11]

Proliferation markers such as the Ki67 labelling index may supply additional useful information, particularly if the assay can be standardized.[12,13] Alternatively, these biological markers can be assessed in the definitive surgical specimen if primary systemic therapy is not planned. However, fixation is better controlled for core biopsies, allowing safer antigen preservation for IHC.[14] In case of negativity of ER/PgR and HER2 in the biopsy specimen, it is advisable to retest for them in the surgical specimen to account for the putative tumor heterogeneity.[15] In case of discrepancy, the results from the surgical specimen are considered definite. In case of a HER2-positive test on biopsy, retesting for HER2 on the surgical specimen is mandatory for invasive carcinoma NOS grade I, ER- and PgR-positive (including special types such as tubular, mucinous, cribriform) or adenoid cystic carcinoma or secretory carcinoma (both usually triple negative).[11] For the purpose of prognostication and treatment decision making, tumors should be grouped into surrogate intrinsic subtypes (Table 1), defined by routine histology and IHC data.[16,17]

Table 1. Surrogate definitions of intrinsic subtypes of breast cancer (adapted from the 2013 St. Gallen Consensus Conference).

Intrinsic subtype	Clinicopathological surrogate definition
Luminal A	'Luminal A-like' ER-positive HER2-negative Ki67 low[a] PgR high[b] Low-risk molecular signature (if available)
Luminal B	'Luminal B-like (HER2-negative)' ER-positive HER2-negative and either Ki67 high or PgR low High-risk molecular signature (if available) 'Luminal B-like (HER2-positive)' ER-positive HER2-positive Any Ki67 Any PgR
HER2	'HER2-positive (non-luminal)' HER2-positive ER and PgR absent
'Basal-like'	'Triple-negative'[c] ER and PgR absent[c] HER2-negative[c]

[a]Ki67 scores should be interpreted in light of local laboratory median values; [s]uggested cut off value is 20%; quality assurance programs are essential for laboratories reporting these results. [t]here is about 80% overlap between 'triple negative' and intrinsic 'basal' subtype, but 'triple negative' also includes some special histological types such as carcinoma with a rich lymphocytic stroma (former medullary), secretory carcinoma, low-grade metaplastic carcinoma and adenoid cystic carcinoma. ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; PgR, progesterone receptor.

Luminal A-like tumors are typically low grade, strongly ER-positive/PgR-positive, HER2-negative and have low proliferative fraction. Luminal B-like tumors are ER-positive but may have variable degrees of ER/PgR expression, are higher grade and have higher proliferative fraction.[16]

Tumor-infiltrating lymphocyte (TIL) scoring is demonstrated to have a prognostic value in triple-negative breast cancer (TNBC) and HER2-positive BC. It has been described as a predictor of pathological complete response (pCR) to chemotherapy in many prospective neoadjuvant clinical trials and its increase appears linked to an improved prognosis after adjuvant therapy. TIL scoring can be used as a prognostic marker, as shown in a variety of clinical trials (e.g. BIG-2-98, FinHER, Cleopatra), providing a typically 15-20% relative improvement in survival per 10% increase in TILs[18,19] and its use as a prognostic factor is endorsed by the 2019 St Gallen Consensus. However, TIL scoring should not be used to take treatment decisions nor to escalate or de-escalate treatment.

Genetic counselling and testing for germline BRCA1 and BRCA2 mutations should be offered to breast cancer patients in high-risk groups, *i.e.* those with:

• strong family history of breast, ovarian, pancreatic and/or high grade/metastatic prostate cancer;

• diagnosis of BC before the age of 50;

• diagnosis of TNBC before the age of60;

• personal history of ovarian cancer or second BC or male sex.[20]

1.3 Staging and risk assessment

Disease stage should be assessed according to the eighth edition of the AJCC TNM staging system.[9] In early breast cancer, routine staging evaluations are directed at locoregional disease. Asymptomatic distant metastases are rare, and most patients do not benefit from comprehensive laboratory tests (including tumor markers[21]) and radiological staging. Minimum blood work-up (a full blood count, liver and renal function tests, alkaline phosphatase and calcium levels) is recommended before surgery and systemic (neo)adjuvant therapy. A computed tomography (CT) scan of the chest, abdominal imaging (US, CT or MRI scan) and a bone scan can be considered for patients with: clinically positive axillary nodes; large tumors (*e.g.* 5 cm); aggressive biology; clinical signs, symptoms or laboratory values suggesting the presence of metastases.

Dual imaging methods combining functional and anatomical information such as fluorodeoxyglucose (FDG) positron emission tomography (PET)-CT may be useful when conventional methods are inconclusive. PET-CT scanning can also replace traditional imaging for staging in high-risk patients,[22] although in cases of lobular cancers and low-grade tumors, PET-CT may be less sensitive. Current evidence does not support the use of FDG-PET-CT in the staging of locoregional disease, due to its limited sensitivity when compared with the gold standard, sentinel lymph node biopsy and axillary lymph node dissection.[23] In patients planned for (neo)adjuvant systemic treatment with anthracyclines and/or trastuzumab, evaluation of cardiac function with a cardiac US or a multigated acquisition scan is essential. The post-operative pathological assessment of the surgical specimens should be made according to the pathological TNM system.[9] This assessment should include:

• the number, location and maximum diameter of the tumors removed;

• the total number of removed and positive lymph nodes, as well as the extent of metastases in the lymph nodes [isolated tumor cells, micrometastases (0.2–2 mm), macrometastases];

• the histological type and grade of the tumor(s) using a standard grading system;

• evaluation of the resection margins, including the location and minimum distance of the margin;

• vascular invasion;

• biomarker analysis.

For small tumors diagnosed by core biopsy, measuring only the residual tumor in the excision may result in understaging. It is recommended to correlate imaging, clinical and gross findings to microscopic observation if necessary.[9] The most important prognostic factors in early breast cancer are the expression of ER/PgR, HER2 and proliferation markers (e.g. Ki67), the number of involved regional lymph nodes, tumor histology, the size, grade and the presence of peritumoral vascular invasion. Additionally, in patients undergoing breast-conserving therapy, the ipsilateral breast recurrence risk is related to the status of the surgical margins and the presence of DCIS. Immunohistochemically detected tumor markers known to have great practical treatment importance are now incorporated into the eighth edition of the AJCC TNM staging system to refine prognosis, which also uses genomic assays, when available, to downstage some ER-positive, lymph node-negative tumours.[9] Clinical parameters (age, tumor stage, ER, PgR and HER2 expression and histological grade) have also been integrated into scoring systems, allowing a relatively accurate estimation of the probability of recurrence and death from BC.

Gene expression profiles, such as MammaPrint (Agendia, Amsterdam, The Netherlands), Oncotype DX Recurrence Score (Genomic Health, Redwood City, CA), Prosigna (PAM50; NanoString Technologies, Seattle, WA), Endopredict (Myriad Genetics Salt Lake City, UT) and Breast Cancer Index (Biotheranostics, Inc., San Diego, CA), may be used to gain additional prognostic and/or predictive information to complement pathology assessment and to predict the benefit of adjuvant chemotherapy.[12] All tests except MammaPrint were designed for patients with ER-positive early breast cancer only. The clinical utility of MammaPrint and Oncotype DX has been or is still being prospectively evaluated in large randomized clinical trials such as MINDACT for MammaPrint, TAILORx for Oncotype DX.[24,25] The prognostic value of MammaPrint has been validated in the RASTER trial, a prospective but non-randomized, clinical trial.[26] Furthermore, both MammaPrint and Oncotype DX are able to identify patients with an ultra-low risk of death from breast cancer at 10 or 20 years.[27,28]

ER/PgR and HER2 are the only validated predictive factors allowing the selection of patients for endocrine therapy (ET) and anti-HER2 therapies, respectively. High ER expression is usually associated with lesser absolute benefit of chemotherapy.[29] It must be stressed that IHC/ISH determination of intrinsic phenotype does not have a 100% concordance with the molecular determination. The prerequisite for using such a surrogate assessment is the use of standardized assays and a meticulous quality control.

After neoadjuvant systemic treatment, the response to treatment and the amount of residual disease are important prognostic factors but need as much standardization as any of the other biological markers. A multidisciplinary international working group developed practical recommendations for the systematic, standardized evaluation of the post-neoadjuvant surgical BC specimen.[30] If a pCR was achieved (defined as no invasive disease both in the breast and axilla), this must be clearly stated.[31] In addition, the presence or absence of residual DCIS must be described. In case of residual invasive carcinoma, a comment must be made as to the presence or absence of chemotherapy effect in the breast and the lymph nodes. The Residual Cancer Burden (RCB) is the preferred method for quantifying residual disease in clinical trials. Post-treatment tumor staging, using the TNM system, should also be included.[9]

1.4 Treatment

1.4.1 Local treatment

The major change in the surgical treatment of primary breast cancer has been a shift towards breast conservation techniques, which started more than 30 years ago. Currently, in Western Europe, 60%–80% of newly diagnosed cancers are amenable to breast conservation (wide local excision and radiotherapy), at diagnosis or after primary systemic therapy. A neoadjuvant approach should be preferred in subtypes highly sensitive to chemotherapy, such as triple-negative and HER2-positive, in tumors >2 cm, and/or a positive axilla. In some patients, mastectomy is still carried out due to:

- tumor size (relative to breast size);
- tumor multicentricity;
- inability to achieve negative surgical margins after multiple resections;
- prior radiation to the chest wall/breast or other contraindications to RT;
- unsuitability for oncoplastic breast conservation; and
- patient choice.

However, the breast-conserving surgery (BCT) is the primary surgical choice for BC. For patients undergoing wide local excision, greater emphasis is now placed on achieving acceptable cosmesis. Despite the overall trend towards breast conservation, increasing numbers of breast cancer patients are opting for bilateral mastectomy (incorporating contralateral risk-reducing surgery) rather than the preferred breast conservation and mammographic surveillance of the irradiated breast.[32] This must be confronted with data demonstrating that patients with early-stage breast cancer who opt for BCT might have an even better survival compared with those who have a mastectomy.[33] Margin status should be reported according to the recommendations of the College of American Pathologists (CAP); for example, a margin is positive and should be reported as such, when there is ink touching invasive cancer or DCIS; the anatomic location of the positive margin should be specified in oriented specimens. For negative margins (i.e. ink not touching invasive cancer or DCIS), the distance of invasive cancer and/or DCIS from the margin(s) should be reported. No tumor at the inked margin is required and >2 mm for *in situ* disease is preferred.[34]

Currently achievable low local recurrence rates [<0.5% per year (with a target of <0.25%) and ≤10% overall at very long-term follow-up] should be maintained. Regional lymph node status remains one of the strongest predictors of long-term prognosis in primary BC. Sentinel lymph node biopsy (SLNB) delivers less morbidity in terms of shoulder stiffness and arm swelling and allows for a reduced hospital stay. With appropriate training in the dual radiocolloid/blue dye technique or others (indocyanine green fluorescence technique or superparamagnetic iron oxide), high identification rates (over 97%), low false-negative rates and favorable axillary recurrence rates following SLNB are achievable. There is no definite consensus for the pathological assessment of SLNB. Micrometastatic spread and isolated tumor cells are prognostically equivalent to N0 disease, with local as well as systemic treatment options selected based on other tumor- and patient-based parameters. For cases with macrometastatic spread in the SLN, the randomized controlled trial ACOSOG- Z0011 (10 years of median follow-up) reported non-inferior rates of Overall Survival (OS), Disease-Free Survival (DFS), for patients with clinical T1–T2 cN0 invasive breast cancer and 1–2 SLNs containing metastases (treated with BCS, tangential adjuvant RT including part of the axilla and adjuvant systemic therapy). Therefore, all patients with micrometastatic spread and patients with limited involvement of the SLN, who are undergoing tangential breast RT and adjuvant systemic treatment and meet the criteria of the randomized trials, do not need any further axillary surgery. For patients who do not meet those criteria, an axillary lymph node dissection needs to be considered. Another option in patients with cN0 and SLN metastases (irrespective of the risk factors) is axillary RT, as demonstrated by the AMAROS study.[35]

Regarding at the surgery for *in situ* malignancy (intraepithelial neoplasia), DCIS may be treated with total mastectomy or BCT, provided that clear resection margins can be achieved. There is no general agreement on what is considered an optimal margin; however, recent consensus has determined that a 2 mm margin is adequate in DCIS treated with whole-breast radiotherapy, because it is associated with lower rates of ipsilateral local recurrences and improved cosmetic outcomes.[34] The risk of a positive SLN with pure DCIS is small (7%–9%) and most of the metastases found are micrometastases or isolated tumor cells, detected by IHC. The decision to carry out an SLNB procedure should be based on the underlying risk of invasion. The invasive breast cancer underestimation rate is reported to be 20%–38%, and increases with the presence of: an associated density on the mammogram; poorly differentiated DCIS in the biopsy; younger age; and larger extent of microcalcifications. Lobular neoplasia [formerly called lobular carcinoma *in situ* (LCIS)], unlike DCIS, is considered a non-obligate precursor to invasive cancer. It is regarded as a risk factor for future development of invasive cancer in both

breasts [relative risk: 5.4–12] and does not require active treatment. The pleomorphic variant of lobular neoplasia may behave similarly to DCIS and should be treated accordingly, after multidisciplinary discussion.

1.4.2 Radiotherapy

Post-operative RT is strongly recommended after surgery. Whole breast radiation treatment alone reduces the 10-year risk of any first recurrence (including locoregional and distant) by 15% and the 15-year risk of breast cancer-related mortality by 4%.[36] Boost RT gives a further 50% relative risk reduction and is indicated for most patients who have unfavorable risk factors for local control such as age <50 years, grade 3 tumors, presence of vascular invasion or extensive intraductal component and non-radical tumor excision.

Whole breast radiation treatment after BCS for DCIS decreases the risk of local recurrence, with survival equal to that after mastectomy. The decrease in the risk of local recurrence by RT is evident in all subtypes of DCIS. It is recommended in the majority of women with DCIS, on the basis of the substantial reduction in disease recurrence leading to a higher rate of long-term breast conservation and the inability to define subsets of women who do not benefit from RT.[37] However, in some patients with low-risk DCIS (tumor size <10mm, low/intermediate nuclear grade, adequate surgical margins), the risk of local recurrence following excision only is low and omitting radiation can be an option. RT is not warranted for lobular intraepithelial neoplasia, with the exception of the pleomorphic subtype that should be considered from a treatment-perspective point of view as high-grade DCIS.

1.4.3 Adjuvant systemic treatment

The decision on adjuvant systemic treatment should be based on the predicted sensitivity to particular treatment types, the benefit from their use and an individual's risk of relapse. The final decision should also incorporate the predicted treatment short- and long-term toxicities, the patient's biological age, general health status, comorbidities and preferences. Adjuvant systemic therapy should be started without undue delays, as data show an important decrease in efficacy when it is administered >12 weeks after surgery.[38]

Endocrine Therapy (ET) should be used in all luminal-like cancers. Indications for chemotherapy (ChT) within this subtype depend on the individual's risk of relapse, considering the tumor burden and features suggestive of biological aggressiveness (grade, proliferation,

vascular invasion), presumed responsiveness to ET and patient preferences (Table 2). Features associated with lower endocrine responsiveness include low steroid receptor expression, lack of PgR expression, high tumor grade and high expression of proliferation markers. The majority of luminal A-like cancers do not require chemotherapy, except those with high disease burden. Data from neoadjuvant studies have demonstrated that chemotherapy sensitivity depends on the intrinsic phenotype, the highest being for HER2-positive (when combined with anti-HER2 therapy) and TNBC. However, even assuming the relative benefit would be similar, the absolute benefit derived from adjuvant chemotherapy varies substantially, depending on the individual risk of relapse, which is determined by both the biology and the burden of the disease. For example, the absolute benefit of adjuvant chemotherapy for a low-burden, luminal A-like breast cancer is extremely small. When balanced against the known short- and long-term side-effects, chemotherapy is not recommended in this setting.

Table 2. Systemic treatment recommendations for early breast cancer subtypes

Subtype	Recommended therapy	Comments
Luminal A-like	ET alone in the majority of cases	Consider ChT if high tumour burden (≥ 4 LNs, T3 or higher)
Luminal B-like (HER2-negative)	ChT followed by ET for the majority of cases	
Luminal B-like (HER2-positive)	ChT + anti-HER2 followed by ET for all patients	If contraindications for the use of ChT, one may consider ET + anti-HER2 therapy, although no randomised data exist
HER2-positive (non-luminal)	ChT + anti-HER2	
Triple-negative (ductal)	ChT	

For special histological types, the authors recommend following the St Gallen recommendations[16] that propose ET for endocrine-responsive histologies (cribriform, tubular and mucinous), ChT for high-risk endocrine-nonresponsive histologies (medullary, metaplastic) and no systemic therapy for low-risk endocrine nonresponsive histologies (adenoid cystic and apocrine). ChT, chemotherapy; ET, endocrine therapy; HER2, human epidermal growth factor receptor 2; LN, lymph node.

Several decision-making tools, such as PREDICT Plus, exist to help predict recurrence risk and potential benefit from systemic treatments.[39] In cases of uncertainty regarding indications for adjuvant chemotherapy, gene expression assays, such as MammaPrint, Oncotype DX, Prosigna, Endopredict or Breast Cancer Index, may be used. These assays can help determine the individual's recurrence risk and potentially predict the benefit of chemotherapy in general.[12,40] Genomic tests are not recommended in case of: low-risk tumors (pT1a, pT1b, G1, ER high, pN0), and/or comorbidities that not allow adjuvant ChT; and/or special types of luminal-like BC, such as low-grade encapsulated papillary carcinoma and solid papillary carcinoma (which should be considered as DCIS), and invasive tubular carcinoma may be treated with

locoregional treatment only, as the prognosis is excellent, 1–3 involved nodes coexisting with many other high-risk factors, or with ≥4 positive nodes for whom adjuvant ChT is indicated.[40] For premenopausal women, tamoxifen for 5–10 years is a standard of care. In patients becoming postmenopausal during the first 5 years of tamoxifen, a switch to letrozole should be considered, depending on predicted risk of late recurrence. In patients requiring ChT and who recover menses (in particular in the first year but acceptable within the first 2 years), addition of Ovarian Function Suppression (OFS) to ET should be strongly considered. The role of replacing tamoxifen with an aromatase inhibitor (AI) can be considered in high-risk patients; if used, it mandates effective OFS, with regular biochemical control of estrogen levels. The role of OFS in patients <35 years not requiring ChT is not clear, but inferior outcomes of young luminal early breast cancer patients suggest the use of the most effective ET (*i.e.* combination with OFS). OFS during ChT provides some protection of ovarian function and has no negative impact on oncological outcomes; thus, it should be proposed to patients. It should not, however, be the sole fertility preservation method used, in case of desired pregnancy. For postmenopausal women, AIs (both non-steroidal and steroidal) and tamoxifen are considered standard treatments. AIs can be used upfront (non-steroidal AI and exemestane), after 2–3 years of tamoxifen (non-steroidal AI and exemestane) or as extended adjuvant therapy, after 5 years of tamoxifen (letrozole and anastrozole). Extended adjuvant therapy should be discussed with all patients, except those with a very low risk of relapse, but the optimal duration and regimen of adjuvant ET are currently unknown. There is only a minimal benefit for the use of AIs for more than 5 years. Patients undergoing OFS and those taking AIs should be advised to have adequate calcium and vitamin D3 intake and undergo periodic assessment of bone mineral density.

ChT is recommended in the vast majority of triple-negative, HER2-positive breast cancers and in high-risk luminal-like HER2-negative tumors. The absolute benefit of ChT is more pronounced in ER-negative tumours.[41] The most frequently used regimens contain anthracyclines and/ or taxanes, although in selected patients cyclophosphamide/ methotrexate/5-fluorouracil (CMF) may still be used. Four cycles of doxorubicin and cyclophosphamide (AC) are considered to have equal efficacy to 6 cycles of CMF. There is no place for routine use of 6 cycles of three-drug anthracycline-based regimens, possibly except in patients with strong contraindications to taxanes.[42] Randomized phase III data have shown that 5-fluorouracil (5-FU) can be dropped from anthracycline-based regimens because it does not add efficacy and it increases toxicity; therefore, the standard anthracycline-based regimens are AC or epirubicin plus cyclophosphamide (EC).[43]

The addition of taxanes slightly improves the efficacy of ChT, independently of age, nodal status, tumor size or grade, steroid receptor expression or tamoxifen use, but at the cost of increased non-cardiac toxicity; most importantly it allows for the use of a lower total dose of anthracyclines through the use of sequential regimens.[42] Sequential use of anthracyclines and taxanes is superior to concomitant use[44] and is also much less toxic. Some data suggest that a taxane/anthracycline sequence may be slightly more effective than the traditionally used anthracycline/taxane order but both are acceptable. Overall, ChT regimens based on anthracyclines and taxanes reduce breast cancer mortality by about one-third.[42,45] Non-anthracycline, taxane-based regimens, such as 4 cycles of docetaxel and cyclophosphamide (TC), may be used as an alternative to 4 cycles of anthracycline-based ChT, although such an approach has proven inferior to a combination of anthracyclines and taxanes. No robust, prospective randomized data exist on the use of platinum compounds in the adjuvant setting, either in unselected triple-negative tumors or in BRCA1/2 mutation carriers and they cannot therefore be recommended.

Trastuzumab combined with ChT in patients with HER2 overexpression/amplification approximately halves the recurrence and mortality risk, compared with ChT alone, translating into a 10% absolute improvement in long-term DFS and 9% increase in 10-year OS.[46] Trastuzumab is approved in patients with node-positive disease and in N0 patients with tumors >1 cm. Due to the relatively high relapse risk, even in patients with N0 tumors <1 cm, it should also be considered in this patient group, particularly in ER-negative disease.[47] If a HER2 test result is ultimately deemed to be equivocal, even after reflex testing with an alternative assay, HER2-targeted therapy may also be considered, although the true benefit from trastuzumab in those patients is still unknown. In most studies, trastuzumab was administered for 1 year. No additional benefit was demonstrated for 2-year trastuzumab administration in the HERA trial.[48] A few studies compared shorter versus standard 12-month administration of trastuzumab, but only the largest Persephone trial was able to show the non-inferiority of the shorter 6-month regimen, although this could not be demonstrated in the other studies.[49] Therefore, a duration of 1 year remains the standard, although in highly selected low-risk patients, who receive anthracycline/taxane-based ChT, shortening trastuzumab duration to 6 months may be discussed. Further data and longer follow-up are needed and several questions are still open regarding de-escalation of anti-HER2 therapy, ChT or both in HER2-positive early breast cancer. Trastuzumab is usually well-tolerated, although cardiac dysfunction may occur, usually reversible. Baseline cardiac function (expressed by the left ventricular ejection fraction) is indispensable before the start of treatment and periodic monitoring of cardiac function (usually

every 3–4 months) during treatment is necessary. Due to its cardiotoxicity, trastuzumab should not be routinely administered concomitantly with anthracyclines. Combination with taxanes is safe and has been demonstrated to be more effective than sequential treatment.[46] Trastuzumab may also be safely combined with RT and ET.

In the neoadjuvant setting, dual anti-HER2 blockade associated with ChT (trastuzumab/lapatinib, trastuzumab/pertuzumab) has led to improvements in the pCR rate when compared with ChT associated with one anti-HER2 agent.[50] However, this did not translate into statistically significant improvement in long-term outcomes for the combination of trastuzumab/lapatinib, and such a treatment cannot be recommended.[51] For the trastuzumab/pertuzumab combination, after reviewing potential risks and benefits (including the financial impact), in selected higher-risk cases it is an acceptable option as primary systemic therapy. In the adjuvant setting, the addition of pertuzumab resulted in a very small (0.9%) improvement in invasive DFS in the intention-to-treat (ITT) population and a higher benefit (2.5%) in the high-risk population (defined as N-positive or ER-negative), leading to its approval in the latter setting by the European Medicines Agency (EMA) and the United States Food and Drug Administration (FDA). This combination can therefore be considered in high-risk patients. It is currently unknown if dual blockade in the neoadjuvant setting should be continued for a total of 1 year in patients for whom a pCR is achieved or if this treatment should be stopped at surgery. For this reason, and until new trials are concluded, it is recommended to decide on the administration of 1 year of trastuzumab/pertuzumab based on the risk assessment at diagnosis; the treatment may start before or after the surgery, in accordance with the approval wording by the regulators.

For patients with HER2-positive early breast cancer who had residual invasive disease after completion of neoadjuvant ChT combined with anti-HER2 therapy, substitution of adjuvant trastuzumab with trastuzumab emtansine (T-DM1) decreases the risk of recurrence of invasive breast cancer or death by 50% and is recommended, once approved and where available.[52] Extended adjuvant anti-HER2 therapy with neratinib in patients who completed 1 year of trastuzumab demonstrated additional improvement in DFS, in particular in the ER-positive/ HER2-positive subgroup, albeit at the cost of significant toxicity, mostly diarrhea. It can be considered in some selected high-risk patients, with appropriate diarrhea prophylaxis and management. It is unknown, however, if this benefit is maintained for patients who have previously received dual blockade with trastuzumab/pertuzumab. In small, node-negative, mostly ER-positive, HER2-positive tumors with no other risk factors, the combination of

single-agent paclitaxel and trastuzumab provided excellent outcomes in a single-arm phase II study.[53] No randomized data exist to support omission of ChT in this group. However, in cases of contraindications for ChT or patient refusal, it is acceptable to offer the combination of targeted agents (ET and trastuzumab).

1.4.4 Personalized medicine

Breast cancer was the pioneer of personalized medicine in oncology. ER, PgR and HER2 status have been used for many years as predictive factors to select patients for targeted ET or anti-HER2 treatment (Figure 1). In recent years, surrogate intrinsic tumor phenotypes, based on biomarker expression, have also been used for treatment individualization. Molecular signatures for ER-positive BC such as MammaPrint, Oncotype DX, and Prosigna are commercially available and may help with (neo)adjuvant ChT decision making, in conjunction with all clinicopathological factors, in cases where decisions are challenging, such as luminal B-like/HER2-negative and node-negative/nodes 1–3-positive breast cancer.[40]

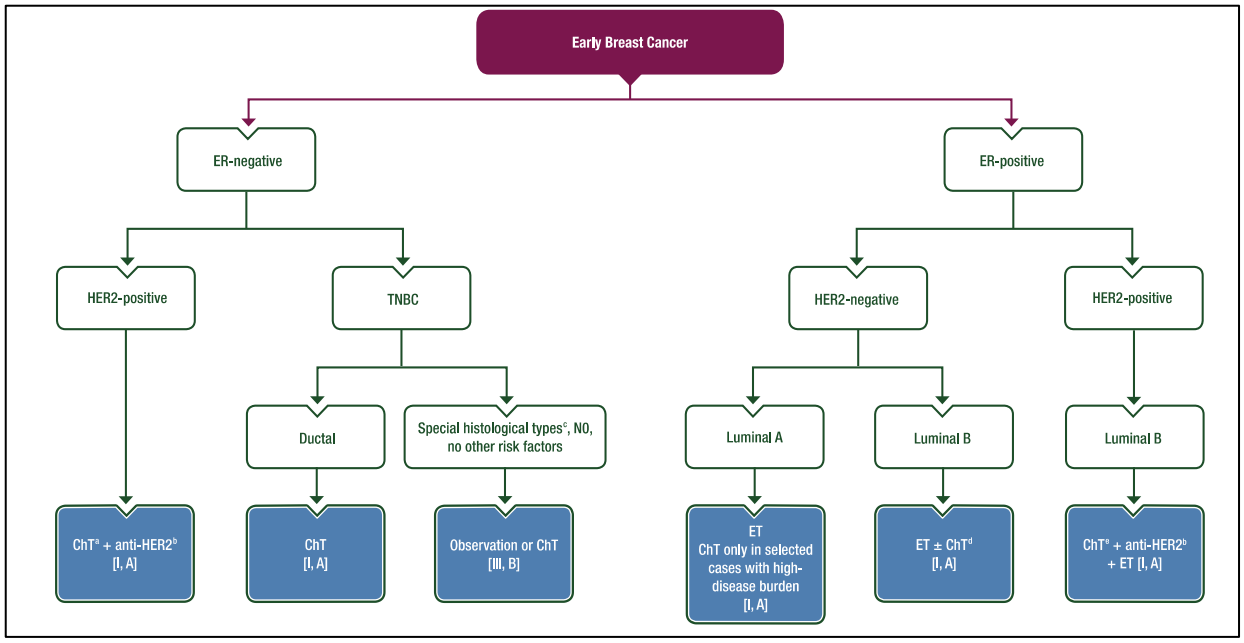

Figure 1. Adjuvant systemic treatment choice by marker expression and intrinsic phenotype. *Reproduced by Cardoso F. et al.* 2019.[54]
[a]With possible exception of selected cases with very low risk T1abN0.
[b]Anti-HER2: trastuzumab with or without pertuzumab.
[c]Adenoid cystic or apocrine, secretory carcinoma, low-grade metaplastic carcinoma
[d]Depending on level of ER and PgR expression, proliferation, genomically assessed risk, tumor burden and/or patient preference.
[e]Except for very low-risk patients T1abN0 for whom ET/anti-HER2 therapy alone can be considered.
ChT, chemotherapy; ER, estrogen receptor; ET, endocrine therapy; HER2, human epidermal growth factor receptor 2; N0, node-negative; PgR, progesterone receptor; TNBC, triple-negative breast cancer.

2. ANDROGEN RECEPTOR

2.1 AR structure and functions

Androgens (testosterone and dihydrotestosterone (DHT)) are the male sex hormones required for development of the reproductive system and secondary sexual characteristics.[55] Testosterone can be converted to its more biologically active form, DHT, by 5α reductase, and to estradiol by aromatase. Testosterone and DHT mediate their actions via the AR, a ligand-dependent nuclear transcription factor.[56] AR belongs to the steroid receptor superfamily and is classically considered a hormone-regulated transcription factor made up of 919-aminoacids encoded from a 180 kb gene located at the chromosome Xq11-12. Other members of the steroid hormone nuclear receptor family include the estrogen receptor (ER), progesterone receptor (PgR), glucocorticoid and mineralocorticoid receptors. The AR is expressed in a diverse range of tissues and as such androgens have been documented to have significant biological actions in bone, muscle, prostate, adipose tissue and the reproductive, cardiovascular, immune, neural and haemopoietic systems.[57]

The receptor has three functional domains: an N-terminal domain (NTD, residues 1–555), containing activation functional domains; a DNA binding domain (DBD, residues 555–623); and a carboxyl-terminal domain (CTD, residues 665–919) which including the ligand-binding domain (LBD) (Figure 1). The N-terminal domain of the AR is the most variable, whilst the DBD is the most highly conserved region between the different members of the steroid hormone nuclear receptor family. The DBDs of all steroid hormone nuclear receptors consist of two zinc fingers that recognize specific palindromic consensus sequence 5′-GGTACAnnnTGTTCT-3′ called androgen response element (ARE). These zinc fingers facilitate direct DNA binding of the AR to the promoter and enhancer regions of AR-regulated genes, thereby allowing the activation functions of the NTD and LBD to stimulate or repress the transcription of these genes. The DBD is linked to the ligand binding domain by a hinge region. The ligand binding domain also has a similar structure between the nuclear receptors and mediates the interaction between the AR and heat shock and chaperone proteins, whilst also interacting with the N-terminus of the AR to stabilize bound androgens.[58] Moreover, a nuclear localization signal (NLS), which is the responsible for AR nuclear import, and a hinge region are located between DBD and CTD.

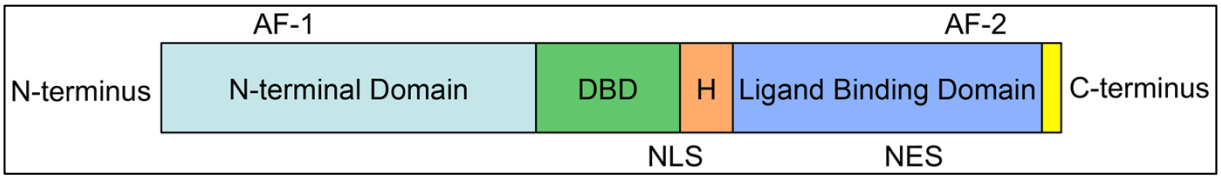

Figure 1. Functional domains of the androgen receptor: N-terminal domain, DNA binding domain (DBD), Ligand binding domain. (H – hinge region, AF-1 – transcriptional activating function 1, AF-2 – transcriptional activating function 2, NLS – nuclear localization signal, NES – nuclear export signal) *Reproduced from Davey & Grossmann (2016).*[59]

Two transcriptional activation functions have been identified: the ligand-independent AF-1, located in the N-terminal domain which is required for maximal activity of the AR, and the ligand-dependent AF-2, located in the ligand binding domain which is important for forming the coregulator binding site as well as mediating direct interactions between the NTD and LBD. AF-1 includes two separable transcription activation units, Tau-1 and Tau-5. The two Tau domains are required for the full activity of AR as well as the ligand-dependent interaction between the NTD and the LBD of the receptor. This interaction stabilizes the AR dimer complex and regulates the transcription of some AR- regulated genes.[60] Key differences in the contribution of specific conserved residues in the AF-2 core domain between the AR and other steroid hormone nuclear receptors have been identified, which likely account for the observed differences between the AF-2 regions of the AR and other steroid hormone nuclear receptors with respect to their structure and function as well as the co-regulatory proteins they interact with.[61] The NTD includes a poly- glutamine (CAG) sequence with a variable number of repetitions. The poli-Q length influences the folding and the structure of this domain and affects the AR-transcriptional activity, as minor length corresponds with a major AR activity.[62]

There are two distinct mechanisms of ligand-dependent AR action, either dependent or independent of DNA binding (Figure 2).

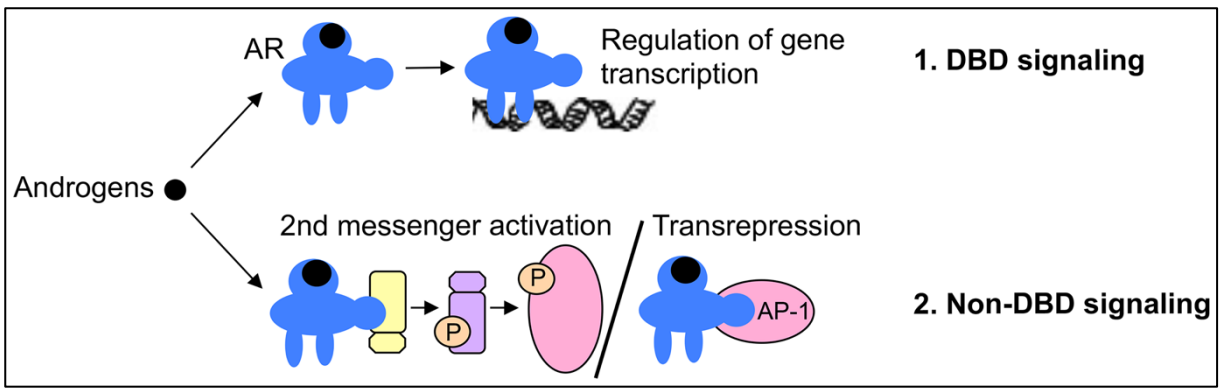

Figure 2. Mechanisms of ligand-dependent androgen receptor (AR) action: (1) DNA binding-dependent (DBD) and (2) non- DNA binding (DBD)-dependent. (AP-1 – activator protein 1) *Reproduced from Rana K. et al. (2014).*[57]

2.1.1 DNA binding-dependent signaling

The DNA binding-dependent actions of the AR are also commonly referred to in the literature as 'genomic', 'classical' or 'canonical' AR signaling. In the absence of ligand, the AR is cytoplasmic, associated with heat-shock and other chaperone proteins. The binding of AR with androgens leads to a conformational change with the dissociation of chaperone proteins and the exposure of NLS. The translocation of androgen/AR complex to the nucleus causes its dimerization and the binding to AREs, within classical target genes to modulate gene transcription. The transcriptional activity of the androgen-bound AR is modulated by specific proteins known as coregulators.[63] Coregulators bind to the activated AR in a ligand-dependent manner to either enhance (coactivator) or repress (corepressor) its ability to transactivate the target gene through chromatin remodeling and histone modifications, as well as being involved in the recruitment of the basal transcriptional machinery.[59]

2.1.2 DNA binding independent signaling

The DNA binding independent actions of the AR are also commonly referred to in the literature as 'non-genomic', 'non-classical' or 'non-canonical' AR signaling. The androgen/AR complex can also signal through non-DNA binding-dependent pathways. Activation of second messenger pathways including ERK, Akt and MAPK have been identified.[59] These effects occur within seconds to minutes of androgen treatment and are therefore too rapid to have arisen via the DBD actions of the AR to regulate the transcription and translation of target genes. Indirect gene transrepression can also occur, by the AR binding and sequestering transcription factors such as activator protein-1 (AP-1) that are normally required to upregulate target gene expression, in the absence of the AR binding to DNA.[59] Evidence exists to suggest that at least some of the non-DNA binding-dependent actions of androgens are mediated via the activation of membrane-bound protein receptors to initiate intracellular signaling pathways, which can occur even in the presence of low levels of androgens.[64,65] The identification and characterization of cell surface receptors that can mediate the rapid non-DNA binding-dependent actions of estrogen and progestins have been documented in a wide range of tissues and cell types,[59] however, to date, membrane-bound AR receptors have not been studied as extensively. For the most part, investigation into the non-DNA binding-dependent actions of the AR have been limited to in vitro studies. Although the physiological significance of the non-DNA binding-dependent actions of the AR is not yet fully defined, it has been proposed that they may oppose the DNA binding-dependent actions, and serve as a brake to fine-tune

androgen action in target tissues.[59] Similar opposing actions of the DNA binding-dependent and non-DNA binding-dependent pathways have been observed for the ER, with DNA binding-dependent activation of the ERα stimulating AP-1 activity, but activation of mitogen- activated protein kinases (MAPK) by a non-DNA binding- dependent ERα function suppressing AP-1 activity.[66,67] Ligand-independent activation of the AR by a number of different growth factors has been demonstrated, via phosphorylation of the AR or following interaction with co-activators. One such pathway identified is IL-6, commonly expressed by high circulating levels in patients with different cancers, that increases AR activity in a ligand-independent manner via the protein kinase A (PKA), protein kinase C (PKC) and MAPK pathways.[68] Similarly, an enhanced AR activation and nuclear localization by epidermal growth factor (EGF) and insulin-like growth factor (IGF) was seen with subsequent MAPK signaling activation.[69] Specifically, low AR levels have a scant transcriptional output, whereas they consistently activate extranuclear signaling pathways (i.e., Src tyrosine kinase, or PI3K, or the filamin A-dependent pathway) leading to massive proliferation and invasiveness of target cells.[70]

2.2 Cross-talk between hormone receptors and growth factors

Depending on the expression of other hormone receptor proteins and their ligands, the AR pathway may promote or inhibit cell proliferation. The interplay between AR, ER and their ligands is complicated by the possible conversion of androgens to estrogens. Patients with ER and AR-positive tumor have a better outcome than those with ER-positive and AR-negative disease. This has been attributed to the competition between AR and ER at the level of Estrogen Response Elements (EREs) and consequent impairment of ER-dependent gene transcription.[71] So the binding of AR to EREs reduces the estrogen proliferative action, thus inducing anti-proliferative effects. Conversely, ER can bind to androgen response elements (AREs), obtaining the opposite effect.[72] This mechanism could explain the role of AR in the resistance to standard endocrine treatments.[73] In fact, some studies highlighted that in ER-positive BC, AR could compete with ER-dependent transcription for the binding to the same sites or facilitating the ER binding to the DNA. In parallel, also in ER- and PgR-positive BC cells AR seems to compete. Instead, in PgR-negative BC cells, AR increases the ER gene transcription providing a protumorigenic role.

AR could be activated also in ligand-independent manner by different growth factors, by phosphorylation or other modifications, or following interaction with co-activators.[74] Moreover a high AR expression can activate the epidermal growth factor receptor (EGFR), promoting an agonist effect of tamoxifen on ER pathway, and this aberrant mechanism could be blocked by

enzalutamide +/- gefitinib.[75] A cross talk between AR pathway and HER2/neu pathway is also known.[76,77]

2.3 "The issue" of AR detection

2.3.1 Tissues approaches

AR is expressed in the nucleus of the cells but can be present also in the cytoplasm. AR translocates to the nucleus, upon ligand binding, where it can modulate transcription of AR-classical responsive genes. The withdrawal of androgen results in the export of unliganded AR from the nucleus to the cytoplasm, where it is transcriptionally inactive. The tissue approaches permit to detect the AR status at cellular level (nuclear and/or cytoplasmic) distinguishing epithelial cells from inflammatory cells and surrounding stroma. Among the different methods to test AR both in primary tumor and in metastasis, immunohistochemistry (IHC) is the cheapest method and can be performed routinely in all laboratories. Different ways to classify AR-positive cases have been used, as well as different percentage of positive cells cut offs. *H* score has also been used (the product of the percentage of positive cells and the staining intensity) to define AR positivity. On tissue, other methods have been used to assess different features of the receptor, such as the Fluorescence *in situ* hybridization (FISH) to analyze the copy number status, sequencing or PCR-based technologies to assess mutations, and gene expression analyses with *in situ* hybridization of AR transcript.[78]

2.3.2 Liquid biopsy approaches

The need of biomarker assessment by using non-invasive methods lead researchers to study and develop new approaches for AR testing on liquid biopsy. Circulating androgens can be detected with different concentrations in pre- and postmenopausal status. In particular, androgen levels decreased in menopause, even if is less drastic than the decrease in circulating levels of estrogen and progesterone.[79] The correlation between high androgens serum concentrations and BC risk is still controversial. Several studies have been focused on the evaluation of AR aberrations on serum/plasma or urine in prostate cancer setting, highlighted the correlation between copy number changes, mutations and splice variants identification with diagnosis, prognosis, tumor evolution monitoring and outcome prediction.[80,81] Regarding BC, few studies were conducted with the main aim to evaluate AR on liquid biopsy. Of note, as well as in PCa, BC circulating tumor cells (CTCs) were evaluated for the expression of the AR active splice variant of AR, called AR-v7, which lacks the ligand-binding domain. In BC, AR-v7 expression seems to be

related to an increased number of bone metastasis.[82] Given the evidences on PCa, the detection of AR-v7 in CTCs could be is a potential predictive marker for abiraterone and enzalutamide efficacy also in BC setting.[78] Recently, in metastatic BC, AR mRNA expression was evaluated in CTCs finding 31% AR-positive samples. Moreover, 58% of matched CTC and primary tumor samples of different BC subtypes showed a discordance of AR status, concluding that the determination of AR expression in CTCs could help to select metastatic BC patients for AR inhibitors.[83]

2.4 Androgens and AR in breast cancer

Androgen receptors are expressed in 60%–90% of breast cancers, mainly in estrogen receptor (ER)-positive tumors.[84,85] Depending on the subtype, the wild type AR (AR wt) is expressed in 50–70% of BC. In MCF-7 cells and in T47D cells has been reported the expression of a membrane androgen receptor (mAR) and in estrogen receptor negative breast cancer MDA-MB 453 cells, showing a molecular apocrine differentiation, is expressed a mutated form of AR with a glutamine to histidine substitution, called Q865H (Figure 3).[86] This mutant exhibits a reduced sensitivity to 5α-dihydrotestosterone (DHT) and does not respond to non-androgenic ligands or AR antagonists.[86] In BC circulating tumor cells (CTCs), an active splice variant of AR, AR-v7 is expressed (Figure 3). Expression of such mutant correlates with an increased number of bone metastases.[82] Additionally, AR45 represents another splice variants expressed in MDA-MB231 and MDA-MB 453, together with the AR-v7. AR45 lacks of exon 1 and is preceded by an N-terminal extension of 7-amino-acid long that inhibits the AR functions (Figure 3).[80] In sum, the presence of AR and/or its variants makes more complex the molecular scenario of BC.

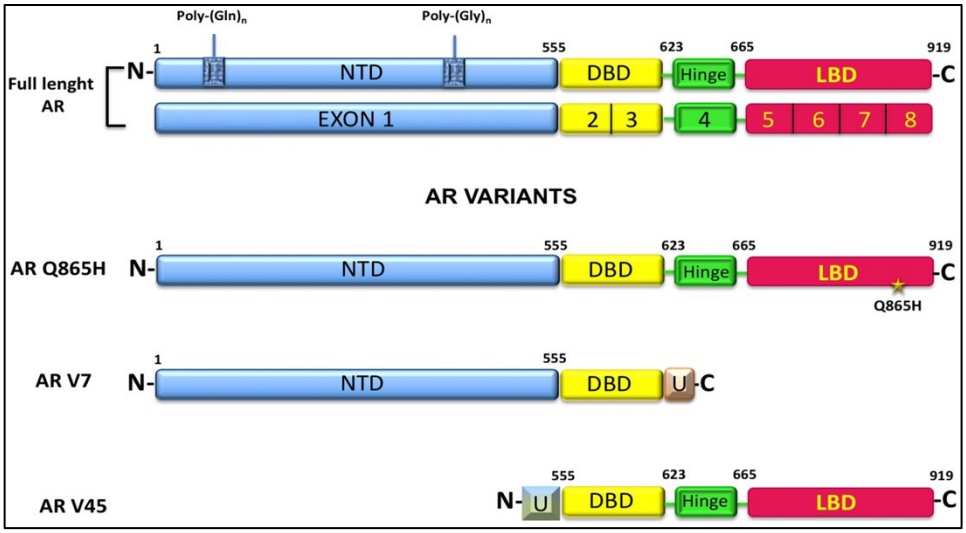

Figure 3. AR structure, alternative splicing variants and mutant commonly expressed in BC.
Reproduced from Giovannelli et al. (2018).[63]

To date, there is not a clear relationship between levels of circulating androgens and BC risk. In women, circulating androgens are dehydroepiandrosterone-sulfate (DHEA-S), dehydro-androstenedione (DHEA), androstenedione (A4), testosterone, and DHT. DHEA, DHEA-S, A4 are secreted by adrenal glands, while testosterone, DHEA and A4 are produced by ovaries (Figure 4).[87,88] Additionally, testosterone, DHT and their metabolites are produced in peripheral tissues, such as brain, bone and breast.[88] All these hormones play key roles in reproductive system, muscle growth and prevention of bone loss. Circulating androgens are detected in pre- and post-menopausal woman with different concentrations. Particularly, the levels of testosterone begin to decline in the mid reproductive years, and the levels of adrenal androgenic steroids (A4 and DHEA) decrease throughout post-menopausal life. Although the levels of androgens decline with menopause, this change, however, is less drastic than the decrease in circulating levels of estrogen and progesterone.[79] This is mainly due to the reduced functionality of the ovaries that decreases the estrogen and progesterone production, but continues to synthesize constant levels of testosterone and, at lesser extent, androstenedione. A huge effort was made to establish a correlation between circulating androgens and BC risk. In pre-menopausal women, high levels of circulating testosterone increase the BC risk, but there are no data that demonstrate a link between high levels of others androgens and BC.[87] In post-menopausal women, high baseline serum testosterone is a strong prognostic factor for local relapse, contralateral BC, and distant metastases.[89] Furthermore, high levels of others androgens (free testosterone, DHEA-S and A4) and SHBG (steroid hormones binding globulin) are correlated with an increased post-menopausal BC risk.[87] However, not all the studies indicated a correlation between increased androgen levels and BC risk. Adly and Colleagues showed that the BC risk is linked only to higher serum levels of estrogens, independently of androgen levels. These hormones might only indirectly influence the BC risk, because of their conversion in estradiol (E2) by aromatase activity.[90] The variability of the presented data might be explained by the different techniques used to measure the testosterone levels in blood or *in situ* as well as the tribulations in interpreting data.[91] To date, it is not clear if circulating androgens are a risk factor *per se* or as substrates for estrogens synthesis in breast tissues and BC. Maybe they can act in both ways in all BC that express ER, but certainly not in ER-negative BC.

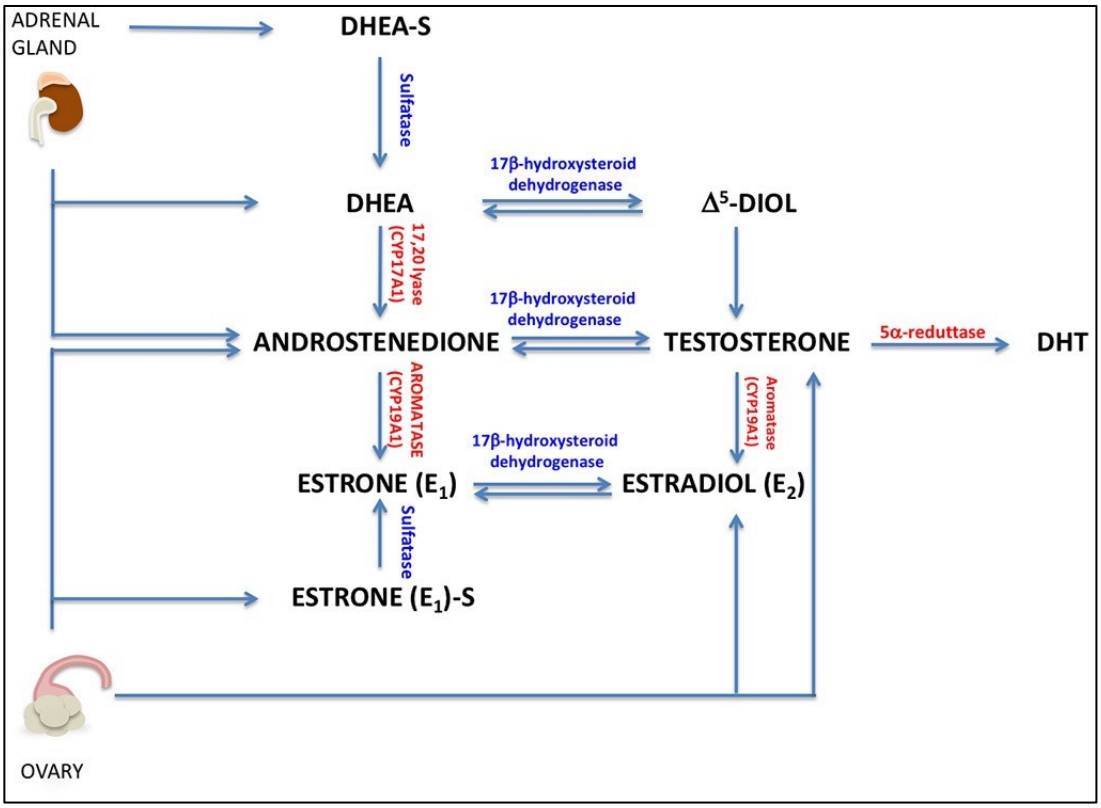

Figure 4. Circulating androgens in women: androgen and estrogen synthesis. (DHEA: dehydroepiandrosterone; DHEA-S: dehydroepiandrosterone sulfate; DHT: dihydrotestosterone). *Reproduced from Giovannelli et al. (2018).*[63]

2.4.1 AR in male breast cancer

BC in male is a rare tumor with biological differences between female BC. Male BC is exclusively hormone receptor positive, also for AR. Male BC showed a prevalence of BRCA2 germline mutations. Di Oto and colleagues showed that X chromosome gain is related to increased AR expression in male BC.[92] X chromosome gain was observed in 74.7% of invasive duct carcinoma, in 20.6% of *in situ* duct carcinoma, and in 14.6% of gynecomastia when associated with cancer, while all cases of tumor-free gynecomastia showed wildtype X chromosome composition. AR IHC expression was observed in 100% of male BC tested. AR gene methylation status revealed low level or absence of methylation. These data suggest that X chromosome can play a role in the neoplastic transformation of male breast epithelium. X chromosome gain is paralleled by AR gene polysomy. Polysomic AR genes showed low methylation levels and high AR protein expression on IHC.[92]

2.5 Anti-androgen therapies: a lesson from prostate cancer

Prostate cancer (PCa) is dependent on AR activation for growth and development; for this reason, androgen deprivation therapy is the gold standard treatment in advanced PCa. AR upregulation is the most common event involved in the progression from hormone sensitive to castration-resistant prostate cancer. In PCa setting several mechanisms responsible for AR transcriptional re-activation have been demonstrated, including mutation, amplification, or rearrangement of the AR gene, and elevated expression of truncated AR variants.[93] Various AR signaling-directed therapies (Figure 5), such as abiraterone, enzalutamide and more recently apalutamide have been developed. Abiraterone is a selective inhibitor of the enzyme cytochrome P450 involved in androgens biosynthesis, reducing the circulating testosterone levels in PCa.[94] Enzalutamide is an anti-androgen with greater affinity for AR than abiraterone.[81] On February 14, 2018, the Food and Drug Administration approved apalutamide for patients with non-metastatic castration-resistant prostate cancer but up to now none demonstrated its role on AR-positive BC. The availability of anti-AR compounds opens the possibility to treat also AR-positive BC patients. Androgens have variable effects in different BC models: often antiproliferative,[95,96] mainly in ER-positive tumors; sometimes pro-proliferative,[97,98] mainly in triple-negative and human epidermal growth factor receptor 2 (HER2)-positive/ER-negative tumors. It appears that in ER-negative BC cells, AR acts in a more homogeneous way as compared to ER-positive BC cells. In these tumors the receptor clearly promotes cell proliferation and spreading by acting at different levels. This evidence depicts AR as a therapeutic target potentially very exploitable for TNBC and provides new opportunities for the treatment of this subtype of BC.[99] The role of AR as a prognostic/predictive biomarker in this subset of patients is controversial, but increasing evidence suggests that AR positive TNBC may respond to therapeutic agents targeting AR. AR-positive TNBC was seen to be more common in older patients and in whom had a higher propensity for lymph node metastases. AR-positive TNBC may represent a BC subtype with unique features that may be amenable to treatment with alternative targeted therapies. The use of first and second generation AR-directed antagonists (bicalutamide and enzalutamide), is the most used therapy for advanced BC (Tamoxifen-resistant BCs and TNBCs).[100,101] Both the antagonists have been used in clinical trials with positive results.[101] The most recent studies were conducted by using in vitro and in vivo experiments with the principal aim to test the dose, efficacy, safety, tolerability of different new potential anti-AR therapies alone and the combination with other drugs. In a phase 1 study of Seviteronel, a selective CYP17 lyase and AR inhibitor, in vitro and in vivo anti-tumor activity was tested. In particular,

the safety, tolerability, pharmacokinetics, and activity of once-daily Seviteronel were evaluated in women with ER-positive or TNBC, showing to be well tolerated.[102] Abiraterone acetate and Seviteronel, CYP17A1 inhibitors, reduce the androgen production and the androgen levels and they are now being tested in phase 2 clinical trials,[103] alone or in combination with AR-directed antagonists. Preclinical and clinical findings, however, have indicated that AR stimulates the growth of TNBC or HER2 positive BC in combination with other effectors.

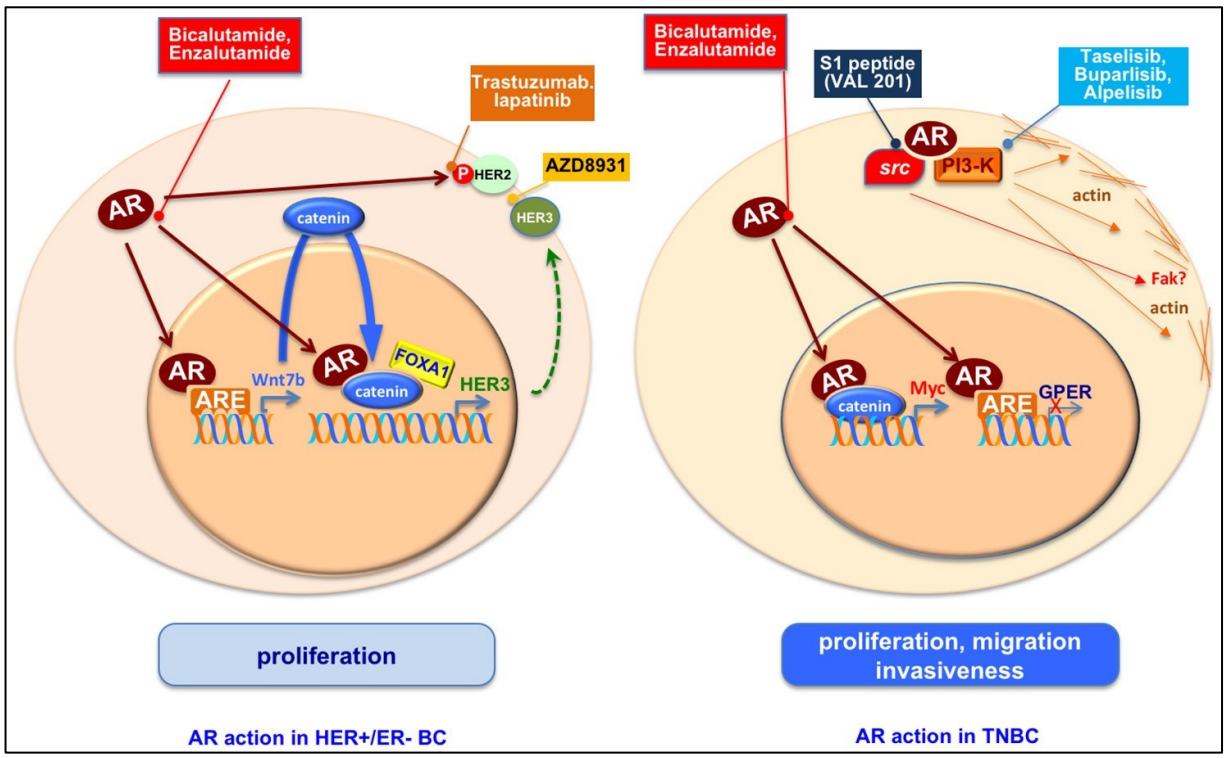

Figure 5. An overview of mechanisms activated by AR in HER+/ER– and TN-BC cells. AR regulates proliferation, migration and invasiveness in ER-BC through genomic and non-genomic pathways. The use of AR antagonists, inhibitors of AR activated proteins as HER2, HER3 or PI3K, or the S1 peptide that disrupts the AR/src association is or could be a starting point to reduce the ER-BC spread. *Reproduced from Giovannelli et al. (2019).*

Optimal results might be obtained by approaches in which AR antagonists are used in combination with inhibitors of these pathways.[104,105] Giovannelli P. and colleagues showed that in TNBC-derived cell lines (MDA-MB231 and MDA-MB453), expressing AR, S1 peptide could be a promising therapeutic option (Figure 5). In fact, it mimics AR proline-rich motif responsible for the interaction of AR with SH3-Src leading to the inhibition of motility and invasiveness of TNBC cells.[105] These in vivo findings suggest also that S1 peptide blocking should be considered as anti-AR strategy. Lehmann's group performed a cell line study showing that AR enriched TNBC cell lines carrying PI3KCA mutations acquire sensitivity to PI3K/mTOR inhibition, promoting the cancer cell growth (Figure 5).[104] Some authors demonstrated that the combination of bicalutamide and PARP inhibitor (ABT-888) could

inhibit cell viability and induce cell apoptosis significantly whatever in vitro or in vivo setting in AR-positive TNBC. For the first time, the analyzed the correlation among AR, PARP1 and BRCA1 in TNBC. After BRCA1 overexpression, the expression of AR and PARP1 were decreased in mRNA and protein levels. Additionally, AR positively regulated PARP1, while PARP1 upregulated AR expression in vitro. They confirmed that BRCA1 expression was negatively correlated with AR and PARP1 in TNBC patients using a tissue microarray with patient samples. These findings highlighted that the combination of bicalutamide and PARP inhibitor may be a potential strategy for TNBC patients and merits further evaluation. These results were recently confirmed by in vivo and in vitro experiments performed by Sang M. and colleagues on sporadic TNBC.[106]

AIM

Androgen Receptor and "5W questions": What, Where, When, Why and hoW

The possibility that a receptor for androgens is expressed in Breast Cancer (BC) is fascinating given that the tumor is predominantly estrogen-dependent. However, the heterogeneity of the disease could explain why not all BC expressing hormones respond to hormonal treatments.[107] AR expression is variable among the different BC subtypes. It is found in about 75% of ER-positive, 50-60% of HER2-enriched, and 20-40% of triple negative BC, respectively.[85,108] In general, AR protein is expressed in about 70-90% of BC. Its levels may however vary depending on the location considered (cytoplasmic and/or nuclear), the cut off of expression (≥1%, ≥10%), and the antibody used for immunohistochemistry (IHC).[84,109]

Despite the heterogeneity of BC, global analyses of tumors using genetic profiles have identified gene expression signatures that characterize many intrinsic tumor subtypes with different biology and clinical behavior. In particular, the role of hormonal status is important to define the prognosis and to predict the response to therapy for BC patients. Currently hormone receptors are widely used as prognostic and predictive factors to manage decision-making in BC patients. Estrogen receptor (ER) expression is mostly important because it can predict about 50-70% of tumor responses under treatment with anti-estrogens, whereas response rate is less than 10% in ER-negative BCs and perhaps 0% in truly ER-absent cases.[107,110–112] Levels of ER affect the time-distribution of BC relapses and ER positivity is associated with more delayed recurrences compared to ER absence.[113] AR appears to have different functions according to the BC subtype, *e.g.* ER-positive or triple negative BC. These data are still controversial because the same authors described a role of AR in predicting response rate and overall survival under hormonal therapy, and at the same time they found no association between AR expression and disease-free survival in ER-positive tumors. In the same works ER status maintained the predominant role as independent prognostic factor for disease-free survival.[114,115] For some authors, AR expression was related to a better survival when it was coexpressed with ER and PgR,[114,116] but not for others.[85] The results from studies, in which the AR/ER ratio was used to estimate prognosis and to predict Tamoxifen failure in patients with primary luminal BC (*i.e.* ER+/HER2-),[73] raises the question as to whether this new parameter will become mandatory for prognostic classification in this BC subset. We can contribute to the debate on the prognostic role of the ratio through a study assessing not only the AR/ER ratio, but also the AR/PgR and ER/PgR ratios both in a subset of patients with a precancerous condition, such as ductal carcinoma *in situ*, and both in an advanced stage of disease, where the meaning of these ratios have never been explored.

AR is emerging as a new biomarker and a potential new therapeutic target in the treatment of BC patients. The recent availability of selective AR inhibitors (e.g. bicalutamide, enzalutamide, apalutamide) approved for the treatment of PCa has opened up the possibility to use them in BC patients whose tumors express AR.[117] To select patients suitable for this kind of treatment, it is necessary to assess AR in tumor tissue. Often only primary tumor samples are available, but not metastatic samples. When we assess AR expression in the primary sample, is it relevant to assess its expression also in metastatic tissue? The concordance of AR expression between primary and metastatic samples is not well defined. Moreover, the time elapsed between the biopsy of the primary tumor and the biopsy of a metastasis could affect the degree of change in AR expression. This difference could make difficult the decision-making process for anti-androgen therapy.

The purposes of this study are the analysis of (WHAT) AR expression (HOW) by IHC in (WHERE) formalin-fixed, paraffin-embedded (FFPE) DCIS samples, primary breast tumors and metastases, and (WHEN) the assessment of changes in AR expression levels over time. In addition, the role of the ratios AR/ER and AR/PgR is analyzed (HOW), since the interplay between steroid hormone receptors is well known but the mechanisms are not yet fully understood (WHY). Furthermore, only few data are available on the biomolecular characterization of Tanzanian breast tumors. To improve cancer control and care in Mwanza (Tanzania), we made an international project involving a non-profit association (Association Vittorio Tison) and the local hospital in Mwanza, Bugando Medical Center (BMC), together with the major local and national health authorities, to open a Medical Oncology Unit and Pathology Laboratory in the hospital. For this research project and since few data exist on the biological features of sub-Saharan Africa BC population (WHY), we carried out a study on the comparison of (WHAT) AR expression between Tanzanian and Italian BCs.

RESULTS

1. ROLE OF ANDROGEN RECEPTOR IN DCIS

1.1 Androgen receptor in DCIS patients treated with surgery

The role of AR in DCIS was retrospectively analyzed on a series of 43 patients, with DCIS diagnosed during the screening, recruited from 2002 to 2009 by the Cancer Prevention Unit and Breast Surgical Unit of Morgagni-Pierantoni Hospital Forlì. The five markers (AR, ER, PgR, Ki67 and HER2) were analyzed for all cases. Patients had surgery alone and followed for up to 13 years; 5 relapsed at 3, 7, 7, 10 and 10 years from diagnosis. Statistical analysis showed that the different markers were not, singly, indicators of relapse; ER was the only marker whose median value was able to predict relapse or not relapse with a p-value near to the statistical significance (Table 1). The proliferative activity as Ki67, or HER2, which are important indicators of aggressiveness in invasive tumors, have no prognostic relevance in DCIS (Table 1). The analyses of ER and AR expression (Figure 1.1) showed that they singly were not indicative of relapse (Table 1). The AR/ER ratio value in relapsed patients was statistically different from that of not relapsed patients (p = 0.011) and, at a cut off of 1.13, showed a sensitivity of 75% and a specificity of 94% for predicting relapse as *in situ* or invasive carcinoma. The ratio AR/PgR at a cut off of 1.00 has a sensitivity of 75% and specificity of 53%, while at a cut off of 3.00 has a sensitivity of 50% and a specificity of 84%. Moreover, while all the biomarkers showed an AUC values ranged from 52% to 77%, the ratio of AR/ER reached a very high AUC (92%) (Table 2).

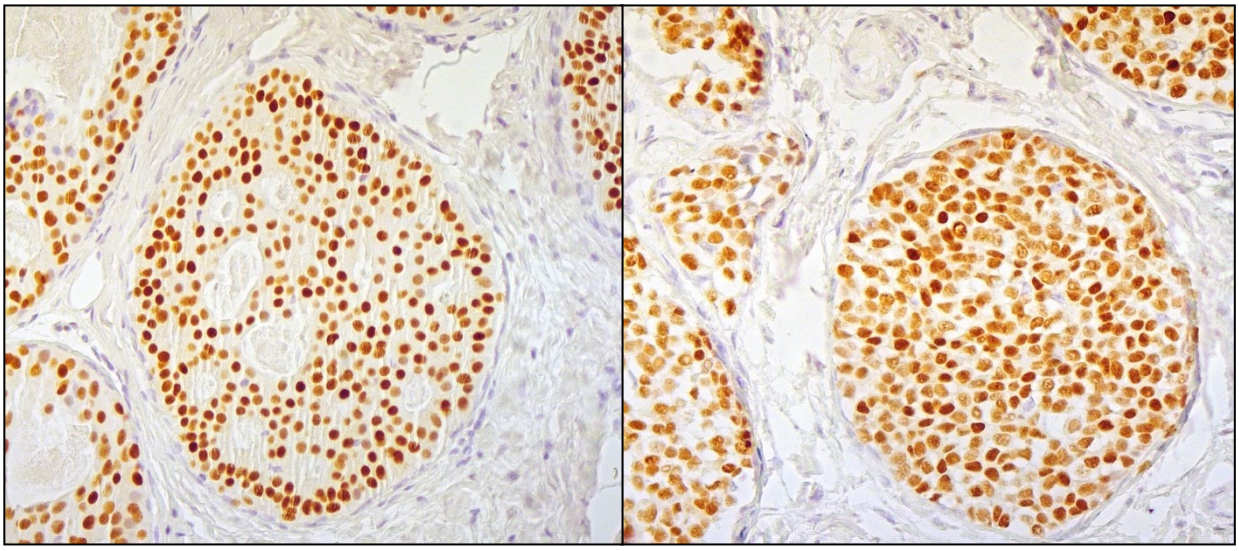

Figure 1.1 Ductal carcinomas *in situ* of the breast immunostained for ER (left side) and AR expression (right side), 20X magnification.

Table 1. Expression of different markers in tumor cells in relapsed and non-relapsed patients

Markers	Overall (n=43)	Non-relapsed (n = 38)	Relapsed (n = 5)	*P*
		Median (range)		
AR	80 (0-100)	75 (0-100)	90 (80-90)	0.125
ER	90 (0-100)	90 (0-100)	80 (0-80)	0.056
PGR	75 (0-100)	80 (0-100)	30 (0-95)	0.265
Ki67	5 (1-25)	5 (1-20)	5 2-25)	0.890
AR/ER ratio	0.95 (0-1.29)	0.89 (0-1.27)	1.12 (1.00-1.29)	**0.011**
		No. (%)		
HER2 positive	15 (35.7)	13 (35.1)	2 (40.0)	
HER2 equivocal	24 (57.2)	21 (56.8)	3 (60.0)	0.801
HER2 negative	3 (7.1)	3 (8.1)	0	

Table 2. Area under the curve (AUC) of biomarkers

Markers	Overall AUC (95% CI)
AR	72 (57-86)
ER	77 (63-92)
PgR	66 (40-92)
Ki67	52 (14-91)
AR/ER ratio	92 (81-100)

1.2 Androgen receptor in DCIS patients treated with surgery and radiotherapy

Forty-two patients diagnosed with DCIS between 2000 and 2009 during screening at the Cancer Prevention Unit and operated on in the Breast Surgical Unit of Morgagni-Pierantoni Hospital, Forlì were enrolled in the study. They underwent quadrantectomy and radiotherapy and were followed up for a median of 95 months. Eleven patients relapsed between 2 and 7 years after the first diagnosis: 6 with DCIS, 3 with IC, and 2 with both DCIS and IC histologies. The relapsed patients were matched 1:3 for age and nuclear grade with non-relapsed patients enrolled in the same period.

Of the 42 DCIS, 3 (7%) were classified as G1 tumors, 19 (45%) as G2 and 20 (48%) as G3 (Table 3). Thirty-one (74%) were unifocal tumors and 11 (26%) were multifocal tumors (Table 3). Thirty-six (86%) lesions showed negative surgical margins and 6 (14%) showed positive margins, the extent of invasion ranging from 0.5 to 2 mm. Only 4 (9.5%) DCIS showed comedonecrosis (Table 3). No differences in age, tumor size, nuclear grade, focality (unifocal versus multifocal), margin status (positive *vs*. negative), and type of DCIS (comedo vs. non comedo) were found between relapsed and non-relapsed patients (Table 3). AR and conventional biomarkers were analyzed in the entire case series (Figure 1.2). HER2 immunostaining was not feasible in 6 patients due to insufficient FFPE material (Figure 1.2).

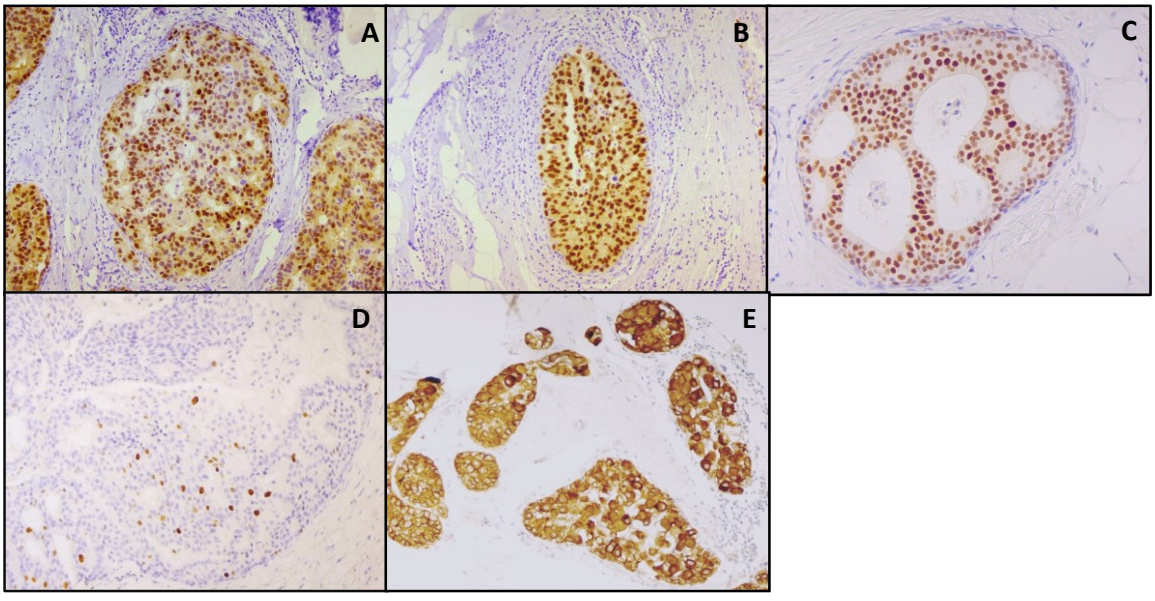

Figure 1.2 DCIS case positive for (A) AR expression; (B) ER expression; (C) PgR expression; (D) Ki67 expression; (E) HER2 expression. All 10x magnification.

Table 3. Patient characteristics

Characteristics	**Overall (*n* = 42)**	**Non-relapsed (*n* = 31)**	**Relapsed (*n* = 11)**	***P***
Median age, years (range)	57 (38-77)	56 (42-76)	57 (38-77)	0.8410
		No. (%)		
Nuclear grade				
1	3 (7.1)	3 (9.6)	0 (0.0)	
2	19 (45.2)	14 (45.2)	5 (45.5)	0.8740
3	20 (47.6)	14 (45.2)	6 (54.5)	
Radiological presentation				
Microcalcifications (M)	36 (87.8)	28 (90.3)	8 (80.0)	
Opacity (O)	3 (7.3)	2 (6.5)	1 (10.0)	0.353
O + M	2 (4.9)	1 (3.2)	1 (10.0)	
Tumor size (mm)				
≤5	10 (27.1)	9 (32.1)	1 (11.1)	
>5 and ≤10	9 (24.3)	8 (28.6)	1 (11.1)	0.3200
>10 and ≤20	9 (24.3)	5 (17.9)	4 (44.5)	
>20 and ≤30	3 (8.1)	2 (7.14)	1 (11.1)	
>30	6 (16.2)	4 (14.3)	2 (22.2)	
Comedonecrosis				
Yes	4 (10.0)	2 (6.7)	2 (20.0)	0.2560
No	36 (90.0)	28 (93.3)	8 (80.0)	
Histological Focality				
Unifocal	29 (72.5)	22 (73.3)	7 (70.0)	1.0000
Multifocal	11 (27.5)	8 (26.7)	3 (30.0)	
Margin status				
Negative	35 (92.1)	7 (87.1)	8 (80.0)	
Positive	6 (7.9)	4 (12.9)	2 (20.0)	0.6220

The evaluation of ER, PgR, Ki67 and HER2 did not bring to light significant differences between relapsed and non-relapsed patients (Table 4). Our findings revealed that AR expression (Figure 1.2) was significantly higher in relapsed patients than in non-relapsed patients (p = 0.0005). Conversely, the expression of estrogen receptors (ER) (Figure 1.2) was higher, albeit not significantly (p = 0.2342), in non-relapsed patients than in those who relapsed. Seven patients (4 relapsed and 3 non-relapsed) were negative for ER expression. The AR/ER ratio value was higher (p = 0.0033) in relapsed patients than in non-relapsed patients (Table 4). For AR/ER ratio the best cut off value of 1.1 showed an 81% accuracy in predicting *in situ* relapse or progression to invasive carcinoma. Moreover, considering the variables separately, AUC values were 0.85 (95% CI: 0.73-0.97) for AR, 0.62 (95% CI: 0.40-0.84) for ER, 0.70 (95% CI: 0.46-0.93) for PgR and 0.80 (95% CI: 0.65-0.96) for the AR/ER ratio, with no significant difference between AR and the AR/ER ratio (p = 0.4170) (Table 5).

Table 4. Marker expression in tumor cells of relapsed and non-relapsed patients

Markers	Overall (n = 42)	Non-relapsed (n = 31)	Relapsed (n = 11)	*P*
		Median (range)		
AR	60 (0-100)	40 (0-95)	80 (40-100)	**0.0005**
ER	80 (0-98)	80 (0-98)	40 (0-95)	0.2342
PgR	40 (0-90)	40 (0-80)	0 (0-90)	0.0869
Ki67	5 (3-25)	5 (3-25)	7.5 (5-20)	0.6936
AR/ER ratio	0.82 (0-95)	0.67 (0-90)	2.5 (0.44-95)	**0.0033**
HER2	Overall (n = 36)	Non-relapsed (n = 25)	Relapsed (n = 11)	
Staining intensity		No. (%)		
0 (absent)	10 (27.8)	9 (36.0)	1 (9.1)	0.2340
1+ (weak)	6 (16.7)	4 (16.0)	2 (18.2)	
2+ (moderate)	8 (22.2)	6 (24.0)	2 (18.2)	
3+ (strong)	12 (33.3)	6 (24.0)	6 (54.5)	

Table 5. Area under the curve (AUC) values of markers in tumor cells

Markers	Overall AUC (95% CI)
AR	0.85 (0.73-0.97)
ER	0.62 (0.40-0.84)
PgR	0.70 (0.46-0.93)
Ki67	0.55 (0.30-0.81)
AR/ER ratio	0.80 (0.65-0.96)

In Cox univariate models, an increase in AR and in AR/ER ratio was related to an increased risk of recurrence (5%, $p = 0.003$, for AR and 2%, $p = 0.015$, for AR/ER) (Table 6). In addition, multivariate analysis identified AR as independent prognostic factor showing an hazard ratio (HR) of 1.06 (95%CI: 1.01-1.11) (Table 5). The relapse-free survival curve showed that 75% of patients were recurrence-free after 5 years (Figure 3).

Table 6. Cox regression models

	Univariate model			***Multivariate model***		
Variables	HR	95%CI	*P*	HR	95%CI	*P*
AR	1.05	1.02-1.09	0.003	1.06	1.01-1.11	0.023
ER	0.99	0.97-1.01	0.072	-	-	-
PgR	0.99	0.96-1.01	0.205	-	-	-
AR/ER ratio	1.02	1.01-1.03	0.015	1.00	0.98-1.02	0.936

ROC curve analysis for AR and the AR/ER ratio is shown in Figure 1.3.

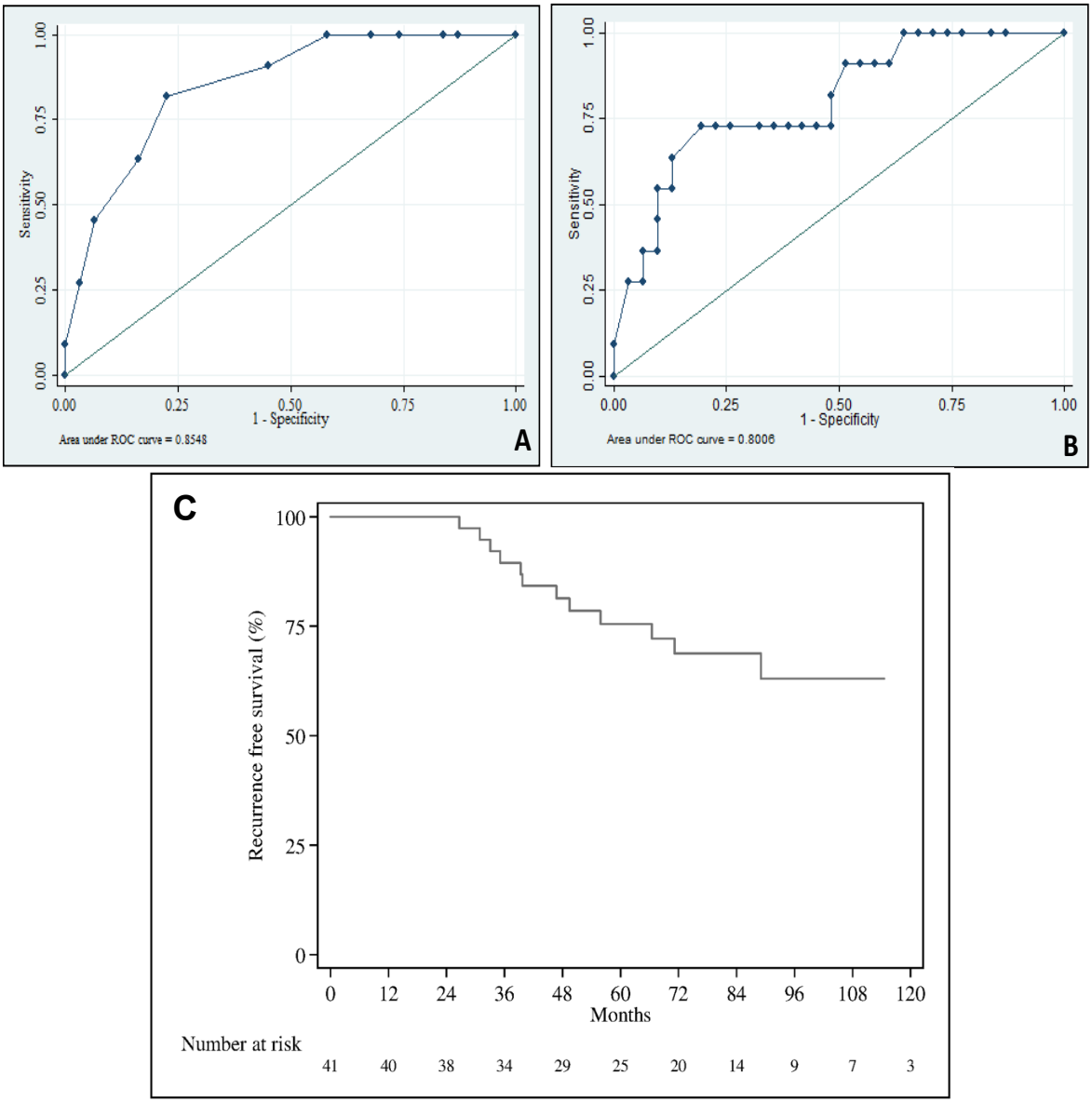

Figure 1.3 ROC curve for (A) AR and (B) AR/ER ratio C) Recurrence free survival curve.

1.3 Comparison of AR role in the two DCIS case series

The analysis was performed on a total of 85 DCIS patients treated with surgery alone or surgery plus radiotherapy. No significant differences in age, tumor size, nuclear grade, focality (unifocal *vs* multifocal), margin status (positive *vs* negative), or type of DCIS (comedo *vs* non comedo) were found between relapsed and non-relapsed patients. The biological profile including the conventional biomarkers is shown in Table. ER and PgR expression was higher in non-relapsed than in relapsed patients ($p = 0.025$, $p = 0.0038$) (Table 7). Seventy-eight (91.7%) samples were AR-positive and expression was higher in relapsed than in non-relapsed patients ($p = 0.0069$) (Table 7). ER assumed greater importance when considered together with AR. We observed that the AR/ER ratio was higher in relapsed patients (p = 0.0012) (Table 7).

Table 7. Marker expression in tumor cells of relapsed and non-relapsed patients

Markers	**Overall (85 cases)**	**Non-relapsed (69 cases)**	**Relapsed (16 cases)**	***P***
		Median (range)		
Ki67	5 (1-25)	5 (1-25)	5 (1-25)	0.53
AR	60 (0-100)	60 (0-100)	80 (40-100)	0.0069
ER	87.5 (0-100)	90 (0-100)	75 (0-95)	0.025
PgR	47.5 (0-100)	60 (0-100)	0 (0-95)	0.0038
AR/ER ratio	0.9 (0-10)	0.9 (0-1.5)	1.1 (0.4-10)	0.0012

Moreover, the accuracy of AR alone in the surgery plus radiotherapy case series was 85% (Table 8), while the AR/ER ratio at the best cut off value of 1.1 showed a sensitivity of 75%, a specificity of 94% with an overall accuracy of 92% in predicting *in situ* relapse or progression to invasive carcinoma in DCIS patients treated with surgery alone. In patients treated with surgery plus radiotherapy, the AR/ER ratio reached an overall accuracy of 80% (Table 8), with a sensitivity of 64% and a specificity of 87%.

Table 8. Area under the curve (AUC) values of tumor markers in relation to type of treatment

Markers	Surgery	Surgery and radiotherapy
	AUC (95%CI)	
Ki67	0.52 (0.14-0.91)	0.55 (0.30-0.81)
AR	0.72 (0.57-0.86)	0.85 (0.73-0.97)
ER	0.77 (0.63-0.92)	0.62 (0.40-0.84)
PgR	0.66 (0.40-0.92)	0.70 (0.46-0.93)
AR/ER ratio	0.92 (0.81-1.0)	0.80 (0.65-0.96)

2. ROLE OF ANDROGEN RECEPTOR IN INVASIVE BREAST CANCER

2.1 Concordance analysis of AR expression between primary tumors and metastases

AR expression was evaluated by IHC in 164 primary tumors and 83 metastatic samples, in order to study the concordance between the primary tumor and the relative metastasis in terms of AR positivity. Two hundred fourteen patients were included in the study (Figure 2.1).

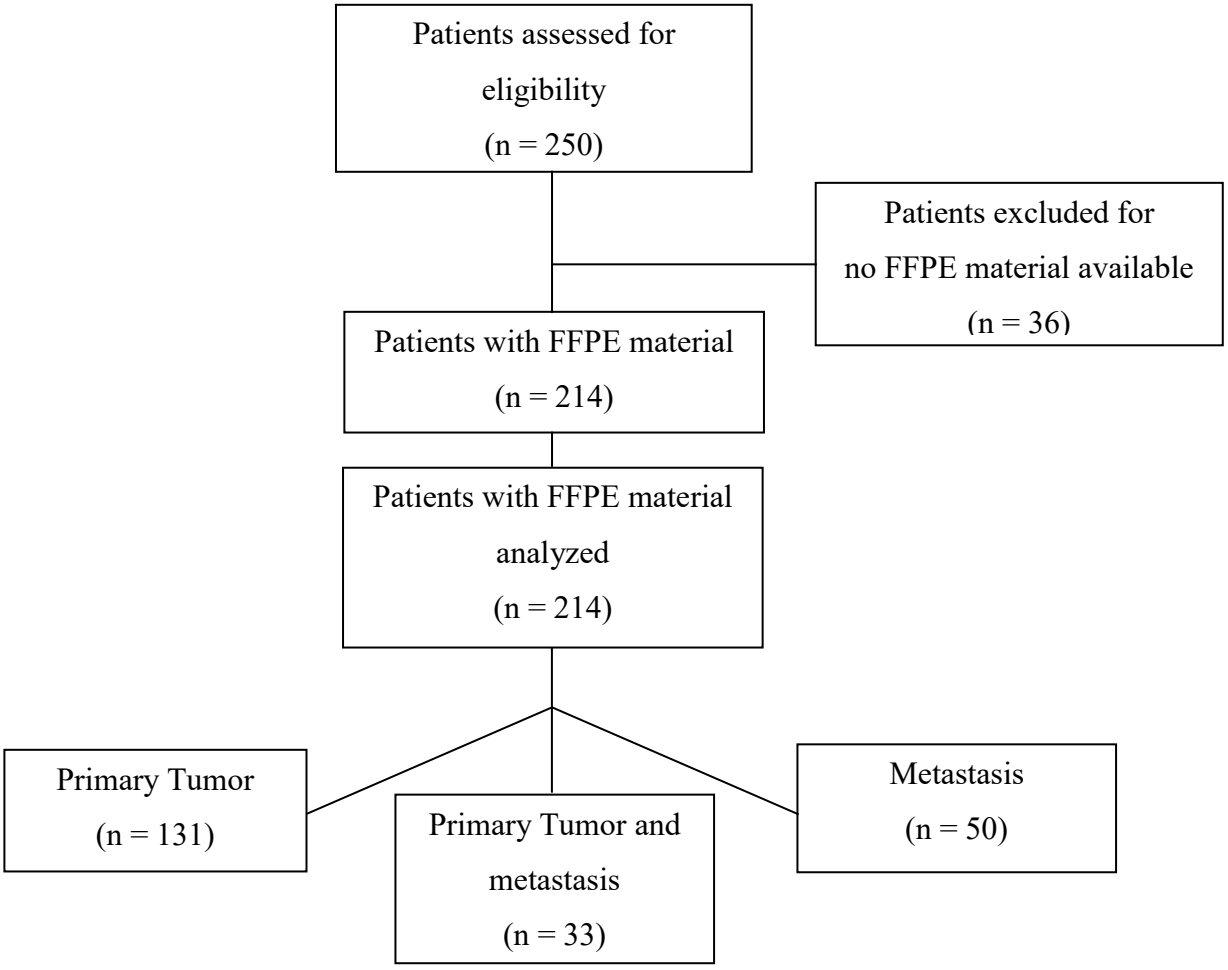

Figure 2.1 Flow chart of the study.

The tumor block material of the primary cancer was available for 164 patients, 154 of whom were completely characterized for BC biomarker (ER, PgR, Ki67 and HER2) and subtype. Eighty-three metastatic tumor material blocks were available and the BC subtype was established for 79 patients based on BC biomarkers. Only 33 patients had both primary and metastatic tumors available. All patients' clinical features are shown in Table 1. The median age was 58 years (range: 26-86).

Table 1. Patient's characteristics

	All patients (n=214), as per clinical practice*
Adjuvant chemotherapy	
No	64 (36.8)
Yes	110 (63.2)
Unknown	40
Adjuvant endocrine therapy	
No	67 (38.5)
Yes	107 (61.5)
Unknown	40
Histotype	
Ductal	169 (82.4)
Lobular	28 (13.7)
Other	8 (3.9)
Unknown	9
Tumor stage	
1	90 (49.2)
2	70 (38.3)
3	7 (3.8)
4	16 (8.7)
Unknown	31
Nodal involvement	
0	72 (40.0)
1	71 (39.5)
2	20 (11.1)
3	17 (9.4)
Unknown	34
Metastases at diagnosis	
Yes	40 (19.1)
No	169 (80.9)
Unknown	5
1st-line endocrine therapy for advanced BC	
Letrozole	72 (46.5)
Anastrozole	32 (20.6)
Exemestane	39 (25.2)
Tamoxifen	9 (5.8)
Fulvestrant	3 (1.9)
Unknown	59

*biomarker expression measured in metastases (when a biopsy was performed on metastases) or in primary tumors (when biopsy on metastases had not been performed)

Regard to the 164 primary tumors, 136 (82.9%) were AR positive (Figure 2) according to a cut off value of ≥ 1%, and 131 (79.9%) with the cut off value of ≥ 10% (Table 2). Similarly, out of 83 metastases, 61 (73.5%) were AR positive according to a cut off value ≥ 1%, and 50 (60.2%) by using ≥ 10% cut off value (Table 2). AR H-score median value was 240 (range 0-300) in primary tumors and 210 in metastases (range 0-300).

Table 2. Tumor biological characteristics

	Primary tumor (n=164)	Metastases (n=83)	As per clinical practice (n=214)
Grade		**No. (%)**	
1	6 (4.6)	0	6 (3.7)
2	49 (38.0)	11 (47.8)	67 (40.8)
3	74 (57.4)	12 (52.2)	91 (55.5)
Unknown	35	60	50
ER status			
<1%	30 (18.7)	8 (9.8)	33 (15.4)
≥1%	130 (81.3)	74 (90.2)	181 (84.6)
Unknown	4	1	0
PgR status			
<1%	49 (30.6)	30 (36.6)	75 (35.0)
≥1%	111 (69.4)	52 (63.4)	139 (65.0)
<20%	81 (50.6)	41 (50.0)	113 (52.8)
≥20%	79 (49.4)	41 (50.0)	101 (47.2)
Unknown	4	1	0
Ki67 status			
<20%	75 (47.8)	48 (62.3)	113 (53.6)
≥20%	82 (52.2)	29 (37.7)	98 (46.4)
Unknown	7	6	3
HER2 status			
Negative	100 (63.7)	71 (88.7)	152 (71.4)
Positive	57 (36.3)	9 (11.3)	61 (28.6)
Unknown	7	3	1
AR status			
<1%	28 (17.1)	22 (26.5)	46 (21.5)
≥1%	136 (82.9)	61 (73.5)	168 (78.5)
<10%	33 (20.1)	33 (39.8)	62 (29.0)
≥10%	131 (79.9)	50 (60.2)	152 (71.0)

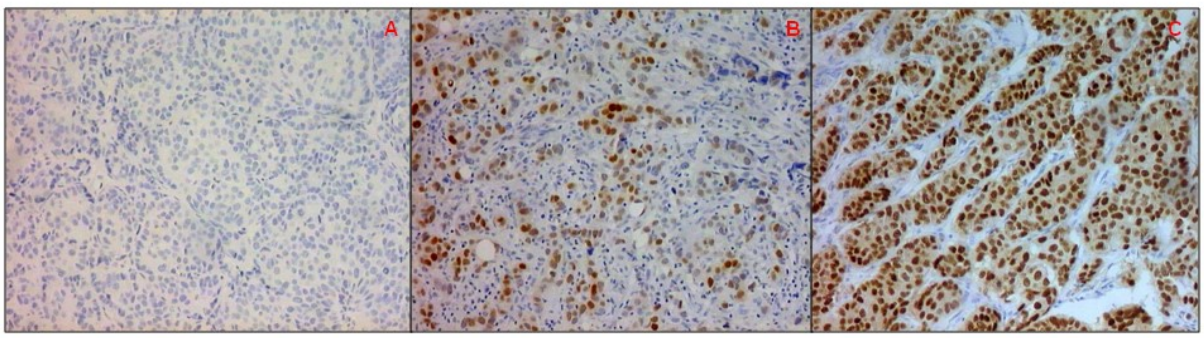

Figure 2.2 Ductal infiltrating carcinomas of the breast showing different AR nuclear expression (10X magnification): A) negative; B) moderate (2+) and heterogeneous positivity; C) strong (3+) and homogeneous positivity.

AR expression was higher in luminal A and luminal B tumors than in HER2-positive and triple negative tumors, both on primary and metastatic samples (Table 3).

Table 3. Distribution of AR expression in the different subtypes

	LA*	LB#	LB-HER2+**	TN^	HER2+ (HR-)@
Primary tumor n=154			**No. (%)**		
AR negative (<1%)	3 (9.4)	8 (14.5)	7 (17.9)	5 (50.0)	5 (27.8)
AR positive (≥1%)	29 (90.6)	47 (85.5)	32 (82.1)	5 (50.0)	13 (72.2)
AR negative (<10%)	4 (12.5)	8 (14.5)	9 (23.1)	6 (60.0)	7 (38.9)
AR positive (≥10%)	28 (87.5)	47 (85.5)	30 (76.9)	4 (40.0)	11 (61.1)
Metastasis n=79			**No. (%)**		
AR negative (<1%)	2 (12.5)	12 (25.5)	2 (25.0)	4 (57.1)	1 (100)
AR positive (≥1%)	14 (87.5)	35 (74.5)	6 (75.0)	3 (42.9)	0
AR negative (<10%)	4 (25.0)	17 (36.2)	4 (50.0)	6 (85.7)	1 (100)
AR positive (≥10%)	12 (75.0)	30 (63.8)	4 (50.0)	1 (14.3)	0

*LA, luminal A-like: ER+, PgR ≥20%, Ki67 <20%, HER2−
#LB, luminal B-like: ER+, PgR <20% or Ki67 ≥20%, HER2−
**LB-HER2+, luminal B-like HER2-positive: ER+, PgR <20% or Ki67 ≥20%, HER2+
^TN, triple-negative: ER−, PgR−, HER2−
@HER2+ (HR−), HER2-positive, hormone receptor-negative: ER−, PgR−, HER2+

The clinical features of the patients with both primary and metastatic tumor are shown in Table 4. The median age was 55, with a range of 33-76.

Table 4. Clinical features of patients with both primary tumor and metastatic samples analyzed

	Patients (n=33)
Adjuvant chemotherapy	**No. (%)**
No	10 (33.3)
Yes	20 (66.7)
Unknown	3
Adjuvant endocrine therapy	
No	8 (26.7)
Yes	22 (73.3)
Unknown	3
Histotype	
Ductal	23 (71.9)
Lobular	6 (18.7)
Other	3 (9.4)
Unknown	1
Tumor stage	
1	14 (48.3)
2	14 (48.3)
3	0
4	1 (3.4)
Unknown	4
Nodal involvement	
0	13 (44.8)
1	11 (37.9)
2	5 (17.3)
3	0
Unknown	4
Metastases at diagnosis	
Yes	3 (9.4)
No	29 (90.6)
Unknown	1
1st-line endocrine therapy for advanced BC	
Letrozole	8 (27.6)
Anastrozole	10 (34.5)
Exemestane	8 (27.6)
Tamoxifen	3 (10.3)
Fulvestrant	0
Unknown	4

The tumor biological characteristics of this case series are reported in Table 5.

Table 5. Tumour biological characteristics of primary and matched metastatic tumors

	Primary tumor (n=33)	**Metastasis (n=33)**
Grade	**No. (%)**	
1	1 (4.5)	0
2	8 (36.4)	5 (50.0)
3	13 (59.1)	5 (50.0)
Unknown	11	23
ER status		
<1%	5 (17.2)	4 (12.5)
≥1%	24 (82.8)	28 (87.5)
Unknown	4	1
PgR status		
<1%	7 (24.1)	18 (56.2)
≥1%	22 (75.9)	14 (43.8)
<20%	13 (44.8)	18 (58.1)
≥20%	16 (55.2)	13 (41.9)
Unknown	4	2
Ki67 status		
<20%	12 (41.4)	18 (58.1)
≥20%	17 (58.6)	13 (41.9)
Unknown	4	2
HER2 status		
Negative	23 (85.2)	28 (90.3)
Positive	4 (14.8)	3 (9.7)
Unknown	6	2
AR status		
<1%	4 (12.1)	11 (33.3)
≥1%	29 (87.9)	22 (66.7)
<10%	4 (12.1)	15 (45.5)
≥10%	29 (87.9)	18 (54.5)

The concordance in terms of AR expression in terms of positivity or negativity between primary tumors and matched metastasis was 66.7% (95% CI 50.6-82.8; p = 0.035) considering the cut off value of 1%. This concordance value dropped to 60.6% (95% CI 43.9-77.3; p = 0.002) by using the cut off value of 10% (Table 6).

Table 6. Concordance of AR evaluated on primary tumor and on metastasis

	Metastasis			
	Negative	**Positive**	**Total**	**McNemar test**
Primary tumor	**N. (%)**	**N. (%)**	**N. (%)**	***P***
AR negative (<1%)	2 (50.0)	2 (50.0)	4 (12.1)	**0.035**
AR positive (≥1%)	9 (31.0)	20 (69.0)	29 (87.9)	
AR negative (<10%)	3 (75.0)	1 (25.0)	4 (12.1)	**0.002**
AR positive (≥10%)	12 (41.4)	17 (58.6)	29 (87.9)	

The relation between the time (months; x-axis) elapsed from surgery of primary tumor to the biopsy of metastasis and the changes in AR expression of the two samples (absolute variation in AR positivity, y-axis) was analysed by univariable linear regression. No association between time and AR expression was observed (R-squared = 0.04 and adjusted R-square = 0.0091, p=0.264) (Figure 2.3).

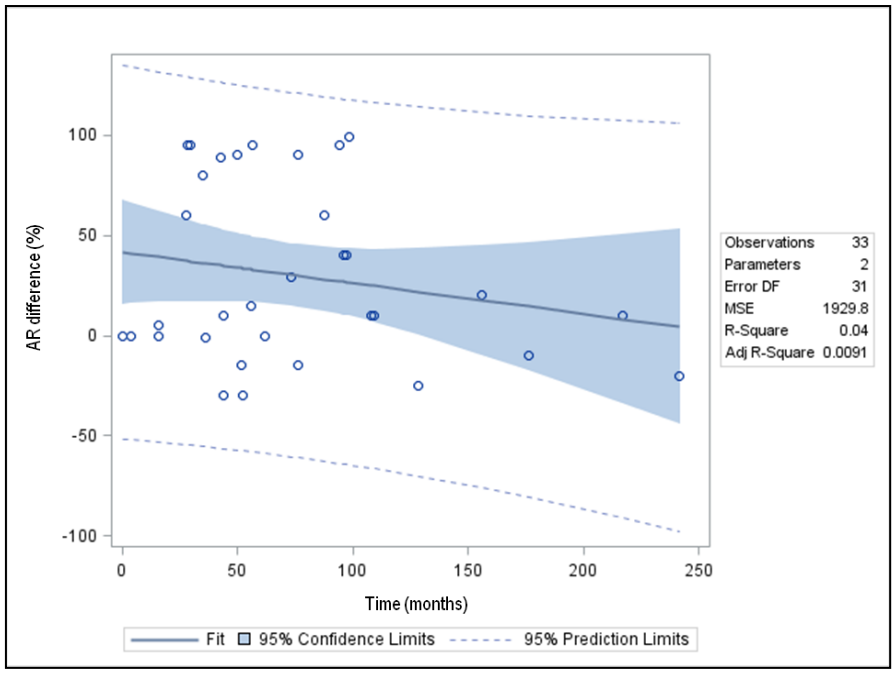

Figure 2.3 Univariable linear regression between the time elapsed from the primary tumor surgery to the metastatic biopsy (months; x-axis) and the changes in AR expression (absolute variation in the % of AR-positive cells between the two samples, y-axis).

2.2 Analysis of the ratio

We analyzed the ratio AR/ER and AR/PgR on 159 primary tumor samples of BC patients, of whom 125 were luminal tumors (defined as ER ≥1% and/or PgR ≥1% with any Ki67 or HER2 values). The ratio was calculated on 113 patients (with AR and ER both ≥ 1%). The images of one patient for whom both primary tumor and metastasis specimens analyzed by IHC for AR, ER and PgR are reported in Figure 1. The ratios AR/ER, AR/PgR, ER/PgR were calculated in each specimen (Figure 2.4). OS analysis was performed on 89/133 patients. The clinical features of 159 patients are reported in Table 1.

The median AR/ER ratio of the primary luminal tumors was 0.95 (range 0.06-95.00) while the median AR/PgR and ER/PgR ratios were 1.55 (range 0.06-95.00) and 1.60 (range 0.08-90.00) respectively (Table 2). The optimal cut off values for AR/ER, AR/PgR, ER/PgR ratios to stratify patients according to prognosis were 0.95, 1.54 and 2 respectively. These values were obtained from receiver operating characteristic (ROC) curve analysis at a median OS of 63 months. We evaluated the impact of the AR/ER, AR/PgR, ER/PgR ratios on OS. Hazard ratios (HRs) and 95% confidence intervals (95% CI) were calculated using the Cox regression model. Median OS was longer for patients with AR/ER values < 0.95 (p-value not significant) in primary tumors (Table 2). OS was significantly shorter when the AR/PgR ratio was ≥ 1.54 for primary tumors (HR = 2.27; 95% CI 1.30-3.97; p = 0.004) (Table 2). Similar results were obtained for ER/PgR ratio ≥ 2 in primary tumors where OS was significantly shorter (HR = 1.89; 95% CI 1.10-3.24; p = 0.021) (Table 2).

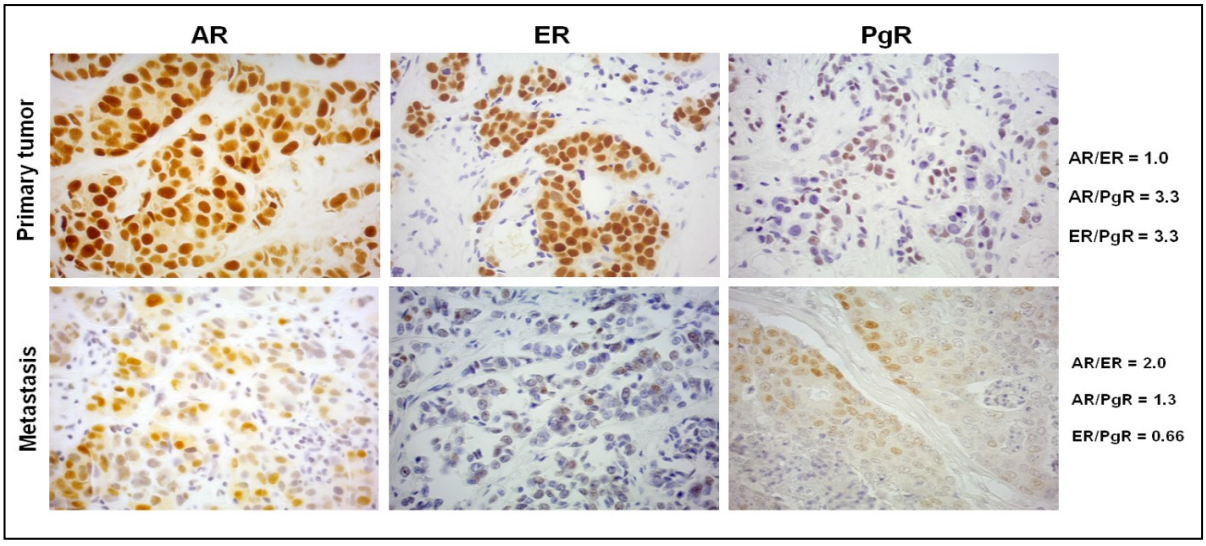

Figure 2.4. Biomarker detection in breast cancer tissue. AR, ER, PgR expression determined by IHC (10X magnification) in primary tumor and metastasis from the same patient, with AR/ER, AR/PgR, ER/PgR ratios determined.

Table 1. Patients' characteristics

Age	**No. (%)**
<50	40 (25.2)
≥50	119 (74.8)
Tumor samples	
Primary	159 (100)
Metastasis	24 (15.1)
Both	24 (15.1)
T at diagnosis	
1	73 (50.0)
2	56 (38.4)
3	5 (3.4)
4	12 (8.2)
Unknown	13
N at diagnosis	
0	59 (41.8)
1	52 (36.9)
2	14 (9.9)
3	16 (11.4)
Unknown	18
M at diagnosis	
0	122 (76.7)
1	37 (23.3)
Grade	
1	6 (4.8)
2	48 (38.1)
3	72 (57.1)
Unknown	33
Adjuvant systemic therapy	
No adjuvant therapy	12 (7.5)
Chemotherapy	84 (52.8)
Hormone therapy	84 (52.8)
Both	58 (36.5)
Unknown	37 (23.3)

Table 2. Impact of AR/ER, AR/PgR and ER/PgR ratios on OS in luminal primary tumors

	Median ratios (range)			
AR/ER	0.95 (0.06 - 95.00)			
AR/PgR	1.55 (0.06 - 95.00)			
ER/PgR	1.60 (0.08 - 90.00)			
	OS according to best cut off ratio Median follow-up: 78 months (range 7 - 155)			
	no. deaths / no. patients	**Median OS (months) (95% CI)**	**HR (95% CI)**	***P***
Overall	55/89	63 (46-76)	-	-
AR/ER				
<0.95	28/47	64 (41-82)	1.00	
≥0.95	27/42	60 (42-83)	1.05 (0.62-1.78)	0.861
AR/PgR				
<1.54	21/39	82 (65-89)	1.00	
≥1.54	34/50	42 (34-56)	2.27 (1.30-3.97)	**0.004**
ER/PgR				
<2.00	25/40	82 (62-88)	1.00	
≥2.00	30/49	42 (34-64)	1.89 (1.10-3.24)	**0.021**

A ratio of AR/ER ≥ 0.90 in the metastases (HR = 0.09; 95% CI 0.01-0.70; p = 0.022) was associated with a longer OS (Table 3). In addition, the difference in both ratios AR/ER and AR/PgR between primary tumor and metastasis was analyzed in relation to prognosis (Table 3). A high AR/ER in the primary tumor that remained high in the metastasis indicated a better prognosis in terms of OS (p = 0.011) (Table 3).

The AR/ER ratio showed a concordance of 45.83% between primary tumors and metastases (95% CI 25.90-65.76) when 0.90 was considered as cut off value, whereas a concordance of 41.67% (95% CI 21.95-61.39) was observed for the AR/PgR ratio by using 0.96 cut off.

Table 3. Impact of AR/ER and AR/PgR ratios on OS in patients with AR and ER detected both in primary tumor and metastasis (no. 24)

OS (HR) according to best cut off of the ratios				
		Primary tumor	**Metastasis**	
AR/ER (cut off 0.90)		0.33 (95% CI: 0.08 – 1.36) p = 0.127	0.09 (95% CI: 0.01 - 0.70) p = 0.022	
AR/PgR (cut off 0.96)		2.56 (95% CI: 0.56 – 11.72) *P* = 0.224	0.53 (95% CI: 0.18 – 1.59) *P* = 0.259	
Median OS (months) according to AR/ER difference between primary tumor and metastasis				
		Metastasis		
		<0.90	≥0.90	
Primary tumor	<0.90	32.4 (95% CI: 23.9 - NR)	36.4 (NR)	*P* = 0.011
	≥0.90	42.2 (95% CI: 14.5 - 87.7)	92.0 (95% CI: 89.4 - NR)	

HR, hazard ratio (AR/ER and AR/PgR ratios < cut off are the reference category);
CI, confidence interval; ND, not determinable; NR, not reached.

The ratios were calculated also in the entire series of 159 patients with any (positive or negative) values for ER, PgR and AR. In case of negative ER or PgR, the AR value was set as the ratio value. Data on follow up and survival were available for 104/159 patients. Median ratios of AR/ER, AR/PgR and ER/PgR were 0.95, 1.59 and 1.35 respectively (Table 4). OS was shorter when the ER/PgR ratio was ≥ 2 in primary tumors (HR= 1.90; 95% CI 1.14-3.17; $p = 0.014$). AR/ER and AR/PgR ratios did not predict OS (Table 4).

Table 4. Impact of the AR/ER, AR/PgR and ER/PgR ratios, assessed on primary tumors, on OS (no. 159)

		Median ratio (range)		
AR/ER		0.95 (0 - 95)		
AR/PgR		1.59 (0 - 100)		
ER/PgR		1.35 (0 - 100)		
		OS according to best cut off ratio		
		Median follow-up: 78 months (range 3-155)		
	no. deaths / no. patients	**Median OS (months) (95% CI)**	**HR (95% CI)**	***P***
Overall	63/104	62 (50-71)	-	-
AR/ER				
<1.30	53/88	63 (50 - 76)	1.00	
≥1.30	10/16	52 (23 - NR)	1.10 (0.56 - 2.16)	0.792
AR/PgR				
<1.70	31/51	66 (53 - 84)	1.00	
≥1.70	32/53	46 (36 - 65)	1.45 (0.88 - 2.39)	0.147
ER/PgR				
<2.00	27/49	81 (62 - 88)	1.00	
≥2.00	36/55	46 (36 - 60)	1.90 (1.14 - 3.17)	**0.014**

2.3 AR Role in predicting the endocrine therapy efficacy in advanced breast cancer

We evaluated the predictive role of AR in the avdanced BC setting, studyng 102 patient cohort. The median age was 60 years (range 33-85). Seventy-eight percent of them were diagnosed as ductal and 14% as lobular histotype. Metastatic disease at diagnosis was found in 26 out of 102 (25.5%). Ninety-two percent were treated with an aromatase inhibitor as first-line endocrine therapy, and letrozole was the most frequent aromatase inhibitor administered (45%) (Table 1). Biomarker's expression (ER, PgR, Ki67, HER2 and AR) was assessed in primary tumors in 70 cases and in metastases in 49, with 17 patients having both determinations (Figure 2.5) (Table 2).

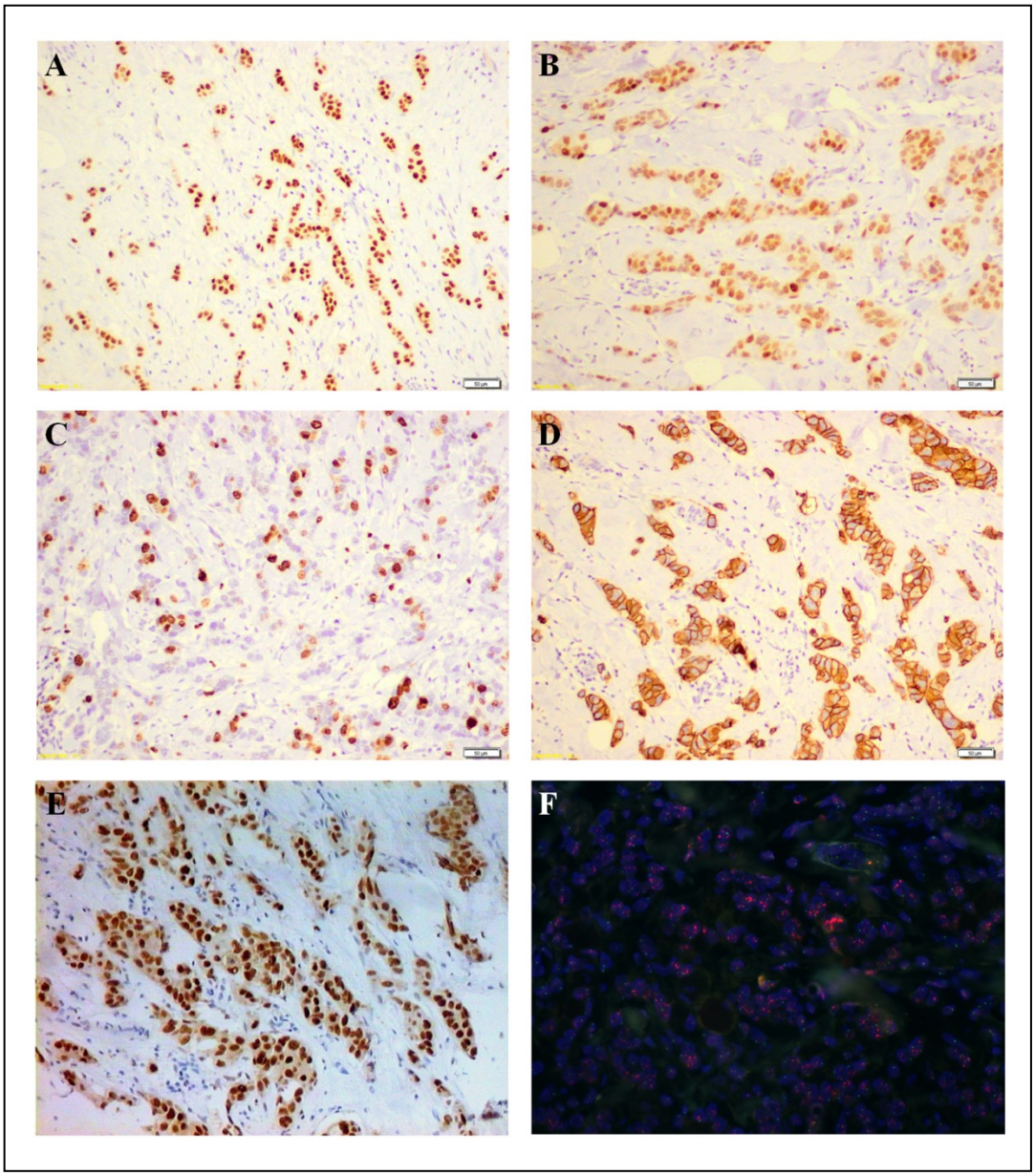

Figure 2.5 Biomarker detection in BC tissue (10X magnification): A) ER; B) PgR; C) Ki67, D) HER2 expression; E) AR; F) HER2 amplified case by FISH, 40X magnification).

Table 1. Patient's characteristics

Adjuvant chemotherapy	**No. (%)**
No	37 (48.7)
Yes	39 (51.3)
Unknown/Not available	26
Adjuvant endocrine therapy	
No	22 (28.9)
Yes	54 (71.1)
Unknown/Not available	26
Histotype	
Ductal	76 (78.4)
Lobular	14 (14.4)
Other	7 (7.2)
Unknown	5
Tumor stage	
1	30 (34.9)
2	42 (48.8)
3	2 (2.3)
4	12 (14.0)
Unknown	16
Grade of primary tumor	
1	2 (2.9)
2	34 (50.0)
3	32 (47.1)
Unknown	34
Metastases (at diagnosis)	
0	76 (74.5)
1	26 (25.5)
First-line endocrine therapy	
Letrozole	46 (45.1)
Anastrozole	22 (21.6)
Exemestane	26 (25.5)
Tamoxifen	5 (4.9)
Fulvestrant	3 (2.9)

Table 2. Biomarker determination

	Primary tumor (n = 70)	Metastases (n = 49)	Clinical practice* (n = 102)
ER status		**No. (%)**	
<1%	3 (4.3)	0	2 (2.0)
≥1%	65 (92.9)	49 (100)	100 (98.0)
Unknown	2 (2.8)	0	0
PgR status			
<1%	10 (14.3)	12 (24.5)	22 (21.6)
≥1%	58 (82.9)	37 (75.5)	80 (78.4)
Unknown	2 (2.8)	0	0
Ki67 status			
<20%	39 (55.7)	30 (61.2)	63 (61.8)
≥20%	27 (38.6)	17 (34.7)	38 (37.2)
Unknown	4 (5.7)	2 (4.1)	1 (1)
HER2 status			
Negative	57 (81.4)	41 (83.7)	88 (86.3)
Positive	10 (14.3)	7 (14.3)	14 (13.7)
Unknown	3 (4.3)	1 (2)	0
AR status			
<1%	5 (7.1)	12 (24.5)	17 (16.7)
≥1%	65 (92.9)	37 (75.5)	85 (83.3)
<10%	7 (10.0)	19 (38.8)	26 (25.5)
≥10%	63 (90.0)	30 (61.2)	76 (74.5)

*biomarker measured on metastatic sample when a metastatic biopsy was available, or on primary tumor when biopsy of metastasis had not been performed.

ER was negative (<1%) in 3 samples (4.3%) of primary tumors and in none of the metastatic samples. PgR was negative (<1%) in 10 (14.3%) primary tumors and in 12 (24.5%) metastases. Ki67 was low (<20%) in 59.1% of primary tumors and in 61.2% of metastases. HER2 status was positive in about 15% of cases both in primary tumors and metastases. In Figure 2.5F, a HER2 FISH amplified case has been reported.

AR status (Figure 2.5A) considered as per clinical practice (biomarker measured on a metastatic sample when a metastatic biopsy is available, or measured on primary tumor when biopsy of a metastasis has not been performed), was negative in 17 (16.7%) cases with cutoff <1% and 26 (25.5%) cases with cutoff <10%. The overall concordance rate between primary tumors and metastases was 64.7% (95% CI 42.0%-87.4%) for AR expression, based on cutoff of 1% (Figure 2.6). Furthermore, we observed a statistically significant association of AR status with a low Ki67, with median value of AR expression as per clinical practice of 80% in patients with Ki67 <20% versus 70% in patients with ki67 ≥20% ($p = 0.017$).

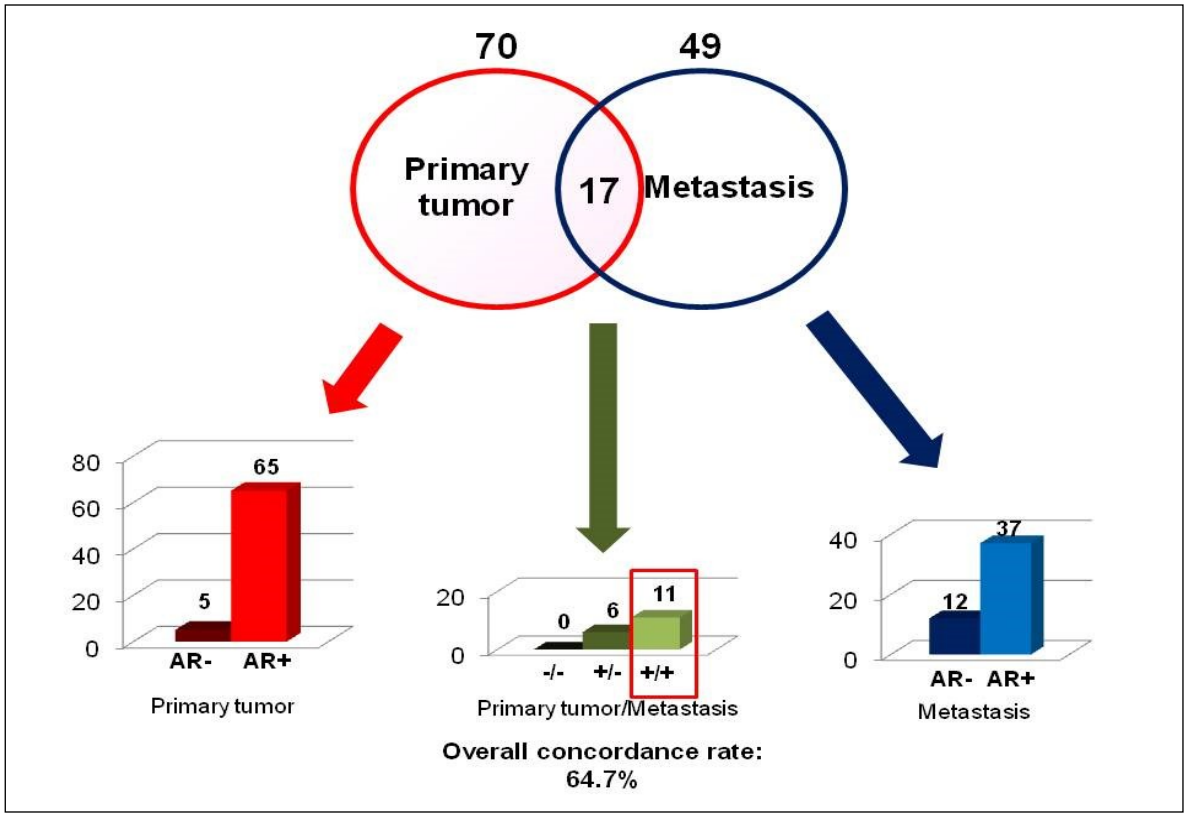

Figure 2.6 Distribution of AR expression in primary tumor and metastasis and concordance.

The predictive value of AR expression, alone or in relation to the other conventional biomarkers, to select the patients responsive to first-line endocrine therapy (ET) was the primary endpoint. The response to endocrine treatment was classified as best objective response (complete response (CR), partial response (PR), stable disease (SD), PD, according to RECIST criteria). The time-to-progression (TTP), meant as the time in months from the beginning of first-line ET until progression or last tumor response evaluation available.

AR status in primary tumors or metastases was not associated with PD (Table 3). Clinical benefit rate, defined as Complete Response (CR) or Partial Response (PR) or Stable Disease (SD), was 88.5% and Progression Disease (PD) 11.5% for AR-positive tumors defined by a cutoff ≥1%, versus 83.3% and 16.7%, respectively, for AR-negative tumors ($p = 0.62$); clinical benefit was 90.7% and PD 9.3% for AR-positive tumors defined by a cutoff ≥10%, versus 79% and 21%, respectively, for AR-negative tumors ($p = 0.18$). Conversely, Ki67 showed a significant association with PD, with 26.9% PD with high Ki67 *vs* 4.3% PD with low Ki67 ($p = 0.009$). PgR-negative status (<1%) showed just a trend in terms of association with PD ($p = 0.079$), while significant results ($p = 0.031$) were obtained by using ≤10% PgR as cut off value. Median TTP was 17 months (95% CI 14-21.5, median follow-up 75 months). Differences in TTP according to AR status were not statistically significant (Table 4). For AR expression ≥1% median TTP was 16.1 months (95% CI 13.0-19.0) *vs* 12 months (95% CI 4.3-48.1) for AR <1%

(p = 0.884); similarly for AR ≥10% median TTP was 16 months (95% CI 13.0-19.0) *vs* 13.8 months (95% CI 11.0-42.1) for AR <10% (p = 0.935). AR/PgR ≥0.96 was associated with a significantly shorter TTP (HR = 1.65, 95% CI 1.05-2.61, p = 0.028) (Figure 2.7). No association was found between AR/ER ratio and TTP. Conversely, a positive PgR status and a low Ki67 (but not HER2 status) were significantly associated with longer TTP (Table 4).

Table 3. Response to treatment in relation to biomarker expressions considered as per clinical practice

		CR or PR or SD	**PD**	
ER status	**N.**	**No. (%)**		***P***
<1%	1	0	1 (100)	
≥1%	72	64 (88.9)	8 (11.1)	0.123
PgR status				
<1%	15	11 (73.3)	4 (26.7)	
≥1%	58	53 (91.4)	5 (8.6)	0.079
Ki67 status				
<20%	46	44 (95.7)	2 (4.3)	
≥20%	26	19 (73.1)	7 (26.9)	**0.009**
HER2 status				
Negative	64	57 (89.1)	7 (10.9)	
Positive	9	7 (77.8)	2 (22.2)	0.306
AR status				
<1%	12	10 (83.3)	2 (16.7)	
≥1%	61	54 (88.5)	7 (11.5)	0.619
<10%	19	15 (79.0)	4 (21.0)	
≥10%	54	49 (90.7)	5 (9.3)	0.182
AR/ER ratio				
<0.90	38	31 (81.6)	7 (18.4)	
≥0.90	35	33 (94.3)	2 (5.7)	0.101
AR/PgR ratio				
<0.96	27	23 (85.2)	4 (14.8)	
≥0.96	46	41 (89.1)	5 (10.9)	0.623

Table 4. Time to progression (TTP) according to biomarker expressions considered as per clinical practice

	N.	HR (95% CI)	Median TTP, months (95% CI)	*P*
AR status				
<1%	17	1.00	12.0 (4.3-48.1)	
≥1%	85	0.96 (0.56-1.66)	16.1 (13.0-19.0)	0.884
<10%	26	1.00	13.8 (11.0-42.1)	
≥10%	76	0.98 (0.61-1.57)	16.0 (13.0-19.0)	0.935
AR/ER ratio				
<0.90	52	1.00	12.9 (11.0-17.1)	
≥0.90	50	0.83 (0.55-1.24)	18.0 (14.0-24.6)	0.362
AR/PgR ratio				
<0.96	34	1.00	16.8 (12.1-47.9)	
≥0.96	68	1.65 (1.05-2.61)	16.0 (12.4-19.0)	**0.028**
PgR status				
<1%	22	1.00	10.5 (4.0-17.0)	
≥1%	80	2.18 (1.34-3.55)	17.0 (14.0-24.7)	**0.001**
Ki67 status				
<20%	63	1.00	17.6 (14.8-22.1)	
≥20%	38	1.60 (1.05-2.45)	12.0 (8.2-16.1)	**0.028**
HER2 status				
negative	88	1.00	15.8 (12.9-19.0)	
positive	14	1.15 (0.65-2.02)	18.0 (7.0-46.0)	**0.929**

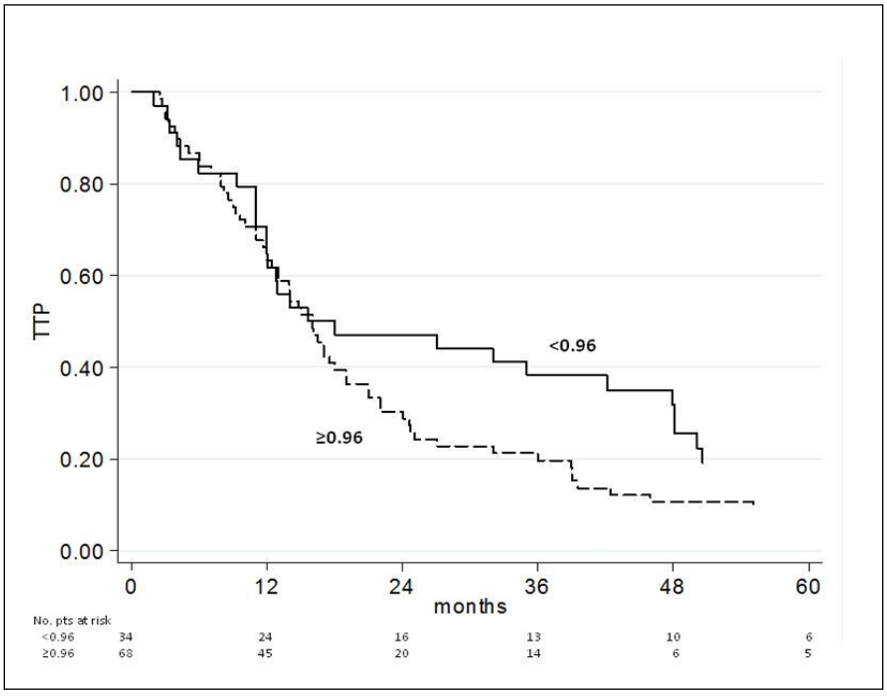

Figure 2.7 TTP as a function of AR/PgR ratio.

2.4 Comparison of AR expression between african and italian population

Androgen receptor expression in BC was compared between African (Tanzanian) and Caucasian (Italian) population, and even among the different tumor subtypes. Tanzanian and Italian BC patients were matched (ratio 1:2) for date and age at diagnosis and the biological characteristics were analyzed: 199 patients were included in the study: 69 patients from Tanzania (100% African ethnicity) and 130 from Italy (100% Caucasian ethnicity). Of 69 Tanzanian BC cases of the overall series only 65 had tumor tissue to assess AR status and were matched with 130 Italian BC patients for age and date of diagnosis. The clinical–pathological features of the two case series are reported in Table 1.

Table 1. Clinical and pathological features of the African and Caucasian population

	African population	**Caucasian population**	***P***
Age (years), median value (range)	51 (29-83)	53 (26-86)	**0.100**
Histological types	**No. (%)**	**No. (%)**	
Invasive ductal carcinoma	60 (92.3)	106 (81.5)	
Invasive lobular carcinoma	3 (4.6)	17 (13.1)	**0.085**
Others	2 (3.1)	7 (5.4)	
Histological grade			
I	3 (4.6)	4 (3.1)	
II	14 (21.5)	45 (34.6)	0.258
III	34 (52.3)	56 (43.1)	
Unknown	14 (21.5)	25 (19.2)	
Clinical stage			
I	4 (6.2)	30 (23.1)	
II	10 (15.4)	49 (37.7)	
III	12 (18.5)	17 (113.1)	**0.004**
IV	12 (18.5)	23 (17.7)	
Unknown	27 (41.5)	11 (8.4)	
Tumor subtypes			
LA	4 (6.1)	23 (19.0)	
LB	28 (43.1)	43 (35.6)	
LB-HER2E	20 (30.8)	34 (28.1)	0.658
TN	13 (20.0)	5 (4.1)	
HER2	-	16 (13.2)	
Unknown-missing	-	9	

The median age of patients at diagnosis was 51 (range 29–83) years for African patients and 53 (range 26–86) years for Caucasian patients. Tumors from patients of the two populations were associated with a higher histological grade (mainly grade 3) even if the differences were not statistically significant ($p = 0.258$). Moreover, the invasive ductal cancer represented the 92.3% of the African BC population and the 81.5% of the Caucasian one. Tanzanian patients presented more frequently disease at advanced stage (III-IV) than Italian patients ($p < 0.004$) (Table 1). Luminal A tumors were 4 (6.1%) and 23 (19%), while luminal B tumors were 28 (43.1%) and 43 (35.6%) in the African and Caucasian population, respectively (Table 1). LB-HER2-enriched tumors were 20 (30.8%) and 34 (28.1%) and TN tumors were 13 (20%) and 5 (4.1%) in the African and Caucasian population, respectively (Table 1).

The median AR expression (% of immunopositive tumor cells in the nucleus) in Tanzanian BC patients was 30 (range 0-100) and in Caucasian population was 80 (range 0–100) ($p < 0.0001$) (Figure 2.8). The median *H* score was 180 (range 10–300) in Tanzanian and 240 (0–300) in Caucasian patients ($p = 0.109$) (Table 2). AR staining intensity differed significantly between the two populations ($p = 0.0003$) (Table 2; Figure 2.8). Significant differences for AR expression between the two populations were observed with a great number of Tanzanian BC patients negative for AR expression considering both ≥ 1% and ≥ 10% as cut off values (Table 2).

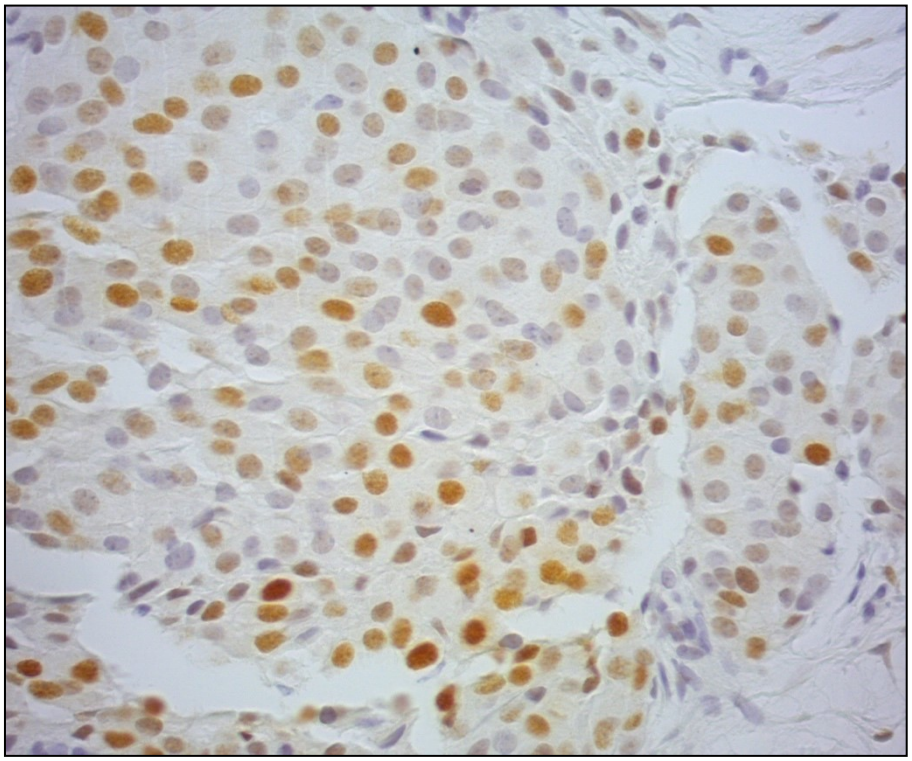

Figure 2.8 A Tanzanian ductal invasive carcinoma showing AR positivity in the nucleus of tumor cells, presenting different staining intensity (40X magnification). This case globally was evaluated as 2+.

Table 2. Median values of androgen receptor (AR) percentage (%), *H score*, and staining intensity in African and Caucasian population

	African population	**Caucasian population**	
	Median value (range)		***P***
AR %	30 (0-100)	80 (0-100)	**<0.0001**
H score	180 (10-300)	240 (0-300)	0.109
AR intensity	**No. (%)**		
0	22 (33.9)	22 (16.9)	**0.0003**
1+	4 (6.1)	0	
2+	13 (20.0)	20 (15.4)	
3+	26 (40.0)	88 (67.7)	
AR % cut off			
AR < 1%	22 (33.8)	22 (16.9)	
AR ≥ 1%	43 (66.2)	108 (83.1)	**0.008**
AR < 10%	25 (38.5)	27 (20.8)	
AR ≥ 10%	40 (61.5)	103 (79.2)	**0.009**

Androgen receptor positivity was more frequently observed in luminal A and B tumors than TN and HER2-enriched tumors in Tanzanian population (Table 4).

In addition, we evaluated the correlation between AR and Ki67 status in primary tumors. In the overall series of African and Caucasian tumors taken together, the *r*s is −0.24 ($p = 0.002$), for the former the *r*s is −0.21 ($p = 0.209$) and the latter showed an *r*s of −0.23 with a p-value of 0.010.

Table 4. Androgen receptor distribution in the different tumor subtypes of African and Caucasian populations

African population	**LA**	**LB**	**LB-HER2**	**TN**	**HER2**	
AR median value (range)	80 (10-100)	60 (0-100)	0 (0-90)	15 (0-90)	-	**<0.0001**
			No. (%)			***P***
AR < 1%	0	1 (3.6)	15 (75.0)	6 (46.1)	0	
AR ≥ 1%	4 (100)	27 (96.4)	**5 (25.0)**	7 (53.9)	0	**0.0001**
AR < 10%	0	3 (10.7)	16 (80.0)	6 (46.1)	0	
AR ≥ 10%	4 (100)	25 (89.3)	4 (20.0)	7 (53.9)	0	**0.0005**
Caucasian population						
AR median value (range)	90 (0-100)	90 (0-100)	70 (0-100)	5 (0-90)	30 (0-80)	**<0.0001**
AR <1%	3 (13.0)	6 (14.0)	6 (17.6)	2 (40.0)	5 (31.2)	
AR ≥ 1%	20 (87.0)	37 (86.0)	**28 (82.4)**	3 (60.0)	11 (68.8)	0.070
AR < 10%	4 (17.4)	6 (13.9)	7 (20.6)	3 (60.0)	7 (43.7)	
AR ≥ 10%	19 (82.6)	37 (86.1)	27 (79.4)	2 (40.0)	9 (56.3)	**0.010**

2.5 Analysis of AR expression data in Cancer Genome Atlas collection

We studied AR expression in different categories of breast invasive carcinoma, interrogating the online database PanCancer (version 2018) Atlas, included in the Tissue Cancer Genome Atlas (TCGA), analyzing RNA-sequencing data.[118] We found no significant difference between pre- and post-menopausal status, inferred as an age cut off of 50 years (Figure 2.9A). Therefore, we classified the samples in more age categories and we observed a slight increase ($p = 0.025$) of AR expression between 60 and 70 years (Figure 2.9B). In regard to cancer type, we observed a lower expression in the less differentiated cancers, the medullary and the metaplastic carcinoma ($p < 0.0001$) (Figure 2.9C).

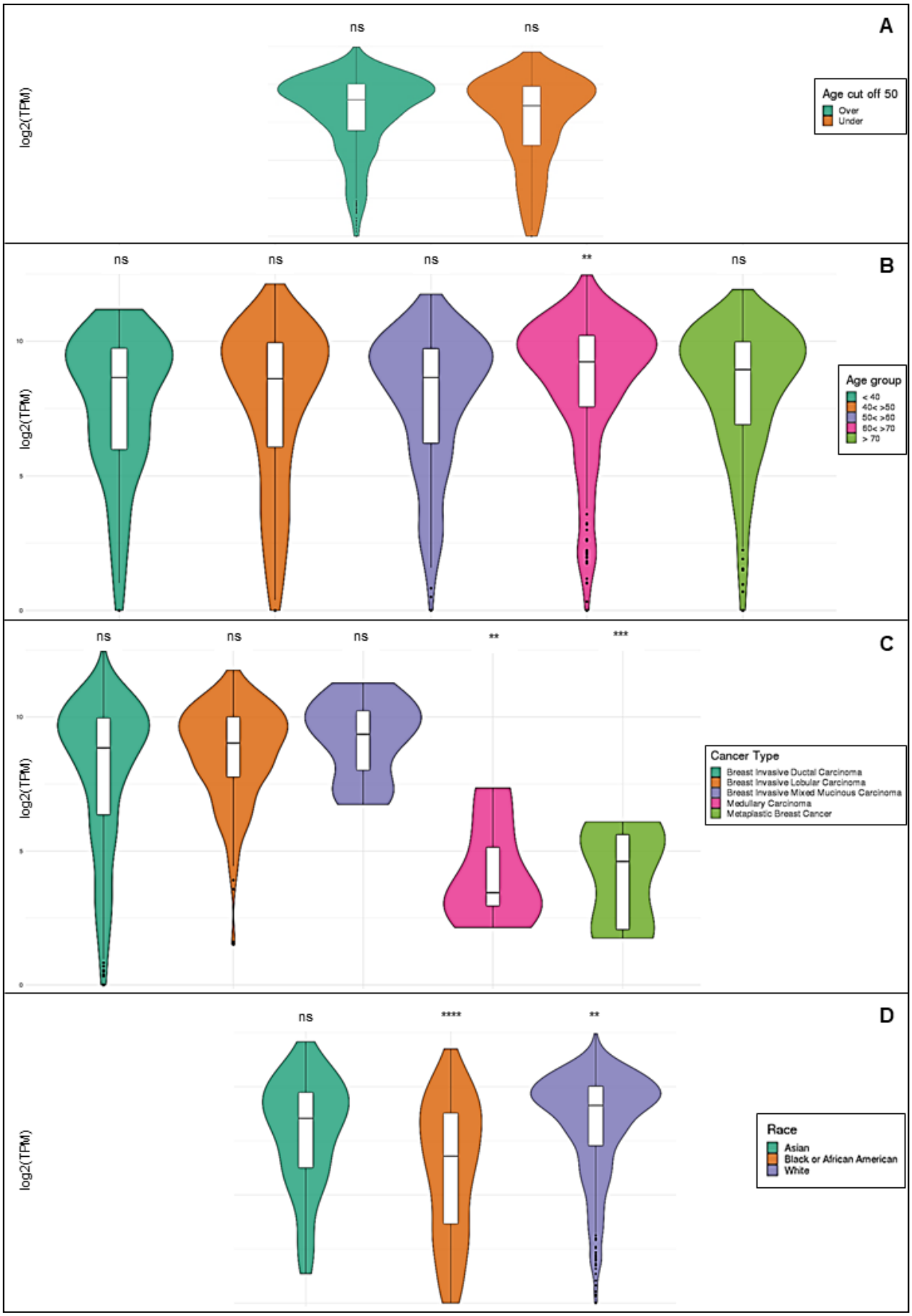

Figure 2.9 AR expression defined as Transcripts Per Million (TPM) in different categories of breast invasive carcinoma samples, included in the PanCancer Atlas: A) and B) age of BC diagnosis; C) cancer types; population type.
ns: not significant

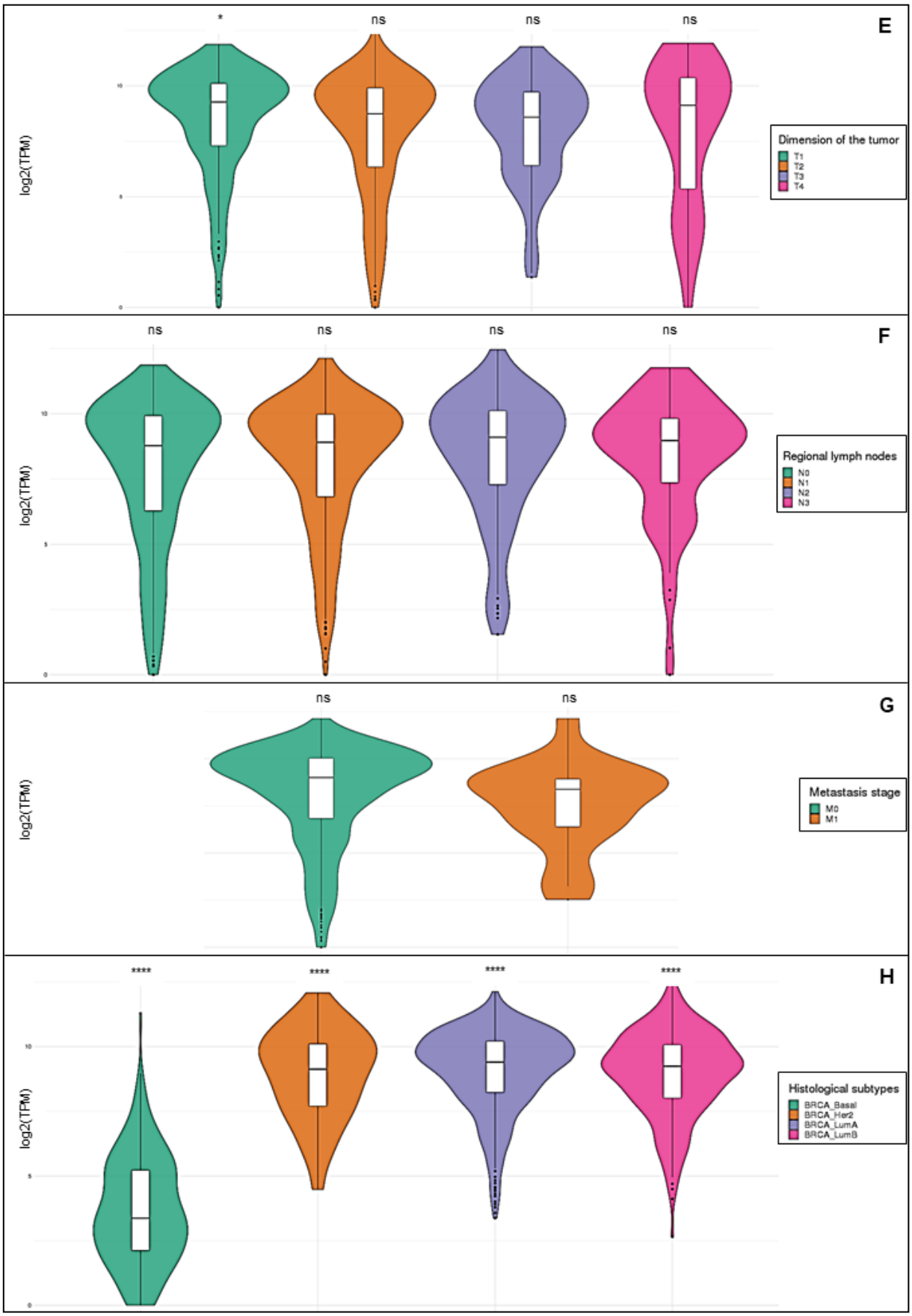

Figure 2.9 AR expression in different categories of E) tumor size F) lymph node involvement G) metastasis stage H) histological subtype.
ns: not significant

In addition, AR was significantly overexpressed in cancers of Caucasian population and downregulated in the African-American BC patients ($p < 0.0001$) (Figure 2.9C). AR did not seem to differ between BC stages (TNM) (Figure 2.9F and 2.9G). Only a slight higher expression level was observed in the smallest tumors (T1) ($p = 0.015$) (Figure 2.9E). The most important and significant differences were found between BC subtype, whereas Luminal A and B tumors showed the highest expression levels, HER2-positive BC had a lower expression than luminal cancers, and the basal ones showed the lowest levels in terms of AR transcripts ($p < 0.0001$) (Figure 2.9H).

3 AR AS THERAPEUTIC TARGET

3.5 Androgen Receptor as Target for Therapy of pre-treated post-menopausal patients with AR-positive metastatic breast cancer: ARTT trial

This multicenter, single-arm, two-stage phase II study evaluated the safety and activity of the androgen precursor DHEA, sodministered 100 mg/day orally continuously, in combination with an AI (anastrozole 1 mg/day, letrozole 2.5 mg/day, or exemestane 25 mg/day) to prevent its transformation into estrogens, in two cohorts of patients with AR-positive metastatic BC. One cohort was composed by patients with ER-positive/HER2-negative and one by patients with triple-negative disease. Patients were postmenopausal and, when ER-positive, had documented resistance to both nonsteroidal and steroidal AIs. The primary endpoints were safety and activity (clinical benefit rate: proportion of patients with stable disease or objective response after 16 weeks).

The DHEA dosage was chosen based on the reported saturation of the enzymatic systems that transform DHEA into sex steroids, occurring at serum levels of about 7 ng/mL, and to the reported serum DHEA levels of about 7 ng/mL achieved after oral administration of DHEA 100 mg daily for 6 months. DHEA was produced by the oncology pharmacy laboratory of IRST, whereas AIs were purchased commercially.

Serum levels of DHEA and its glucuronidated metabolites were measured by liquid chromatography-tandem mass spectrometry. The expression of AR and its main phosphorylated forms (Serine650 and Serine10-213) was assessed by immunohistochemistry and AR gene amplification by Fluorescence *In Situ* Hybridization (FISH).

From November 2013 to July 2015, 12 patients were enrolled in the ER-positive and 6 in the triple-negative cohort; the last closed early, due to emerging preclinical evidence of tumor stimulation by androgens. Patients' characteristics are reported in Table 1. In the ER-positive cohort, the median age was 74 years, Eastern Cooperative Oncology Group (ECOG) performance status 0–2; nine patients had visceral metastases, five underwent 1–2 lines of chemotherapy and all with 1–4 lines of endocrine therapy for advanced disease. The median duration of treatment was 71 days (range 55–697).

All patients in the ER-positive cohort had developed resistance to both nonsteroidal and steroidal AIs. Seven patients had received an AI as their last line of treatment before entering the trial and, after progressing on the AI, had continued the same AI but with the addition of DHEA. Conversely, five patients received DHEA in combination with an AI to which they had

developed resistance in the past, but which was not the last line of therapy they received before entering this trial.

Table 1. Clinical and pathological features of patients

Variable	Cohort 1 (*n* = 12), *n* (%)	Cohort 2 (*n* = 6), *n* (%)
Median age, years (range)	74 (58–90)	76 (50–86)
Performance status (ECOG)		
0	9 (75)	5 (83)
1	2 (17)	1 (17)
2	1 (8)	—
Hormone receptors[a]		
Androgen-positive	12	6
Estrogen-positive	12	—
Estrogen-negative		6
Progesterone-positive	9 (75)	—
Progesterone-negative	3 (25)	6
Negative HER2 status[a]	12	6
Number of metastatic sites		
1	2 (16.67)	1 (17)
2	2 (16.67)	2 (33)
3	6 (50.00)	2 (33)
4	2 (16.67)	1 (17)
Sites of metastases		
Soft tissues (only)	2 (17)	0
Bone (± soft tissue)	1 (8)	1 (17)
Viscera (± other)	9 (75)	5 (83)
Previous lines of hormone therapy for MBC		
1	1 (8)	—
2	6 (50)	—
3	3 (25)	—
4	2 (17)	—
Previous lines of chemotherapy for MBC		
0	7 (58)	1 (17)
1	3 (25)	1 (17)
2	2 (17)	2 (33)
3	—	2 (33)
Chosen aromatase inhibitor		
Exemestane	6 (50)	4 (67)
Anastrozole	4 (33)	2 (33)
Letrozole	2 (17)	—
Last line of therapy before enrollment into this clinical trial		
Same AI, continued within this study	7 (58)	
Other treatment	5 (42)	

Seven patients showed progressive disease (PD) at 8 weeks, four had stable disease (SD) at 8 weeks and PD at 16 weeks, and one had SD lasting >16 weeks (692 days). Median time to progression (TTP) was 63 days (95% CI 57–126) and median overall survival (OS) 559 days (95% CI 134–not reached; Figure. 3.1). Two patients died within 30 days of the end of therapy, one after 8 days and one after 21 days, all due to tumor progression. No virilizing effects were registered. The study closed after the first stage for poor activity. All patients in the triple-negative cohort had PD.

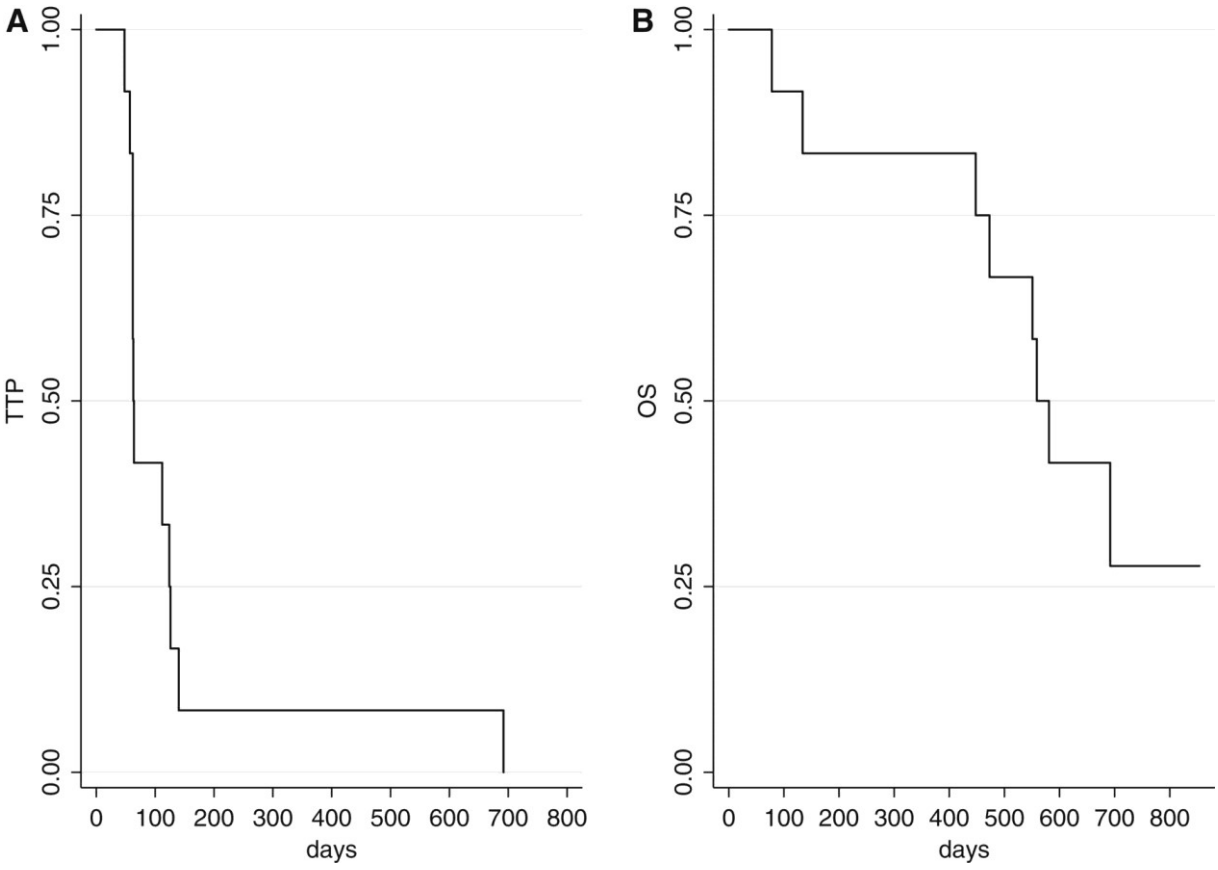

Figure 3.1 (A) Time to progression and (B) overall survival of the estrogen receptor-positive cohort.

The four serious adverse events reported were not attributed to DHEA. DHEA-related adverse events, reported in four patients, included grade 2 fatigue, erythema, and transaminitis, and grade 1 drowsiness and musculoskeletal pain. Toxicities related to DHEA were (worst grades) G2 fatigue, facial erythema, and increase in transaminases (the last required temporary treatment interruption) and G1 sleepiness and joint/muscular pain. Other toxicities, attributable to AIs or the underlying disease included four serious adverse events: uncontrolled pain, trauma, seizure, and constipation, and all but the last were not treatment related.

Although well tolerated, only one out of 12 ER+ MBC patients obtained a clinical benefit with prolonged SD for almost 99 weeks. She had previously received letrozole for 4 years for a regional relapse and then tamoxifen for 8 months upon progression. Following further progression, she was enrolled in the trial and received letrozole + DHEA. The three patients whose tumors showed lower AR expression levels (<50% of positive cells and H-score < 100) had disease progression (PD) after 8 weeks, whereas five of the seven patients with higher AR expression showed SD at this time. AR phosphorylation and AR gene copy number were available for 10 patients (Table 2).

Table 2. Androgen receptor expression, phosphorylation, and gene amplification in 10 patients

		AR (nuclear)			AR	AR p650					AR p210-213					Response
Patient	Site	%	Int	H	FISH	%	N	C	Int	H	%	N	C	Int	H	at 8 weeks
1	M (chest wall skin)	70	3	210	+	45	+	–	2-3	135	10	+	–	1	10	SD
2	P	90	3	270	–	90	–	+	3	270	0					PD
3	P	90	3	270	–	80	+	+	3	240	35	+	+	1	35	SD
4	P	30	2	60	–	95	–	+	3	285	0					PD
5	P	90	3	270	–	50	–	+	2-3	150	30	+	–	1	30	PD
6	M (chest wall skin)	95	3	285	–	100	–	+	3	300	0					SD
7	P (relapse)	85	3	255	–	90	–	+	3	270	0					SD
8	M (mediastinum)	80	3	240	–	90	–	+	3	270	0					SD
9	P	25	2	50	–	70	+	–	1	70	0					PD
10	P	30	3	90	–	30	+	+	2	60	0					PD

Abbreviations: %, percentage of stained cells; AR, androgen receptor; AR FISH, AR gene amplification by fluorescence in situ hybridization; C, cytoplasm; H, H-score (= % * Int); Int, staining intensity; M, metastasis; N, nuclear; P, primary tumor; p650, phosphorylation at serine 650; p210-213, phosphorylation at serine 210-213; PD, progressive disease; SD, stable disease.

Remarkably, the patient with clinical benefit was the only one whose tumor harbored an AR gene amplification, with AR gene clusters observed in 20% of tumor cells (Figure 3.2). All tumor samples showed AR phosphorylation at serine 650 (p650) in variable amounts and at different locations (cytoplasm or nucleus). The two patients with lower p650 H-scores (<100) had PD at 8 weeks, whereas of the eight patients with intermediate/high H-scores, five had SD and three had disease progression at 8 weeks. The patient who experienced prolonged SD had a nuclear expression of p650, whereas in most cases p650 was found in the cytoplasm. AR phosphorylation at serine 210-213 was present, mainly in the nucleus, in only three patients, one of whom was the patient with prolonged SD (Figure 3.2).

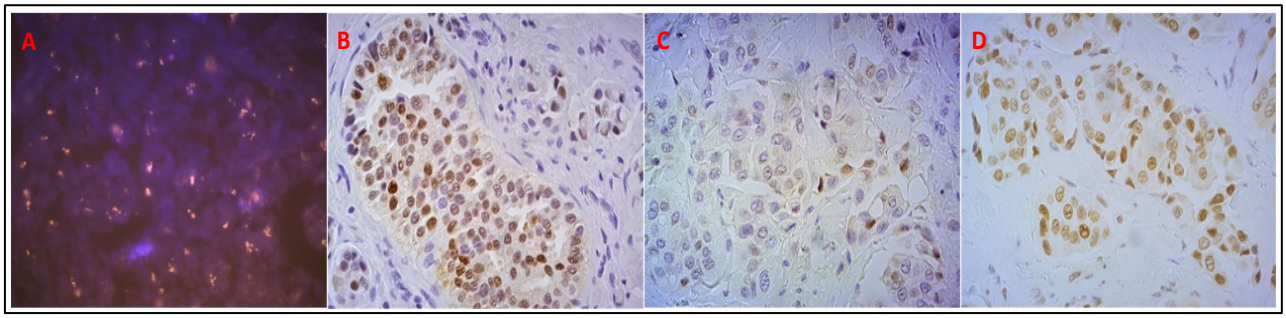

Figure 3.2 Analysis of AR status in the patient who had SD with DHEA: A) AR copy number, evaluated by FISH, showing clusters of orange signals; B) AR positive nuclear expression by IHC; C) AR pSer210-213 positive but weak nuclear and cytoplasmic expression; D) AR pSer650 positive nuclear expression.

Serum levels of DHEA and its glucuronidated metabolites androstane-3alpha,17beta-diol-3-glucuronide (3α-diol-3G), androstane-3alpha,17beta-diol-17glucuronide (3α-diol-17G), and androsterone glucuronide (ADT-G) were measured at baseline, at 8 weeks, and at the end of treatment in 10 patients. DHEA was assessable at all three time-points in four patients, 3α-diol-3G in two patients, 3α-diol-17G in seven patients, and ADT-G in eight patients. There was wide intra- and interpatient variation in DHEA serum levels (Figure 3.3), but no significant changes over time were observed, probably because of the small number of patients with all measurements p = 0.333). Only one patient had DHEA values constantly above the target threshold of 7 ng/mL and progressed after 8 weeks. The patient with prolonged disease stabilization had a median DHEA serum level of 4.01 ng/mL. Among the glucuronidated metabolites, median serum levels of 17α-diol-17G and ADT-G showed significant changes over time (p = 0.020 and p = 0.007, respectively, Friedman test). No clear pattern of metabolite levels emerged in relation to response to treatment at 8 weeks.

The combination DHEA-AI was well tolerated but poorly active in ER-positive metastatic BC. Serum levels of DHEA and its metabolites showed high inter- and intra-patient variability. Although dose and patient selection could be further studied, variability in serum levels and in tumor intracrinology (the intracellular formation of sex steroids from DHEA) may hamper further DHEA development in BC.

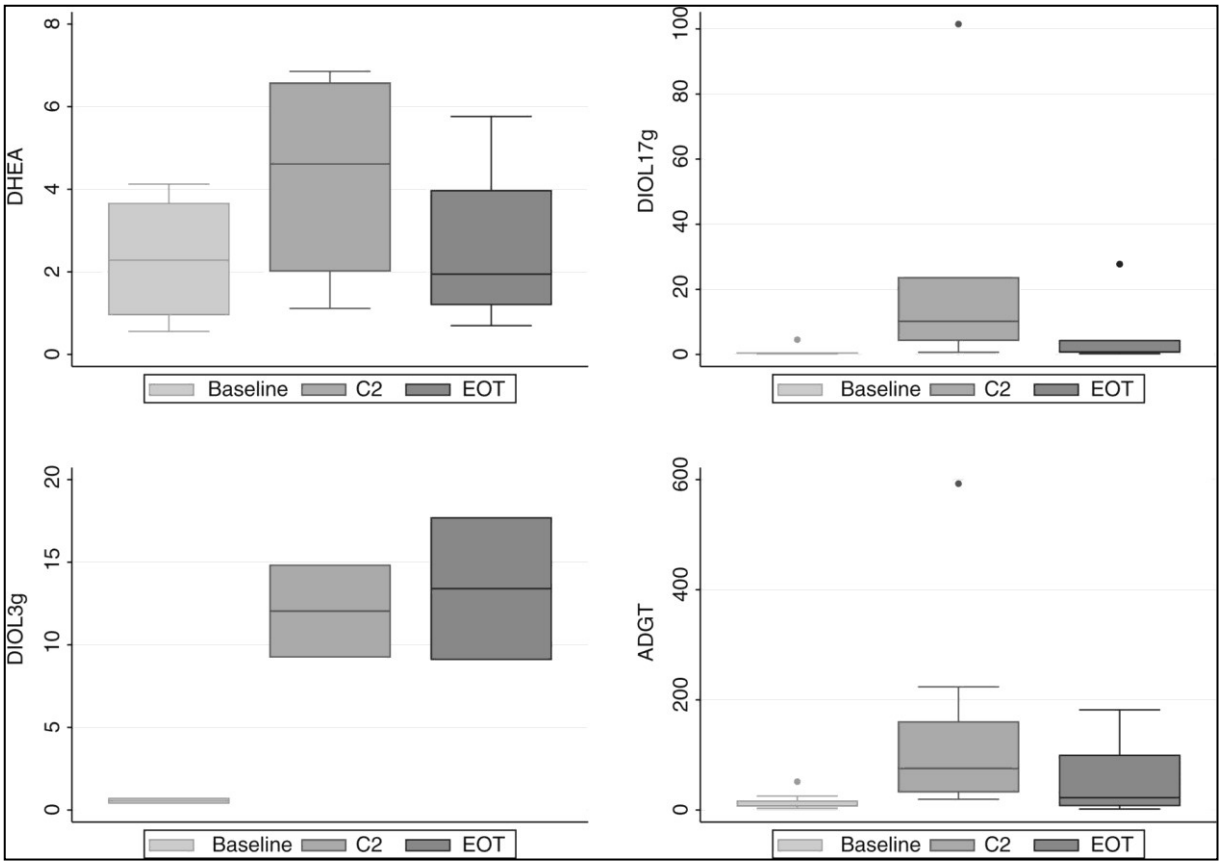

Figure 3.3 Boxplots of serum concentrations of DHEA and metabolites. Box and whisker plots, showing the median, interquartile range, and the highest and lowest values for each analyte at three time points (baseline, cycle 2 day 1, and end of treatment.

DISCUSSION

1. AR IN DUCTAL CARCINOMA *IN SITU* OF THE BREAST

AR is expressed in normal breast tissue, and it seems that its expression decreases starting from a condition where the proliferation is within the duct (DCIS) to the invasive cancer growth. AR has recently been shown to play an oncogenic or oncosuppressive role in cancer. Despite some studies in invasive BC have reported that AR expression is related to better survival when it is co-expressed with ER and PgR, its prognostic role in *in situ* BC has been never investigated. Early data from clinical trials evaluating AR antagonists in invasive/metastatic triple-negative BC suggest that some patients may benefit from androgen blockade, but data on the role of AR as therapeutic target in DCIS has to be explored yet. Oshilaja and colleagues recently reported the usefulness of IHC testing and potential clinical trials of AR antagonists for chemoprevention in patients with AR-positive and ER-negative DCIS.[119] AR and ER play an important role in discriminating tumors which will relapse or not and can give important information in planning therapy. As in clinical practice DCIS patients are treated almost exclusively with surgery and radiotherapy, the predictive role of specific markers, especially AR, on the clinical outcome in this population was investigated. We retrospectively analyzed series of matched DCIS relapsed and non-relapsed patients, treated with quadrantectomy alone and/or quadrantectomy plus radiotherapy. AR and AR/ER in DCIS patients showed to have an unfavorable prognostic role independently of the treatment. Seventy-eight (91.7%) samples were AR-positive and expression was higher in relapsed than in non-relapsed patients ($p = 0.0069$). AR expression was seen in all grades of DCIS, but the majority of the AR-positive cases were high grade, and the most common histological subtype in this subset was a solid growth pattern with apocrine features. Of the 78 AR positive cases, 21 (27%) were ER negative. ER assumed greater importance when considered together with AR. We observed that the AR/ER ratio was statistically higher in relapsed patients of both case series, independently of the treatment. Moreover, while the single variables showed an AUC values from 52% to 77%, the ratio of AR/ER reached very high AUC values of 92% and 80%, in the case series of patients treated with surgery and surgery plus radiotherapy, respectively. The hormonal variables together with AR, could be important prognostic tools able to increase the accuracy in terms of relapse prediction in this setting.

2. AR IN INVASIVE BC

Luminal BC have been reported to be positive for AR expression with higher levels in Luminal A than Luminal B tumors, more often than HER2 enriched and Triple Negative BC

(TNBC).[63,115] These findings are controversial because some of them described a role of AR status in predicting response rate and overall survival (OS) under hormonal treatment and at the same time, they reported no association between AR expression and disease free survival in ER-positive tumors. In the same works, ER status maintained the principal role as independent prognostic marker for disease free survival (DFS).[114,120] However, for Cochrane and colleagues it seems to be an independent prognostic marker if hormone receptor are expressed while for Vera-Badillo and colleagues its prognostic role seems to be independent from the expression of the hormonal receptors.[73,85] Kraby and colleagues demonstrated that AR was an independent predictor of good prognosis in BC, particularly in grade 3 and Luminal A tumors.[121]
Collins and colleagues reported that AR is most commonly expressed in luminal A and B invasive BC and it is present in approximately one-third of basal-like cancers.[84] Our results are in agreement with these findings because in our case series AR is more frequently expressed in luminal than the other subtypes, both in primary tumors and metastases. Nonetheless, the low number of HER2-positive and triple negative BC in our study precludes firm conclusions about the distribution of AR expression in different molecular subtypes. In this study we classified tumors according the conventional immunohistochemistry panel (Hormone receptors, HER2 and Ki67 expression) despite the increasing use of gene expression profiles, such as Oncotype Dx, PAM 50. In fact, recent work has showed that molecular assays do not furnish additional prognostic value over tumor morphology and immunohistochemistry.[122,123]

2.1 AR concordance between primary tumors and metastases

Only few studies have been performed with the attempt to evaluate AR expression in primary tumor and metastasis.[124,125] As reported by Kraby et al. discordant AR expression data between primary tumor and lymph node metastases were observed in 21.4% of cases and most often there was a switch from AR-negative primary tumor to AR-positive axillary lymph node metastases.[121]

We have highlighted an overall concordance of AR detection between primary tumor and metastasis greater than 64%, using two cut off values (1% and 10%). This implies that a clinician who need the AR value to give anti AR therapy should have the data on both the tumor materials available given that AR status in primary tumor could be different respect to that of metastasis. Due to the retrospective nature and potential selection bias of our study, we did not evaluate the prognostic or predictive role of AR expression in these two types of specimens. Some authors observed that hormone receptor status (ER and PgR) may change several times

over the course of the disease. These changes could be associated with prognostic worsening. Hence, they suggest to repeat the hormone receptor determination in metastatic BC patients.[126] For this reason we assessed the association between the time-interval from primary tumor removal to biopsy of the metastatic site, and the change in AR expression between the two samples. We found that the variation in the sampling time of the two types of specimens does not explain the difference of AR expression between primary and metastatic lesions, because R-squared value of a linear regression of time to AR change is close to 0. This finding might reflect the high spatiotemporal variability of AR expression, with intratumor spatial heterogeneity exceeding temporal heterogeneity. Although our results must be interpreted cautiously, due to the low number of paired primary tumor and metastasis samples analyzed in our study, they suggest that the evaluation of AR by IHC, in order to plan a potential anti-AR therapeutic approach, should be performed in all the biological material available for each patient regardless of the time between sampling.

2.2 AR expression in the different ethnicities

Information on BC biomarkers is poor in the majority of low resource countries, such as Sub-Saharan Africa. It is worthy of note that health infrastructures are insufficient in most parts of Sub-Saharan Africa.[127–129] A different biology in terms of biomarker expression was previously seen in between Caucasian and Tanzanian BC patients.[127] HER2-positive, ER, and PgR negative, highly proliferating tumors were more frequently observed often in Tanzanian women than in Caucasian patients.[127] These highly aggressive biological patterns, with very advanced stage at diagnosis, could be considered the principal reasons for the high BC mortality rate in African population. Then, the search for new biomarkers that could be used in the clinical practice is still an open issue and even more the identification of biomarkers to optimize the treatment choice.

Even if we know that our results are preliminary due to low number of cases analyzed, in our knowledge this is the first study that compares the pathological and the biological features and AR expression in invasive BC in African (Tanzanian) and Caucasian (Italian) case series. We demonstrated that AR expression in Tanzanian BC patients was lower than the Caucasian population in terms of percentage, *H* score, and staining intensity. These findings were in agreement with Thike and colleagues, showing that the lower AR expression reflects the higher aggressiveness of tumors, but their study was performed in an Asiatic case series.[130] The lower AR expression in African than Caucasian patients might be a consequence of a major tumor aggressiveness (low hormonal receptor expression and highly proliferating tumors) and

probably of a different carcinogenesis. In Caucasian patients, AR was seen to be more expressed in luminal tumors than TN tumors, and its presence seems to be related to a better prognosis in ER-positive tumors.[120,131–133] The AR expression for Tanzanian patients had the same trends to that observed in Caucasian population among the different tumor subtypes.

Davis and colleagues demonstrated in African American women that AR-negative triple negative or "quadruple negative" tumors have an enriched basal and immune signature, suggesting that AR could be used as prognostic marker in this specific BC subtype.[134] Another study evaluated AR expression in Ghanaian BC patients. They found a lower percentage (24%) of AR-positive tumors (defined by ≥10% cutoff) among TN BCs.[135] AR expression was evaluated among internationally diverse patient populations by Jiagge and colleagues.[136] AR expression was higher in White American patients and decrease in African American, Ethiopian and Ghanaian patients albeit the difference was not statistically significant. In a clinicopathological study from Jordan on AR expression in invasive ductal BC, a significant relationship between AR and ER was found. AR expression was significantly associated with smaller tumor size. Although AR status was not independently associated with survival, their data suggest that AR is a good prognostic factor.[137] This area needs further investigation as the data on the differences of gene expression profiles in BC patients of various ethnicities are still controversial.[138,139]

In the Tanzanian clinical practice, all patients underwent adjuvant hormonal therapy without testing the receptors. It means that only the fraction of ER-positive patients would benefit from this type of treatment. On the other hand, the majority of the patients (about 70%) were exposed to hormonal therapy unnecessarily, with subsequent side effects and additional costs in a low-income country. In addition, the use of expensive drugs, such as monoclonal antibodies, is prohibitive and the availability of cheaper drugs, such as anti-AR compounds, could open new therapeutic options in this low economic income population. Given the high proportion of AR-positive TNBC, AR could represent a valid therapeutic target, reducing recurrence and mortality rates, and costs.[127,128]

Our findings in this population highlighted the importance to introduce in Tanzanian routine testing for these markers before initiation of hormonal therapy and also to consider an anti-AR therapeutic approach. Further analyses are ongoing to evaluate the role of other biomarkers in Tanzanian BCs. In addition, in order to improve the efficacy of the treatment, the evaluation of combined therapeutic approaches, such as anti-AR with PARP, mTOR, HER2 and immune checkpoint inhibitors, have to be better explored.

3. THE IMPORTANCE OF THE RATIO

We highlighted the unfavorable prognostic role of the AR/ER ratio in different subset of patients with ductal carcinoma *in situ* of the breast, independently of treatment (*i.e.* surgery alone or surgery plus radiotherapy). Similarly, Rangel et al. and Cochrane et al. reported a poorer prognosis when the AR/ER ratio was higher in the primary tumor of early BC patients.[73,140] Then, considering that these data on the role of AR/ER ratio as unfavorable prognostic marker have been reported only in primary tumor of early BC patients, we performed a study in a different BC population, who presented disease relapse. Furthermore, different cut off values have been used for the ratios and the reason could be the different subset of patients analyzed. In our study, we found in the luminal case series that the AR/ER ratio in primary tumor is not associated with prognosis and a significantly worse prognosis was observed when AR/PgR and ER/PgR were high. In both luminal and overall series, the HRs went in the same direction for all the three ratios, even if the statistical differences obtained were not the same. AR/PgR ratio was statistically different for the luminal case series, whereas ER/PgR ratios for both. PgR is an independent prognostic biomarker as previously demonstrated,[141] and for this reason it may have a stronger prognostic impact than AR and ER in the ratios. The finding of a risk of relapse 10-fold lower for patients with higher AR/ER values on metastases must be taken with caution, because it refers to a subgroup of patients whose AR/ER ratio on primary tumor differs from that of the entire case series, for the small sample size and the large confidence intervals. Patients who presented a high AR/ER ratio both in primary tumor and metastasis had a better prognosis. Although our study was based on a small case series, it had the advantage of being able to compare primary tumor and metastatic samples from the same patients, which is fairly unusual in this setting. In conclusion, our findings indicate that a prospective study is needed to better clarify the role of AR/ER ratio in different BC settings (*i.e.* adjuvant and metastatic). The relation between AR and PgR has to be better understood even if a high AR/PgR ratio in luminal tumors could be prognostically unfavorable and used as an additional risk-stratification marker.

4. THE PREDICTIVE ROLE OF AR

4.1 AR is not useful to predict the efficacy of endocrine therapy

AR seems to have different functions depending on BC subtypes (e.g., luminal or TN). It seems that in ER negative BCs AR expression does not have a clear prognostic effect,[85] but it can

predict response to AR inhibitors.[117,142] Most of ER-positive BCs are AR-positive (about 80-90% of them) and the coexpression of AR, ER and PgR is associated with a better prognosis and well-differentiated phenotype.[114,115,132] The cross talk between AR and ER (alpha or beta) in human breast and prostate cancer cells has been known for long time and it is exerted at the level of estrogen responsive elements.[76] It occurs also at non-genomic levels. Migliaccio and colleagues demonstrated that a non-genomic interplay between AR and ER can occur at protein level involving Src tyrosine kinase and epidermal growth factor receptor.[143,144] Several coregulators balance the activity of these two hormone receptors and their interactions in different clinical settings. Some therapeutic approaches can be based on blocking this cross talk.[145] Some authors suggested that the levels of expression of AR and its relation to ER expression levels in primary tumors predict benefit from adjuvant endocrine therapy with tamoxifen.[73] Cochrane et al.[73] evaluated nuclear protein expression levels of AR and ER because previous studies reported that AR mRNA and protein decrease in tumors responsive to neoadjuvant endocrine therapy.[146,147] We evaluated, for the first time, both AR/ER ratio and AR/PgR ratio in a subset of metastatic BC patients, to assess their predictive potential for efficacy of endocrine therapy. These ratios were measured as the percentages of tumor positive cells for each receptor, through immunohistochemical staining. In our study, the ROC analysis identified 0.9 as the best cut off value for AR/ER ratio, which differed from the one calculated by Cochrane et al. (cut off = 2.0), probably due to the different subset of patients analyzed, and was not associated with outcome. Moreover, we evaluated AR/PgR ratio and we found a potential predictive value of this parameter at the cut off of 0.96. This finding could be explained by the stronger predictive value of PgR alone in comparison with AR alone, as PgR <10% and Ki67 ≥20% showed a significant association with PD as best response, and PgR ≤1% and Ki67 ≥20% were significantly associated with shorter TTP.

Our findings suggest that AR expression does not predict the efficacy of first-line endocrine treatment in ER- or PgR-positive advanced BC, both in term of TTP and PD as best response. This study was not powered to determine whether AR expression could predict response to tamoxifen or fulvestrant, as the majority of patients received aromatase inhibitors and very few patients received those therapies. Our results might be influenced by the limited amount of AR negative cases in this subtype of BC.

In conclusion, PgR and Ki67 seem to be useful to select patients with a higher probability of being responsive to first-line endocrine therapy for metastatic BC and their stronger predictive effect could make the role of AR expression less evident, as suggested by the predictive

significance of the AR/PgR ratio. The AR expression could acquire more relevance when anti-androgen therapy will be available for BC patients.

4.2 ARTT trial

Androgen receptors are commonly expressed in BC, but androgens have variable effects in different BC subtypes, and both AR agonists[148–151] and antagonists are being studied as antitumor agents in BC.[117,152–154] Dehydroepiandrosterone (DHEA) is a steroid produced mainly by the adrenal cortex and transformed into sex hormones (androgens and estrogens) within peripheral target tissues.[88,155–157] The action of sex steroids is confined within the cells in which they are synthesized (a process called "intracrinology"), with little or no release into the extracellular spaces or the general circulation. This process also occurs within BC cells, and there is preclinical evidence of antitumor activity of DHEA in BC.[158–160] The administration of an aromatase inhibitor (AI) prevents the conversion of DHEA into estrogens and favors its conversion into androgens. To investigate the role of androgens in BC, avoiding the virilizing effects of available androgenic agents, we conducted a two-stage, phase II, prospective clinical study to evaluate the safety and activity of DHEA in combination with an AI in two cohorts of patients with AR-positive metastatic breast cancer: ER-positive cohort and TN cohort.
The poor activity of DHEA in our study may partly be due to heavy pretreatment, which may have compromised hormone sensitivity. Variability in adrenal function,[161] in DHEA disposition after oral administration especially in elderly patients,[162–164] and in BC cells intracrinology may further be involved.[165] The AR gene amplification present in the only patient who showed a prolonged clinical benefit is intriguing, prompting to hypothesize the potential value of AR gene amplification as a predictive biomarker of response to androgenic treatments in breast cancer. However, the small number of patients involved in the study and the low rate of clinical benefit prevents any definitive conclusions from being drawn. Similarly, the role of phosphorylated AR remains to be ascertained.

5. CONCLUSIONS AND FUTURE PERSPECTIVES

PCa studies suggested AR as prominent prognostic and predictive marker. Given that the prognostic and predictive role of AR in BC is matter of debate, AR detection is not routinely performed. The standardization of IHC methods could render AR an easily detectable biomarker in DCIS and in primary invasive and metastatic BC.

We demonstrated the prognostic role of AR expression detected by IHC and the ratio AR/ER in DCIS patients, independently of the treatment (surgery or surgery plus radiotherapy), indicating that the analysis of this biomarker could be worthy to assess patient prognosis and to predict disease relapse. The differences of AR expression found between primary and metastatic tumors suggest that AR has to be detected in all biological materials available for the patient, considering also the different role of this biomarker in the different subsets of disease. The results obtained on AR expression in different populations, such as the Tanzanian one, give the possibility to treat patients at low economical income with anti-AR compounds, considering the low cost and the high incidence of AR-positive TN Tanzanian BCs. Despite we did not found any role of AR in predicting the response to ET, the ratios with hormone receptors have to be considered, given their importance for patient risk assessment. The possibility to treat AR positive TNBC patients with new anti-AR compounds, such as Apalutamide, opens new perspectives in this prognostically unfavorable subset. However, in this field, additional studies are needed to verify the in vivo efficacy of the combination of anti-AR strategies, together with PARP inhibitors, CDK4/6 and PI3K inhibitors.

MATERIALS & METHODS

1. PATIENT AND SAMPLE SELECTIONS

The studies were carried out on patients enrolled from 2000 to 2011 in clinical and/or biological studies performed at Istituto Scientifico Romagnolo per lo studio e la cura dei Tumori (IRST) IRCCS (Meldola, Italy), in collaboration with the Cancer Prevention Unit and the Breast Surgery Unit of Morgagni-Pierantoni Hospital (Forlì, Italy). Patients aged ≥18 years with a histological diagnosis of DCIS and/or BC were eligible. All the patients had to be followed up for at least 5 years, unless they had relapsed earlier.

The study protocols were reviewed and approved by the IRST and AVR (Area Vasta Romagna) Ethics Committee (approval n. 1164 and n. 3692) and patients provided written informed consent according to Italian privacy's law. We collected data from medical records of these patients. Subsequently we gathered tumor tissue samples of these patients for biomarker assessments. The original hematoxylin and eosin stained sections were reviewed by the pathologist in order to select the most representative inclusion of tumor tissue for each patient.

1.1 DCIS pathological features

The original hematoxylin- and eosin-stained sections were reviewed for the analysis of clinical pathological features, such as nuclear grade, presence of comedonecrosis and margin status. Multifocality is a pathologic feature defined as more than one distinct focus of DCIS, with at least 5 mm of intervening healthy tissue confined to a single quadrant of the breast. The size of the largest focus was recorded in the event of multifocal DCIS. Recurrent disease in patients was defined as a DCIS or IC lesion occurring more than 12 months after surgery. The resection margin status was reported as positive when DCIS was present at the inked or cauterized edge of the specimen and negative if there was no DCIS within 2 mm of the inked margin, as recommended by the most recent guidelines endorsed by the Society of Surgical Oncology (SSO), the American Society for Radiation Oncology (ASTRO), and the American Society of Clinical Oncology (ASCO).[34] The final margin status (positive or negative) refers to the resection margin status of the first surgical specimen.

1.2 Tanzanian case series

We compared the biological characteristics of 69 consecutive Tanzanian patients who underwent biopsy or surgical resection of primary BC from 2003 to 2010 at the BMC (Mwanza, Tanzania) and Italian BC patients matched (ratio 1:2) for date and age at diagnosis. The Medical Scientific Committee of IRST IRCCS, the Ethical Committees of Area Vasta

Romagna (Italy) and BMC (Tanzania), and the National Institute for Medical Research (Tanzania) approved the study. The informed written consent from the participants was obtained. The clinical and pathological assessments of African cases were performed at the Oncology Unit and Pathology Laboratory of BMC, while FFPE African tissues were analyzed for AR and conventional biomarker expressions at the Biosciences Laboratory of IRST - IRCCS in Meldola, Italy.

Breast cancers from Caucasian patients were randomly extracted from an electronic database (Log80) of the Pathology Unit of Morgagni-Pierantoni Hospital (Forlì, Italy) and matched with Tanzanian patients for year of diagnosis and age at diagnosis (maximum difference of 2 years). The former stratification factor was chosen to avoid biological material alteration due to the long enrollment period, and the latter was chosen because age can affect the analysis of biomarkers in BC.

2. BIOMARKER DETERMINATION

2.1 Immunohistochemistry

Tumor material obtained during surgery was fixed in neutral buffered formalin and embedded in paraffin. Four-micron sections were mounted on positive-charged slides for each patient (Bio Optica, Milan, Italy). Biomarker determinations were performed according to European Quality Assurance guidelines. Immunostaining for conventional biomarkers and AR expression was performed using the Ventana Benchmark^{XT} staining system (Ventana Medical Systems, Tucson, AZ, USA) with the Optiview DAB Detection Kit (Ventana Medical Systems). ER, PgR, Ki67 (all monoclonal by Leica, Novocastra, Newcastle, UK), HER2 (polyclonal by Dako, Carpinteria, CA, USA) and AR (SP107, monoclonal by Cell Marque, Ventana Medical Systems) antibodies were used. For ER, PgR, Ki67 and HER2 detection, tissue sections were incubated for 60 minutes with antibodies diluted 1:80, 1:40, 1:100 and 1:350, respectively, in antibody diluent (Ventana Medical Systems). AR antibody, pre-diluted by the supplier, was used. Regard to AR phosphorylated forms, monoclonal antibodies anti-AR pSer210-213 (NR3C4, 156C135.2) and -ARpSer650 (NR3C4) were used (Novus biological, Centennial, Colorado, USA), respectively diluted 1:500 and 1:250 in antibody diluent (Ventana Medical Systems). Sections were incubated for 16 minutes and automatically counterstained with hematoxylin II (Ventana Medical Systems). Positive and negative breast tissues were used as intra- and inter-assay controls for AR expression. Biomarker positivity was detected and semiquantitatively quantified as the percentage of immunopositive tumor cells. All samples

were evaluated by 2 independent observers and any disagreement (>10% of cells) was resolved by consensus after joint review using a multihead microscope. As clear guidelines for AR expression have not been available until now, we used two different cut off values ≥1% and >10% of immunopositive tumor cells to assess AR positivity. Staining intensity (*i.e.,* 0 absent, 1+ weak, 2+ moderate, and 3+ strong) was also analyzed in order to calculate the H-score, defined as the product of the percentage of AR-positive tumor cells and staining intensity. In order to calculate the AR/ER and AR/PgR ratios, AR expression value was considered as a continuous variable (% of immunopositive tumor cells ranging 0-100%) and in case of ER or PgR negativity (0%), the ratio was set as the AR value.

2.2 Classification of BC subtypes

Molecular subtypes were defined according the status of ER, PgR, Ki67 and HER2 biomarkers. ER-positivity and PgR-positivity were considered as ≥1% tumor cells staining for ER and PgR, respectively; Ki67 was considered high when detected in ≥20% of tumor cells; HER2-positivity was defined as 3+ staining intensity by IHC or as HER2 amplification (HER2/Chromosome 17 centromere ratio ≥2.0, or mean HER2 gene copy number ≥6 per tumor cell). The expression of these biomarkers allowed to classify samples according to the St. Gallen expert consensus and the ASCO-CAP guidelines.[11,16] Luminal A-like (ER-positive, PgR≥20%, low Ki67 (<20%), HER2-negative), luminal B-like (ER-positive, PgR <20%, high Ki67 (≥20%), HER2-positive or HER2-negative), HER2-positive non-luminal (ER-negative, PgR-negative, HER2-positive), and triple-negative (ER-negative, PgR-negative, HER2-negative).

2.3 Fluorescence *In Situ* Hybridization

The copy number of loci Xq12 (AR) was assessed on FFPE sections using a locus specific Spectrum Orange labelled probe (Abbott Vysis, Illinois, USA). Sections were pre-treated using the Vysis Paraffin Pretreatment IV. (Abbott Vysis). After denaturation at 78°C for 3 minutes and hybridization at 37°C for 17 hours, the stringency washes were performed using the Post-Hybridization Wash Buffer Kit at 73°C. The slides were finally counterstained with DAPI I (Abbott Vysis) and evaluated under an epifluorescence microscope (Zeiss, Oberkochen, Germany) equipped with the corresponding wavelength filter, CCD camera, and image capturing and analyzing system. A pathologist selected the area for analysis on the hematoxylin-eosin stained section. Cells displaying more than two signals were

classified as amplified. Copy number signals were counted blindly on at least 60 non-overlapping nuclei per sample by 2 trained technologists.

3 STATISTICAL ANALYSES

All the data were summarized using descriptive statistics. Frequency tables were performed for all categorical variables. Continuous variables were presented using median and range. Chi square or Fisher exact tests were used to evaluate the relationship between clinical characteristics and categorical variables and the relapse status or best response, as appropriate. Spearman's correlation was used to investigate the relationship between the different biomarkers considered as continuous variables. The accuracy of single or combined biomarkers, considered as continuous variables, was measured using the area under the curve (AUC). In the Receiver Operating Characteristic (ROC) curves, true positive rates (sensitivity) were plotted against false positive rates (1-specificity) for all classification points. The optimal cut off values were obtained from receiver operating characteristic (ROC) curve analysis.

Concordance of AR expression was defined as either positive or negative in both tumor and metastasis, while discordance was defined as positivity at one site and negativity at the other or vice versa. The concordance rate was calculated as the proportion of concordant cases with respect to the total number of patients. McNemar's test was performed in order to compare AR status between the primary tumor and paired metastatic sites.

Univariable linear regression was used to assess and graphically display the relationship between the time elapsed from the removal of the primary tumor to sampling of the metastasis and the difference of AR expression between the two samples.

The prognostic role of biomarkers with regard to the survival endpoints together with Hazard ratios (HR) and their 95% confidence intervals (95% CI) were analyzed using Cox proportional regression models. Given the co-linearity issues between AR, ER and the AR/ER ratio, separate models were performed. Departures from the proportional hazard assumption were assessed on the basis of Schoenfeld residuals. AR expression was analyzed in relation to the other conventional biomarkers (ER, PgR, HER2 and Ki67), best response to therapy (CR, PR, SD, PD), and time to progression (TTP) (months).

OS, TTP and Recurrence-free survival were estimated using the Kaplan-Meier method and compared with the log-rank test. OS was calculated as the time from the date of the start of first-line treatment for metastatic disease to the date of death from any cause or the date of the last follow-up visit.

All p-values were based on two-sided testing and values lower than 0.05 were considered statistically significant. Statistical analyses were performed using SAS statistical software version 14 (SAS Inc., Cary, NC, United States of America).

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With love... S

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