"*NEW MOLECULAR APPROCHES USING AR, FOXA1 AND HER2 FOR OUTCOME CLASSIFICATION OF ESTROGEN RECEPTOR-POSITIVE (ER+) BREAST CANCERS*"

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“To my dad,….. because I know that he guides me and takes care of my steps”

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LIST OF ABBREVIATIONS

AIC	Akaike Information Criterion
AR	Androgen Receptor
AR-	Androgen Receptor - Negative
AR+	Androgen Receptor - Positive
ASCO/CAP	American Society of Clinical Oncology / College of American Pathologists Guideline Recommendations
AT	Adjuvant Therapy
AUC	Area Under the Curve
AURKA	Aurora Kinase A
BC	Breast Cancer
ER	Estrogen Receptor
BIRC5	Baculoviral IAP Repeat Containing 5 / Survivin
CCNB1	Cyclin B1
cDNA	complementary Desoxiribonucleic Acid
CEP17	Centromeric Probe Chromosome 17
ChBU	Comittee for human Biospecimen Utilization
ChIP-seq	Chromatin Immunoprecipitation sequencing
CIs	Confidence Intervals
CPI	Cell Proliferation Index
ct	Cycle threshold
CTRL	Control group
DASL	cDNA-mediated Annealing, Selection, extension and Ligation
DCIS	Ductal carcinoma “in situ”
DFI	Disease Free Interval
DNA	Desoxiribonucleic Acid
DSS	Disease Specific Survival
E	Equivocal
EGFR	Epidermal growth factor receptor
EQV	HER2 double-equivocal carcinomas
ER-	Estrogen Receptor - Negative
ER+	Estrogen Receptor - Positive
EREs	Estrogen response elements
FFPE	Formalin-Fixed Paraffin-Embedded
FISH	Fluorescence *In Situ* Hybridization
FOXA1	Forkhead-box A1
FOXA1-	Forkhead-box A1 - Negative
FOXA1+	Forkhead-box A1 - Positive
H&E	Hematoxilin and Eosin
HER2-	human epidermal growth factor receptor 2 - Negative

HER2/*ERBB2*	human epidermal growth factor receptor 2
HER2+	human epidermal growth factor receptor 2 - Positive
HR	Hazard Ratio
HT	Hormonal Therapy
IEO	European Institute of Oncology
IgG	Immunoglobulin G
IHC	Immunohistochemistry
ILC	Invasive Lobular Carcinoma
ISH	In Situ Hybridization
Kip	Cyclin dependent kinase inhibitors
MGB	Minor Groove Binder
MKI67	Marker of proliferation – Ki67
MMPs	Matrix Metalloproteinases
mRNA	messenger Ribonucleic Acid
N	Negative
Neg	Negative
NFQ	Nonfluorescent Quencher
ns	Non Significant
NST	Non Special Type - Histotype
NT	Normal Tissue
P	Positive
PAM50	50-gene classifier. Prediction Analysis of Microarray
PCR	Polymerase Chain Reaction
pCR	Pathologic Complete Response
PDR	Probability of Distant Recurrence
PgR	Progesterone receptor
PMEA	Prognostic multigene expression assays
pNR	Pathologic No Response
Pos	Positive
pPR	Pathologic Partial Response
qPCR	Quantitative Polymerase Chain Reaction
RNA	Ribonucleic Acid
ROC	Receiver Operating Characteristic analysis
ROR	Risk of Recurrence
St. Gallen	Saint Gallen Consensus meeting
TAM	Tamoxifen
TCGA	The Cancer Genome Atlas
TMAs	Tissue Microarrays
TNBC	Triple Negative Breast Cancer
UBE2C	Ubiquitin Conjugating Enzyme E2 C
Δct	Delta - Cycle threshold

SUMMARY

Nowadays, the diagnoses of Breast Cancers (BCs) are usually assessed before the spreading to lymph nodes (early-stage breast cancer) and 75% of these cases are Estrogen Receptor - positive (ER+), classified as luminal tumours following Saint Gallen Consensus meeting (St. Gallen) and American Society of Clinical Oncology/College of American Pathologists Guideline Recommendations (ASCO/CAP). ER+ tumours are characterized by a more favorable outcome than Estrogen Receptor - negative (ER-) tumours and they are candidates for treatment with drugs targeting ER signaling (endocrine therapy). Although anti-estrogen therapy is considered the gold standard treatment, approximately 30 - 50% of all ER+ tumours display resistance and recurrences will occur after the first 5 years of starting therapy. To overcome this problem, patients may be eligible for chemotherapy in association with hormonal treatment, however the actual benefit of chemotherapy in ER+, node-negative and human epidermal growth factor receptor 2 - negative (HER2-) cases is not clear. Currently, prognostic multigene expression assays - PMEA (*e.g. Prosigna)* may give further information about tumour biology and risk of recurrence, which can be taken into account for therapeutic decisions. However, the use of these tests in routine clinical practice is limited. Hence, in order to take correct and efficient therapy decision it is necessary to study and standardize new prognostic and predictive easy access strategies (markers), for better classify ER+ BC disease, which might provide additional information and be complementary to the clasical clinical-pathological prediction models.

Accordingly, this document is divided in three (3) chapters, each one describing a new strategie to try to better classify ER+ BC patients using new and clasical markers for evaluation of BC prognosis. Thus, in Chapter 1 and 2 it is presented the potential role of Androgen Receptor (AR) and the pioneer factor Forkhead-box A1 (FOXA1), as emerging markers for outcome evaluation in BC patients, but even more important that AR and FOXA1 are correlated between them and with the ER status, allowing a better prognosis classification of ER+ BC

disease. In chapter 1, the assessment of AR levels relative to ER levels (AR/ER ratio) by immunohistochemistry (IHC) provided evidence that in ER+ BC, higher levels of AR (AR/ER ratio ≥ 2) identifies a subgroup of patients with aggressive biological features (*e.g* higher proliferation) and worse prognosis. Besides, the prognostic multigene expression assay – Prosigna, indicated that a significant number of cases with AR/ER≥2 could be non-luminal tumours. On the other hand, in chapter 2 expression level analysis of FOXA1 mRNA (RT-qPCR), positively correlated with levels of both AR and ER mRNA and in ER+ BCs, FOXA1 positivity (IHC) was associated with a good prognosis independently of AR expression.

In Chapter 3 it is described a specific group of ER+ BCs characterized by equivocal protein expression and gene copy number of HER2 (named HER2 double-equivocal carcinomas). Here we aimed to stratify double-equivocal carcinomas by transcriptomics, showing that these cases are preferentially luminal B tumours with a high risk of recurrence. Moreover, they clustered with ER+/HER2-negative (HER2-) carcinomas, and a subgroup overexpressed genes with a mutually exclusive pattern of expression to HER2 (*AGTR1*, *TPRG1*, *NOVA1*).

1. GENERAL INTRODUCTION

1.1 JUSTIFICATION

According to recent estimates, breast cancer (BC) remains one of the leading causes of cancer death in women (1). Various clinical (age, menopausal status, tumour size, lymph node status, histological grade) and pathological factors, (Estrogen Receptor-ER, Progesterone Receptor-PgR, human epidermal growth factor receptor 2 - HER2 status, Ki67) have been carefully evaluated as indicators of clinical course; however these markers still shown limited ability to predict individual patient outcomes, and patients with the same clinical-pathological features (*e.g* ER-Positive/HER2-Negative cases) can have largely different prognostic and predictive outcome (2, 3). Between the aforementioned factors, hormone receptor status (ER/PgR) and HER2 are the most important features in predicting outcome and guiding treatment options. In fact, about 75% of BCs are classified as ER - positive (ER+), and these tumours are characterized by a more favorable prognosis. Among them, about 10% are also HER2 – positive (HER2+) (4), but these tumours usually present worse survival. Although ER+ and HER2+ tumours are good candidates for treatment with drugs targeting both ER and HER2 signaling (*e.g* Tamoxifen and Trastuzumab, respectively), evidence has demonstrated that in approximately 30 - 50% of cases, therapy fails to reduce tumour size and to prevent recurrences, and even, indicate that patients may be subjected to side-effects (e.g toxicity and drug resistance) without benefit from such interventions (5-8).

The above suggest ER+ BC is a heterogeneous disease accompanied by differences not only in clinical, but also in molecular and biological features (9, 10). As a matter of fact, Prognostic multigene expression assays - PMEAs (*e.g.* Prosigna), have identified two distinct intrinsic molecular subtypes of ER+ BC (Luminal A and B subtypes). Although Luminal B tumours have poorer outcomes and some of them can be identified by their expression of HER2, its major biological distinction is proliferation, which is higher in luminal B than in luminal A

tumours (11). However, whilst several PMEAs and subtype classification models have been developed, there is little agreement between prognostic signatures generated from different studies (12); furthermore, translation of PMEAs in routine clinical practice is still limited, mainly by their elevated costs (3, 13). Even though vast technological improvements has increased the understanding of BC as a heterogeneous disease, current clinical-pathological and molecular parameters, are not sensitive enough to stratify and identify ER+ BC patients at high risk, which creates a challenge for prognosis and treatment of this common disease.

Since BC is a hormone-dependent disease, the study of markers related to this component could prove a greater prognostic and predictive effectiveness. Within them, the nuclear hormone receptor androgen receptor (AR), and the main co-transcriptional factor modulating nuclear steroid receptor activity in breast, the pioneer factor Forkhead-box A1 (FOXA1), both seems play an important role in ER+ breast carcinogenesis and therefore might be useful in a further classification of ER+ BCs. On the other hand, assessment of HER2 status is a key step to predicting BC outcome, but new guidelines for its evaluation have increased the number of cases with an equivocal result (equivocal protein expression and gene copy number). Hence, study new approches, like transcriptomics, could help to better stratify “ER+/HER2-equivocal” cases. Further, the identification of subgroups of ER+ BC patients, with differences in prognosis, might guide clinicians in the selection of the optimal therapeutic approach (type and time of treatment - endocrine therapy, chemotherapy or both) in order to reduce over or under-treatments in this subset of patients.

1.2 BREAST CANCER OVERVIEW

Breast cancer (BC) is the second most common cancer in the world and, by far, the most frequent cancer in women, accounting for 25% (1.67 million) of the total new cancer cases and 14.7% (522,000) of the total cancer deaths in 2012 (1). Factors related with increased risk of disease are reported in Table 1.

Table 1. Factors that increase risk of developing BC.

RISK FACTOR	RISK GROUP	RELATIVE RISK
Age	Elderly	>10
REPRODUCTIVE FACTORS		
Menarche*	Before 11 years of age	3
First child	Nulliparity	3
Menopause*	After 54 years of age	2
LIFESTYLE		
Diet	High intake of saturated fats	1.5
Body weight (post menopause)	Body mass index >35	2
Alcohol	Excessive intake	1.2
HORMONAL STATUS		
Oral contraceptives	Common use	1.24
Hormone replacement therapy	Use for ≥10 years	1.35
RADIATION	Abnormal exposure after 3 years of age	3
FAMILY HISTORY	BC in first degree	≥2

Classification of BC

There are several criteria to classify BC, from the histologic (ductal, lobular, mucinous, tubular, medullary, and papillary), which is the oldest technique, to those that have been proposed with the use of molecular techniques developed in more recent years. Perou's Molecular Classification is perhaps the most cited classification system developed from microarray gene profiling, which classifies tumours into four main intrinsic molecular subtypes: Luminal A, Luminal B, HER2-enriched and Basal-like (9). The expression profile provides additional information about the tumor biology, thus Luminal A (35-40%) and Luminal B (25-30%) tumors

usually have intermediate to high expression of ESR1 (ER protein) and ER regulated genes and rarely have high ERBB2 gene expression (HER2 protein); conversely, HER2-enriched tumors (10-20%) usually have intermediate to high expression of the ERBB2 gene and intermediate to low expression of ESR1 and ER regulated genes. Finally, Basal-like tumors (10-20%) usually have low or negative expression of ESR1, ERBB2, and ER-regulated genes, but have high expression levels of genes associated with cell proliferation (9).

Prognostic and predictive factors

Prognostic factors are routinely used to estimate the likelihood of BC; these clinical parameters are useful for predicting early (at 0-5 years) and late (at 5-10 years) recurrence after diagnosis and treatment (14, 15). The most frequently used prognostic factors in routine clinical practice include:

- Tumour size: Tumours sizes up to 15-20mm are usually correlated with bad prognosis.
- Lymph node status: Presence of metastasis in axillary lymph nodes is considered a bad prognostic indicator in patients with BC in early stages.
- Histological grade: Lower levels of tumor differentiation (Higher histological grades), are related to metastasis and short survival.
- Hormone receptors status: Estrogen (ER) and Progesteron Receptor (PgR) status helps guide treatment for BC. These markers are, not only, prognostic but also predictive factors for response to hormone therapy. Patients with high percentage of hormone receptors can be tailored to endocrine therapies.
- HER2: Overexpression of this protein is observed in poorly differentiated BCs. It predicts resistance to hormone therapy and chemotherapy
- Vascular or lymph invasion: High number of blood or lymphatic vessels are predictive of reduced survival.
- Cellular proliferation markers (Ki-67): A high positivity percentage of this marker (>20%) has been related to poorly differentiated and large tumors, as well as early recurrence and poor survival (16).

- The molecular characterization of BC has enabled greater understanding of the cellular and molecular biology processes underlying this disease and recent evidence indicate that gene expression analysis for tumour molecular characterization, provide not only prognostic (where non-luminal tumours have worse survival), but also predictive information regarding the utility of cytotoxic therapy, alone or in addition to endocrine therapy, for patients with early-stage BC.

1.3 GENERALITIES OF THE ER AND HER2 AS CLASSICAL MARKERS OF BC

Estrogen Receptor (ER)

Higher levels of ER in benign breast epithelium has been particularly associated with increased risk to develop BC, in fact ~75% of all BCs are ER+. As mentioned before, its detection is important as a prognostic marker since predict risk of progression and respons to therapy. When compared with ER negative (ER-), ER+ tumours exhibit stronger clinical response to hormonal treatment (1), better differentiated morphologic appearance (17) and shown low rates of cell proliferation, between other favorable characteristics (18).

ER mediates estrogens (E2) action, mainly. ER it`s encoded by *ESR1* gene on chromosome 6 and normally expressed in low levels in different tissues. ER regulate expression through recognition of a conserved estrogen response element (ERE), usually present in the promoter region of a wide variety of genes involved in development, reproduction, regulation of cell cycle, DNA replication, differentiation, apoptosis, angiogenesis, survival and tumor progression. ER use different mechanism of carcinogenesis which explain the association between ER pathway with BC development; however the classical and most important mechanism involve E2 binding to ER, either to the nuclear or plasma membrane ER. This binding cause conformational changes and nuclear translocation of the ligand-stimulated ER, that allow ER binding to ERE, therefore stimulating gene transcription of its target genes (19).

Human epidermal growth factor receptor 2 (HER2)

HER2 (also called *ERBB2*) is a proto-oncogene located on the long arm of chromosome 17 and encodes a protein of 185 kD. The HER2 protein belongs to a family of growth factor receptors displaying tyrosine kinase activity, whose biological activity affects the activation of cellular pathways involved in cell survival and proliferation (e.g MAPK) (20).

Amplification of this gene, as well as, its protein overexpression is observed in 15% of all BCs (21) and is considered a bad prognostic marker and a predictive factor of response to chemotherapeutics and therapy using specific antibodies to block the receptor function (22). However, several pre-analytical, analytical, and interpretational factors affect the precision and accuracy of HER2 evaluation, which also affects treatment decision-making. Accordingly, recommendations have been published for HER2 testing and evaluation (23), but some authors (24, 25) have highlighted that the guidelines have increased the number of cases with an equivocal result i.e., equivocal protein expression and gene copy number ("double-equivocal" HER2 BC). Therefore, precise definitions of HER2 status its neccesary to better classify ER+ BC with HER2 equivocal characteristics for improve tailor therapy of BC patients.

1. 4 GENERALITIES OF AR AND FOXA1 AS EMERGING MARKERS OF BC

Androgen Receptor (AR)

Both, testosterone (T) and its active metabolite, 5α-dihydrotestosterone, (DHT) are androgens having high affinity for the AR (26). AR gene is located on the X Chromosome and encode a protein of 110kD, which regulates gene expression through recognition of specif androgen response elements on the target promoter gene (ARE) associated with apoptosis, angiogenesis, survival, differentiation and tumor progression (27). Like ER, AR employs different mechanisms in order to regulate its target gene expression, but the most important includes the nuclear translocation of the ligand-stimulated AR and requires AR–DNA interactions or protein–protein interactions – usually involving the interaction of AR with other transcription factors (28).

Increasing data support a role of AR as a marker of prognosis in BC. Several recent studies shown AR expression in luminal tumours (ER+) to be an independent prognostic factor of good outcomes (29, 30), mainly due to the anti-proliferative affects of AR (31). However, contradictory results have been reported, since some studies have found increased proliferation rates in ER+ cell, when AR signaling pathway is stimulated (32, 33). Furthermore, association between AR and its prognostic value in ER- BC is less clear, since there are original clinical research showing no association or even poor prognosis in this type of tumours (34, 35), as well as, in vitro studies showing that AR signalling promotes proliferation of ER- cell. Togheter, these data indicate that the clinical and biological significance of AR expression in BC is not fully defined.

Forkhead-box A1 (FOXA1)

FOXA1 gene, located in long arm of chromosome 14, encode a pioneer factor protein. As such, it has been demonstrated that FOXA1 facilitates ER binding to compacted chromatin DNA (36), which indicate that FOXA1 has an important role in development of normal and BC tissues. Without FOXA1, ER cannot bind to the corresponding EREs even in the presence of E2, and consequently, E2-mediated transcription and BC cell proliferation are abrogated (36). Several studies have investigated the associations between FOXA1 expression and the clinical pathological features of BC; although most of them indicate FOXA1 is marker of good prognosis in ER+ tumours, conclusion of these studies has not always been consistent (37).

Interestingly, the same effects of FOXA1, as pioneer factor, have been reported on AR-mediated transcription in BC tumours; more precisely, a study in ER- BC provided insights about the close clinicopathological and molecular connection between AR and FOXA1 (co-expression of both markers) (38). However, clear implications of these biomarkers on tumour biology and patient prognosis have not been fully explained, mainly in ER+ tumours.

1.5 OBJECTIVES

1.5.1 GENERAL

To study new markers and strategies for better classify ER+ BC disease that might provide additional prognostic information and be complementary to the clasical clinical-pathological prediction models.

1.5.2 SPECIFIC

- To analyse the prognostic impact of AR expression with respect to ER (AR/ER ratio) in a large case series of ER+/HER2- BC patients.

- To evaluate if the AR/ER ratio may identify a subset of ER+/HER2- tumours with different clinical, pathological and biological characteristics.

- To assess the co-expression, at both protein and mRNA levels, of FOXA1 and AR in a group of BC patients.

- To evaluate the prognostic impact of the co-expression of FOXA1 and AR in a large case series of BC patients, particularly in ER+ BC cases.

- To analyse and stratify a group of ER+ “double-equivocal” HER2 BCs using different transcriptomic approaches.

- To evaluate whether the prevalence and pattern of intratumoral *HER2* heterogeneity may affect the definition of the HER2 equivocal category in a group of ER+ “double-equivocal” HER2 BCs.

2. CHAPTER 1

"THE ROLE OF THE AR/ER RATIO IN ER-POSITIVE BREAST CANCER PATIENTS."

2.1 INTRODUCTION

ER and PgR are expressed in most BCs (~75%) and both have wide prognostic and predictive utility (5). In contrast, the clinical and biological significance of AR expression in BC is not fully defined. AR positivity has been detected in up to 61% of primary and metastatic BC lesions (39-41) and approximately 75% of ER+ BCs are also AR positive (AR+). Several studies have shown that AR expression in luminal tumours (ER+) is associated with lower tumour grade, smaller tumour size, lower proliferative index (Ki67 level), and more importantly, AR expression in ER+ tumours is an independent prognostic factor of a good outcome (29, 30, 42, 43). On the other hand, up to 31% of ER negative (ER-) BCs are reported to be AR+ (40, 43), but the prognostic impact of AR expression in this subset of BCs is not clear (34, 39, 44-49).

While the interaction between the signalling pathways of ER and AR are well known (50), it is still ambiguous how the level of AR expression influences ER+ tumours. In vitro studies have shown that AR signalling inhibits estrogen-induced proliferation of ER+ MCF7 BC cells (31, 51-53). This inhibitory effect seems to be mediated by several mechanisms, but the most important is the ability of AR to compete with ER for binding of estrogen response elements (EREs), preventing ER-dependent gene transcription (54). In line with this observation, some studies have reported that increasing AR expression results in a greater androgen-dependent inhibition of ER function (50, 55). However, other studies performed on ER+ MCF7 BC cells described an increase in proliferation when the AR signalling pathway is stimulated (32, 33). Moreover, Cochrane et al (56) recently reported that high AR levels and low ER levels (higher AR/ER ratio) could be associated with a worse prognosis and tamoxifen (TAM) resistance.

Considering these data, the aim of this part of the study was to analyse the prognostic impact of AR expression with respect to ER (AR/ER ratio) in a large case series of ER+/HER2 negative (HER2-) BC patients. We evaluated if the AR/ER ratio may identify a subset of tumours with different clinical, pathological and biological (Cell proliferation index) characteristics. In addition, in the subgroup of BCs with high AR/ER ratio values, we performed Prosigna-PAM50 assays to assess the molecular subtypes of these BCs.

2.2 PATIENTS AND METHODS

Case series

We collected a cohort of 402 ER+/HER2- primary invasive BC patients with available follow-up (Figure 1), who underwent surgery from January 1998 to December 2012 at the Breast Unit of the Città della Salute e della Scienza of Torino, University Hospital of Torino in Turin, Italy. In the diagnostic setting, the cut-off value considered for ER and PgR positivity was ≥1%, as suggested by the St. Gallen and ASCO/CAP Guideline Recommendations (57, 58) and the same cut-off was adopted for AR positivity (42). For all cases, the following clinico-pathological data were obtained from the clinical charts and pathological reports: age, type of surgery (conservative surgery vs radical mastectomy), tumour size (<15 mm vs ≥15 mm), histological type, tumour grade and nodal involvement. An additional group of forty-seven (47) ER+ BCs with matched fresh-frozen samples was included for cell proliferation index assessment. Ethical approval was obtained from the Comittee for human Biospecimen Utilization (Department of Medical Sciences - ChBU). The project provided an informed consent, obtained from the patients at the time of surgery due to the retrospective approach of the study, which did not impact on their treatment. The procedure for collecting the consent was approved by the Committee for human Biospecimen Utilization (Department of Medical Sciences - ChBU). All the cases were anonymously recorded and data were accessed anonymously.

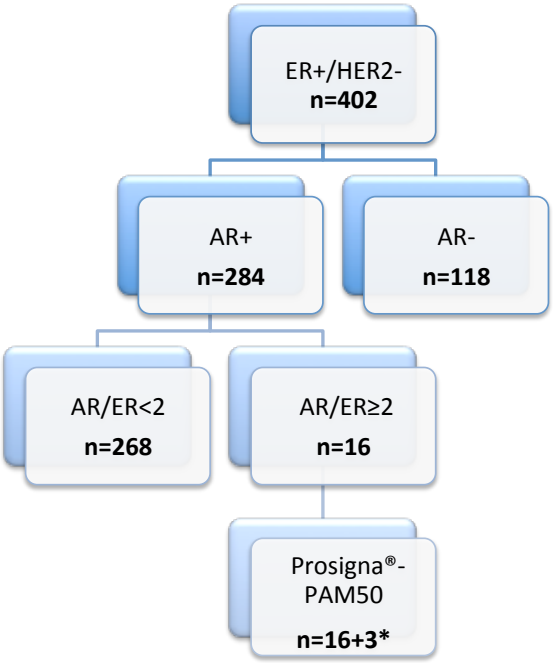


Figure 1. Study flowcharts. *Three additional cases (without follow-up) with a ratio of AR/ER≥2 were included for the Prosigna®-PAM50 assay

Immunohistochemistry (IHC)

For each case, representative blocks were selected and multicore tissue microarrays (TMAs - tissue arrayer Galileo TMA CK 3500, Integrated Systems Engineering Srl, Milan, Italy) were prepared, as previously described (59). IHC was performed using an automated slide processing platform (Ventana BenchMark AutoStainer, Ventana Medical Systems Inc., Tucson, AZ, USA) with the following primary antibodies: prediluted anti-ER rabbit monoclonal antibody (SP1, Ventana Medical Systems Inc); prediluted anti-PgR rabbit monoclonal antibody (1E2, Ventana Medical Systems Inc); anti-AR mouse monoclonal antibody (AR441, diluted 1:50, Dako, Glostrup, Denmark) and anti-Ki67 mouse monoclonal antibody (MIB1, diluted 1:50, Dako). Measurement of HER2 expression was performed by an anti-HER2 polyclonal antibody (A0485, diluted 1:800, Dako). IHC equivocal cases (score 2+) were assessed for HER2 status by fluorescence in situ hybridization (FISH) (60). Positive and negative controls (omission of the primary antibody and IgG-matched serum) were included for each immunohistochemical run. All cases were confirmed as ER+ and HER2-. For statistical analyses and

according to the St. Gallen recommendations (57), a cut-off of 20% for dichotomizing tumours as having low and high levels of PgR and Ki67 was adopted. In addition, this cut-off agrees with the median Ki67 value of our laboratory, previously established to differentiate tumours with a higher proliferative index (57, 61).

AR/ER ratio calculation

AR and ER nuclear staining percentages were compared. Receiver operating characteristic analysis (ROC curve) was used to establish the optimal AR/ER ratio cut-off value that allowed to subdivide the patients into those with good and worse prognosis, as described below.

Statistical analyses

Pearson's Chi square test and Student's t-test were preliminarily performed to compare categorical and continuous variables, respectively, and to evaluate the potential differences in the variable distribution among the groups. The disease-free interval (DFI) was calculated from the date of surgical excision of the primary tumour to the date of the first relapse or last check-up. Disease-specific survival (DSS) was calculated from the surgical excision date of the primary tumour to the date of BC death or last check-up. Survival distribution curves were plotted using the Kaplan-Meier method and the statistical comparisons were performed using the log-rank test. Cox regression analyses were carried out on the DFI and DSS to calculate the crude and adjusted Hazard ratios (HR) and 95% CIs for the different study group. The cases lost to follow-up and cases with non-breast cancer-related deaths were censored at the last follow-up. Models were created to evaluate the prognostic role of different variables. The proportional hazard assumption was assessed with the Schoenfeld residuals. This did not give reasons to suspect a violation of this assumption. The nature of the variables (continue/categorical) included in the models was evaluated considering literature reports and the results of the log-likelihood ratio test. For model selection, the Akaike information criterion (AIC) test was used. All statistical tests were two-sided. P-values <0.05 were

considered significant. Statistical analyses were performed using Stata/SE12.0 Statistical Software (STATA, College Station, TX).

Prosigna - PAM50. Prognostic multigene expression assay - PMEA

Sixteen ER+/HER2- BC cases with an AR/ER ratio ≥2 with long follow-up and 3 additional cases collected during the routine diagnostic assessment of ER and AR, were selected for Prosigna-PAM50 analysis (NanoString Technologies® Inc., Seattle, WA, USA). Briefly, tissue obtained after macrodissection of formalin-fixed paraffin-embedded (FFPE) tumours were processed with a Roche FFPET RNA Isolation Kit (Roche, Mannheim, Germany). The isolated RNA was hybridized to 58 gene-specific probe pairs, plus 6 positive and 8 negative controls (Capture and Reporter Probes – Prosigna CodeSet. NanoString Technologies® Inc.), overnight at 65ºC in a single hybridization reaction. The removal of excess probes, followed by binding of the probe-target complexes on the surface of a specific nCounter cartridge, was performed on the nCounter Prep Station (NanoString Technologies® Inc.). Finally, the nCounter cartridge with immobilized probe/target complexes was read in the nCounter Digital Analyzer (NanoString Technologies® Inc.). The conversion of gene expression measurements into intrinsic molecular subtypes, risk of recurrence (ROR) scores and risk categories used a fully prespecified algorithm has been previously described (62, 63).

Cell proliferation index analysis

RNA was extracted from 47 matched fresh-frozen ER+ BC samples, using TRIzol (Life Technologies, Carlsbad CA, USA) according to the manufacturer's instructions. RNA was used to evaluate the expression of AR, ESR1 and five cell proliferation genes (AURKA, BIRC5, CCNB1, MKI67 and UBE2C) through TaqMan®-qPCR assays. Assays consist of a pair of unlabeled PCR primers and a TaqMan probe with an FAM dye label on the 5' end and minor groove binder (MGB) and nonfluorescent quencher (NFQ) on the 3' end. Primers and probes were ordered from Applied Biosystems (Foster City, CA) as follows: AR (Hs00171172_m1); ESR1 (Hs00174860_m1); AURKA (Hs01582072_m1); BIRC5

(Hs04194392_s1); CCNB1 (Hs01030099_m1); MKI67 (Hs01032442_m1); UBE2C (Hs00964100_g1). AR/ER-qPCR ratio was calculated based on $2^{-\Delta\Delta Ct}$ method. Nine fresh-frozen breast normal tissues and 9 fresh-frozen ER-negative (ER-) BCs samples were used as controls.

2.3 RESULTS

Patients and tumour characteristics

Clinical and pathological features of the 402 ER+/HER2- tumours according to the AR status are shown in Table 2. The median time of follow-up was 8 years. The majority of cases, 70.6% (284/402), were AR+. The distribution plots of IHC ER and AR nuclear staining percentages are presented in Annex 1. According to previous reports (42, 64), AR expression (≥1% nuclear staining) was confirmed to be significantly correlated with a longer DSS (P = 0,0008; Annex 2) of ER+ BC patients.

AR/ER ratio and correlation with histological and immunohistochemical features

The median AR/ER ratio was 0.51. Two was the optimal AR/ER ratio that differentiated the cohort by prognosis (AR/ER≥2: AUC=0.74; P=0.002) (Figure. 2). In addition, the same value was reported by a recent study (56) as a good predictor of DFI and DSS, in a cohort of ER+ BC patients. AR/ER optimal ratio (AR/ER≥2) was further defined and confirmed to predict DFI and DSS in our cohort (ER+/HER2- BC patients) by univariate Cox (HR) analysis. The characteristics of the 284 ER+/HER2-/AR+ BC cases stratified by an AR/ER ratio are reported in Table 3. Of the 284 AR+/ER+ cases, 268 (94%) had an AR/ER ratio <2 and 16 (6%) an AR/ER ratio ≥2 (Figure. 1; Figure. 3). In the descriptive analysis, patients with a higher AR/ER ratio carried larger tumours with a higher histological grade and lower PgR levels, and they frequently had more metastatic lymph nodes and had a higher number of relapse events (P≤0.004) (Table 3).

Table 2. Clinical-pathological characteristics for 402 ER+ BC cases according to AR status.

Characteristics		Total	AR-	AR+	P Value (Fisher test)
Patients		**402**	**118**	**284**	**--**
Age (Median, interval)		62 (31-92)	62 (35-92)	62 (31-88)	**0.958**
Grading	**1**	143	39	104	**0.272**
	2	177	49	128	
	3	82	30	52	
Tumor size	**<15 mm**	203	54	149	**0.358**
	≥ 15 mm	195	60	135	
Metastatic Lymph nodes	**0**	268	77	191	**0.246**
	1-3	87	25	62	
	4-9	29	7	22	
	>9	18	9	9	
Ki67	**<20%**	225	57	168	**0.046**
	≥20%	177	61	116	
PgR†	**<20**	49	7	42	**0.066**
	≥20	329	87	242	
ER%	**Median (interval)**	90 (2-100)	90 (10-100)	90 (2-100)	**0.244**
Relapse	**No**	317	84	233	**0.041**
	Local	7	2	5	
	Distant	78	32	46	
Surgery	**Quadrantectomy**	267	79	188	**0.884**
	Mastectomy	135	39	96	
Therapy*	**HT**	275	77	198	**0.415**
	HT + AT	125	40	85	

*2 patients refused therapy. † Missed data for 24 patient. ‡ Missed data for 4 patient. HT: Hormonal therapy. AT: Adjuvant therapy (Chemotherapy)

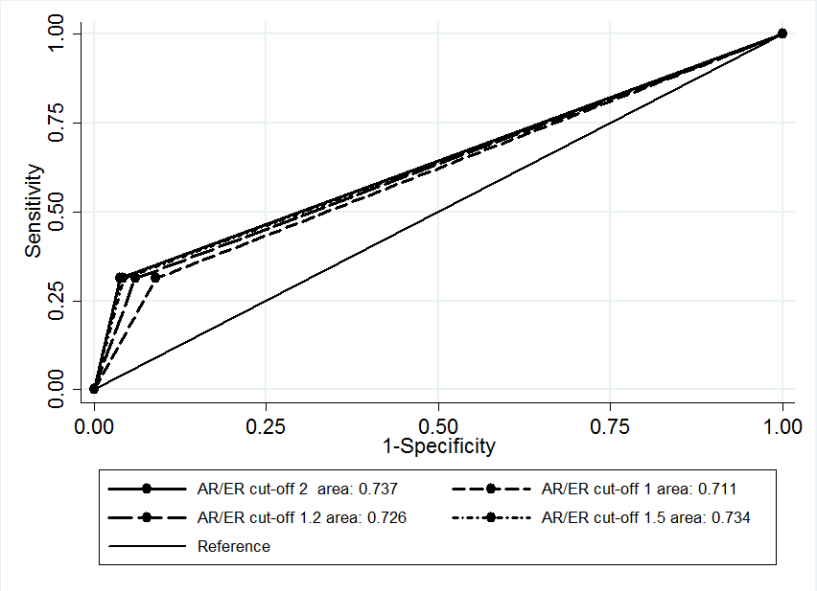


Figure 2. ROC curve comparing different cut-off points for AR/ER ratio. area: Area Under the Curve (AUC).

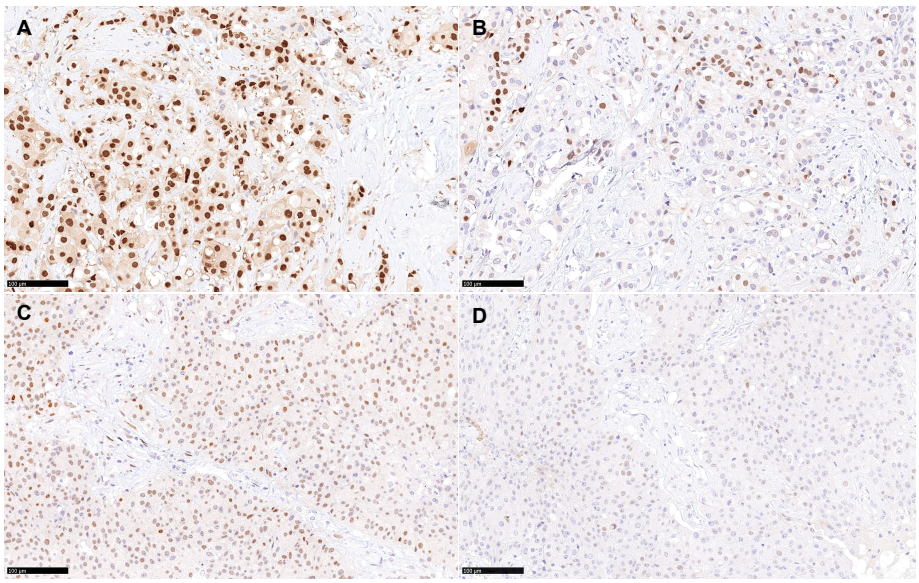


Figure 3. Immunohistochemical staining. Representative IHC for the Androgen Receptor -AR (A, C) and Estrogen Receptor - ER (B, D) in two BC cases with high AR levels with respect to ER (AR/ER≥2). Although ER is expressed at the IHC level in these cases, the molecular test classified them as non-luminal subtypes.

Table 3. Clinical-pathological characteristics of ER+/AR+ BC patients.

Characteristics		Total (%)	AR/ER<2 (%)	AR/ER≥2 (%)	P Value (Fisher test)
Number of patients		284 (100)	268 (94.3)	16(5.7)	--
Median Age (Interval)		62 (31-88)	62 (31-87)	65 (47-88)	0.309*
Grading	**1**	104 (36.7)	103 (38.4)	1 (6.3)	<0.001
	2	128 (45)	122 (45.5)	6 (37.5)	
	3	52 (18.3)	43 (16.1)	9 (56.2)	
Tumor size	**<15 mm**	149 (52.5)	146 (54.5)	3 (18.7)	0.004
	≥ 15 mm	135 (47.5)	122 (45.5)	13 (81.3)	
Metastatic Lymph nodes	**0**	191 (67.2)	183 (68.3)	8 (50)	<0.001
	1-3	62 (21.8)	61 (22.8)	1 (6.3)	
	4-9	22 (7.8)	18 (6.8)	4 (25)	
	>9	9 (3.2)	6 (2.1)	3 (18.7)	
Ki-67	**<20%**	168 (59.2)	162 (60.4)	6 (37.5)	0.075
	≥20%	116 (40.8)	106 (39.6)	10 (62.5)	
PgR	**<20%**	64 (22.5)	50 (20.5)	9 (56.3)	0.001
	≥20%	220 (77.5)	213 (79.5)	7 (43.7)	
ER%	**Median (interval)**	90 (2-100)	95 (30-100)	18 (2-45)	<0.001*
AR%	**Median (interval)**	50 (5-99)	40 (5-99)	80 (25-99)	0.01*
Relapse	**No**	233 (82)	225 (84)	8 (50)	0.001
	Local	5 (1.8)	5 (1.9)	0	
	Distal	46 (16.2)	38 (14.1)	8 (50)	
Surgery	**Quadrantectomy**	188 (66.2)	181 (67.5)	7 (43.7)	0.036
	Mastectomy	96 (33.8)	87 (32.5)	9 (56.3)	
Therapy†	**HT**	198 (69.7)	190 (70.9)	8 (50)	0.073
	AT	85 (29.4)	77 (28.7)	8 (50)	

*****P value from Student's t-test. †1 patient refused therapy. HT: Hormonal therapy. AT: Adjuvant therapy

AR/ER ratio and impact on prognosis

As shown in Table 4, univariate analysis confirmed that an AR/ER ratio ≥2 was one of the most significant markers of poor survival (HR = 7.55 for DFI, and HR = 10.84 for DSS, both P<0.001), together with tumour grade, tumour size ≥15 mm, nodal involvement ≥4 and high Ki67 index. Moreover, the Kaplan-Meier curves and the

Log-rank test showed significant differences in the survival times between the two groups (DFI & DSS P<0.001) (Figure 4A, 4B). In the analyses, were also included ER and AR expression as continuous variables to compare the weight on the prognosis of different levels of the receptor expression with the AR/ER ratio. While the percentage of AR expression did not show any impact on prognosis, the levels of ER were correlated with prognosis although at a lower significance compared to the AR/ER ratio (Table 4). Multivariate analysis confirmed an independent effect on the prognosis of the AR/ER ratio. According to this model, patients with an AR/ER≥2 were five times more likely to relapse (HR=4.96, P<0.001 for DFI) and eight times more likely to die of BC (HR=8.69, P=0.004 for DSS) compared with patients with a ratio <2. Tumour size ≥15 mm, lymph nodes >9, and a high Ki67 index had an unfavourable effect on DFI and DSS (Table 5). The proportionality assumption was satisfied both for the DFI (P=0.1227) and DSS (P=0.3517).

Table 4. AR/ER ratio and impact on prognosis. Univariate analysis

Characteristics		DFI		DSS	
		HR (95% CI)	***P***	***HR (95% CI)***	***P***
Age		0.99 (0.96-1.02)	0.694	0.97 0.93-1.01	0.264
Grading		3.02 (1.96-4.66)	<0.001	5.26 (2.37-11.7)	<0.001
Tumor size ≥ 15		6.97 (3.22-15.06)	<0.001	11.9 (2.74-52)	<0.001
Metastatic Lymph nodes	0	1			
	1-3	2.92 (1.37-6.23)	<0.005	2.85 (0.71-11.4)	0.138
	4-9	5.58 (2.41-12.9)	<0.001	12.2 (3.44-43.3)	<0.001
	>9	15.5 (6.25-38.6)	<0.001	23.17 (5.74-93.3)	<0.001
Ki-67≥20%		7.66 (3.55-16.52)	<0.001	12.25 (2.81-53.3)	<0.001
PgR≥20%		0.65 (0.34-1.25)	0.201	0.77 (0.27-2.16)	0.061
ER%		0.98 (0.97-0.99)	0.027	0.98 (0.96-0.99)	0.016
AR%		1.00 (0.99-1.01)	0.541	0.99 (0.98- 1.02)	0.994
AR/ERα≥2		7.55 (3.31-17.2)	<0.001	10.84 (3.52-33.3)	<0.001
HT vs AT		3.77 (2.02-7.03)	<0.001	3.85 (1.42-10.42)	0.008

To exclude the possibility that prognostic information from the AR/ER ratio was only a consequence of the low ER levels, an additionally cut-off point for ER nuclear staining at 10% was tested. As expected, patients with lower ER levels

(<10%) were associated with worse DFI & DSS (Annex 3). However, according to the Akaike information criterion (AIC) test, which was used for model selection, the AR/ER ratio model received the lowest AIC score (DFI AIC=378.8, DSS AIC=139.7), indicating that this model is more effective at providing prognostic information than the model with an ER cut-off at 10% (DFI AIC=384.6, DSS= AIC=145.9). Furthermore, although patients with lower ER levels were more likely to have AR/ER≥2, 56.2% of tumours (9/16 cases) with a high AR/ER ratio had a high ER level (≥10%).

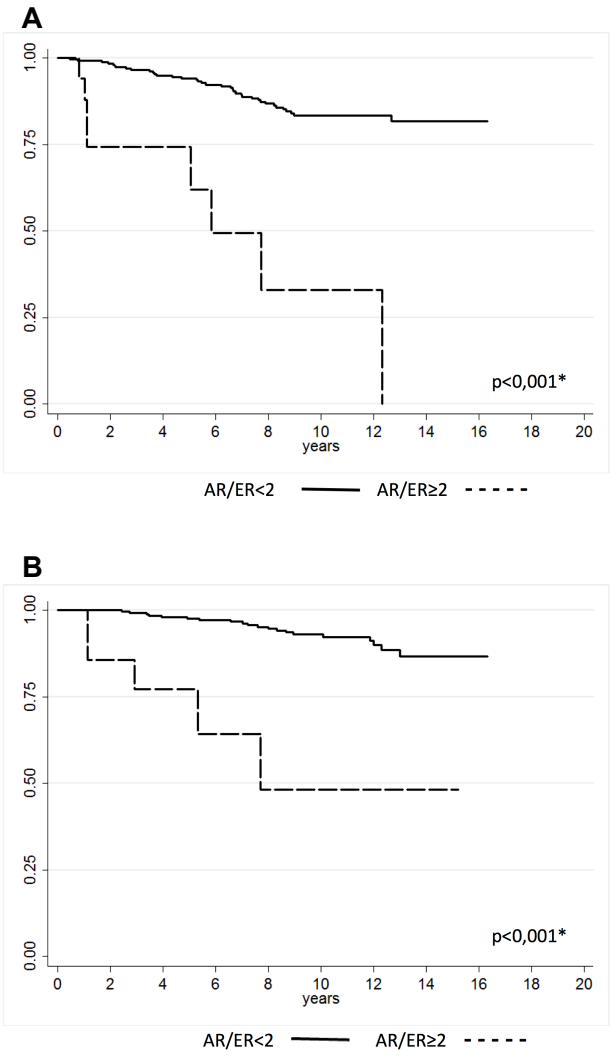


Figure 4. Survival curves for AR/ER<2 Vs. AR/ER≥2. A. Disease-Free Interval (DFI)**. B.** Disease-Specific Survival (DSS)**. ***Log-rank test for equality of survivor functions.

Table 5. AR/ER ratio and impact on prognosis. Multivariate analysis

Characteristics		DFI*		DSS*	
		HR (95% CI)	*P*	*HR (95% CI)*	*P*
Age		1.01 (0.98-1.04)	0.474	0.98 (0.94-1.03)	0.602
Tumor size ≥ 15		4.16 (1.88-9.18)	<0.001	8.87 (1.71-46)	0.009
Metastatic Lymph nodes	0	1			
	1-3	1.41 (0.58-3.40)	0.441	1.28 (0.23-7.1)	0.778
	4-9	1.59 (0.63-3.99)	0.321	3.45 (0.81-14.7)	0.095
	>9	4.42 (1.66-11.79)	0.003	5.71 (1.17-27.7)	0.031
Ki-67≥20%		3.98 (1.78-8.86)	<0.001	5.26 (1.12-24.6)	0.035
AR/ERα≥2		4.96 (1.95-12.68)	<0.001	8.69 (2.02-37.44)	0.004
HT Vs. AT		1.64 (0.72-7.03)	0.234	1.02 (0.25-4.18)	0.974

*Test of proportional-hazards assumption global test DFI p=0.3188, DSS p=0.3871

AR/ER≥2 and association with intrinsic molecular subtypes

A Prosigna-PAM50 assay was performed on the 19 cases with a ratio AR/ER≥2 to evaluate their ROR and molecular subtype. Twelve out of the 19 cases (63.2%) resulted in intermediate or high-risk categories (High probability of distant recurrence at 10 years) (Table 6). Then the IHC-based subtypes (57, 58) were compared with the intrinsic molecular subtype obtained by the Prosigna®-PAM50 assay and the percentage of ER expression. Three cases were classified as IHC Luminal A (15.8%) and 16 cases as Luminal B (84.2%). The concordance between the IHC subtypes and intrinsic molecular subtypes was very low (k=0.0583) since only 2 cases (10.5%) maintained the same subtype (Luminal A) using the Prosigna®-PAM50 assay. Molecular tests classified 47.4% of samples as Luminal A, 5.3% as Luminal B, 10.5% as HER2-enriched and 36.8% as a Basal-like subtype (Figure 5A, Table 6). Thus, gene expression analyses showed that 47.4% of BCs with an AR/ER ratio ≥2 were assigned to non-luminal subtypes (Figure 5A, Table 6). The correlation with the percentage of ER expression showed that 6 of the cases that switched from luminal to not-luminal had an ER<10%, although two cases classified as Luminal A by Prosigna-PAM50 assay had an ER<10% (2% and 5% respectively) (Figure 5B).

Table 6. Characteristics of cases evaluated with Prosigna - PAM50 assay.

Clinical and molecular characteristics		AR/ERα≥2 n(%)
Grading	1	1(5.3)
	2	8(42.1)
	3	10(52.6)
Tumor size	<15 mm	5(26.3)
	≥ 15 mm	14(73.7)
Metastatic Lymph Nodes	0	11(57.9)
	1-3	1(5.3)
	4-9	4(21)
	>9	3(15.8)
Ki67	<20%	8(42.1)
	≥20%	11(57.9)
PgR	<20	12(63.2)
	≥20	7(36.8)
IHC-based subtype	Luminal A	3(15.8)
	Luminal B	16(84.2)
PAM50-Intrinsic molecular subtype	Luminal A	9(47.4)
	Luminal B	1(5.3)
	HER2-Enriched	2(10.5)
	Basal-Like	7(36.8)
Prosigna Risk Category	Low	7(36.8)
	lntermediate	4(21.1)
	High	8(42.1)
Prosigna Risk Category - PDR†	Low	7(4.57)*
	lntermediate	4(10.25)*
	High	8(34.87)*

† Probability of Distant Recurrence. *Mean percentage of PDR to 10 years for each category

Correlation between AR/ER ratio and cellular proliferation

According to IHC and relative quantification - qPCR analysis ($2^{-\Delta\Delta Ct}$), from 47 ER+ cases with matched fresh-frozen tissue, 10 (21,3%) had both higher AR gene and protein expression levels relative to ER expression levels (AR/ER≥2). After divide

cases according to AR/ER ratio levels (≥2 Vs. <2), tumours with ratio higher than two (2) were not associated with clinico-pathological characteristics of bad prognosis, except for a clear relationship with amplification of HER2 gene (Table 7). There were not differences in the expression of the main IHC proliferation marker - Ki67 between groups (AR/ER≥2. Vs. <2.). However a cell proliferation index, evaluated by TaqMan-qPCR, as the mean expression of 5 proliferation genes, showed that patients with AR/ER≥2 have higher proliferation (P<0.05), even when only ER+/HER- cases were studied. Furthermore, the cell proliferation index in cases with AR/ER≥2 was similar to that observed in ER- BC patients (Figure 6), which are characterized for have higher levels of proliferation.

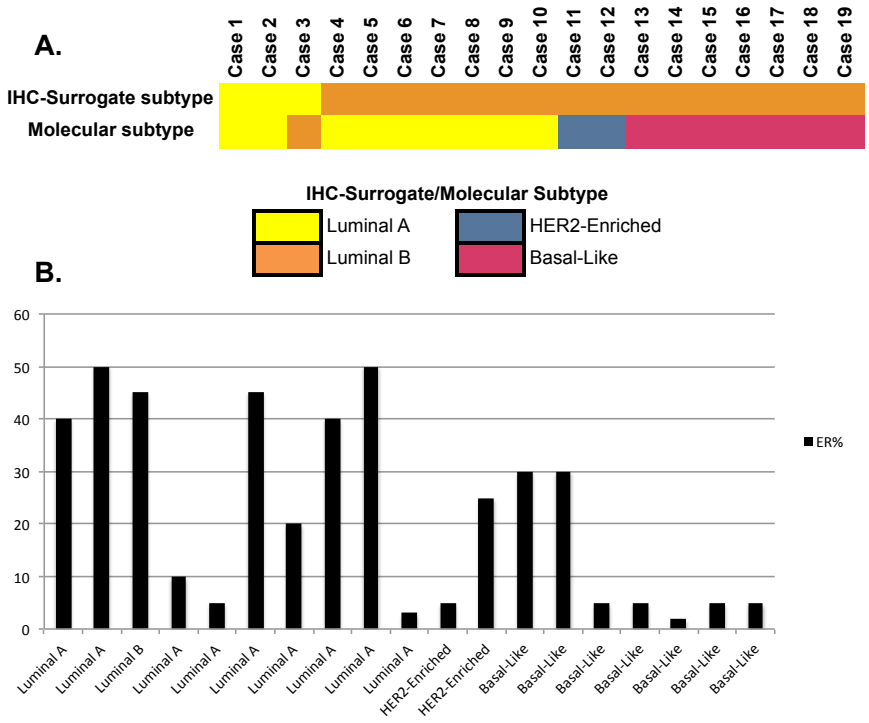


Figure 5. IHC-based Vs. Intrinsic molecular subtypes. **A.** Following guidelines recommendations, all BC with an AR/ER ratio ≥2 (19 cases) were classified as luminal by IHC. However, the Prosigna®-PAM50 assay changed the classification of 17 cases (89.5%), almost half of them to the non-luminal subtypes. **B.** Correlation of the ER percentage (ER%) nuclear staining (IHC) and intrinsic molecular subtypes in BC with an AR/ER ratio ≥2.

2.4 DISCUSSION

In this part of the study, it was demonstrated that within ER+ BCs, the AR/ER ratio may represent an additional independent prognostic marker. Specifically, we showed that BCs with an AR/ER ratio ≥2 had a worse DFI and DSS. This particular subset of tumours is rare within ER+ BCs and, from the molecular point of view; they do not always fit with the luminal subtype and present higher proliferation.

Table 7. Clinical and pathological characteristics of 47 ER+ cases with matched fresh-frozen tissue.

Caracteristicas clínicas		Total n(%)	AR/ER<2 n(%)	AR/ER≥2 n(%)	P Value (Fisher
Number of patients		47 (100)	37 (78.7)	10 (21.3)	--
Grade	1	4 (8.5)	3 (8.1)	1 (10)	0.215
	2	20 (42.6)	18 (48.6)	2(20)	
	3	23 (48.9)	16 (43.2)	7 (70)	
Tumoral size*	< 20 mm	16 (34.8)	12 (33.3)	4 (40)	0.485
	≥ 20 mm	30 (65.2)	24 (66.7)	6 (60)	
Metastatic lymph nodes	0	22 (46.8)	20 (54.1)	2 (20)	0.108
	1-3	14 (29.8)	9 (24.3)	5 (50)	
	>3	11 (23.4)	8 (21.6)	3 (30)	
Vascular invasion	No	7 (14.9)	4 (10.8)	3 (30)	0.155
	Si	40 (85.1)	33 (89.2)	7 (70)	
Ki67	< 20%	14 (29.8)	12 (32.4)	2 (20)	0.366
	≥ 20%	33 (70.2)	25 (67.6)	8 (80)	
PgR	< 20	30 (63.8)	26 (70.3)	4 (40)	0.136
	≥ 20	17 (36.2)	11 (29.7)	6 (60)	
HER2 Status	Negative	36 (76.6)	33 (89.2)	3 (30)	0.000
	Positive	11 (23.4)	4 (10.8)	7 (70)	

Patients were grouped according to AR/ER ratio (<2 Vs ≥2), calculated by relative quantification – TaqMan/qPCR analysis. *Missed data for one patient.

The prognostic role of AR in ER+ BC has been extensively studied. Several authors have reported that AR expression in luminal cancers is associated with a better outcome compared to AR negative BCs (30, 47, 65). However, some reports suggest that AR could be related to BC progression (66), as it is detected in a significantly higher percentage of ductal carcinomas "in situ" (DCIS) that are adjacent to invasive carcinomas than in pure DCIS (41).

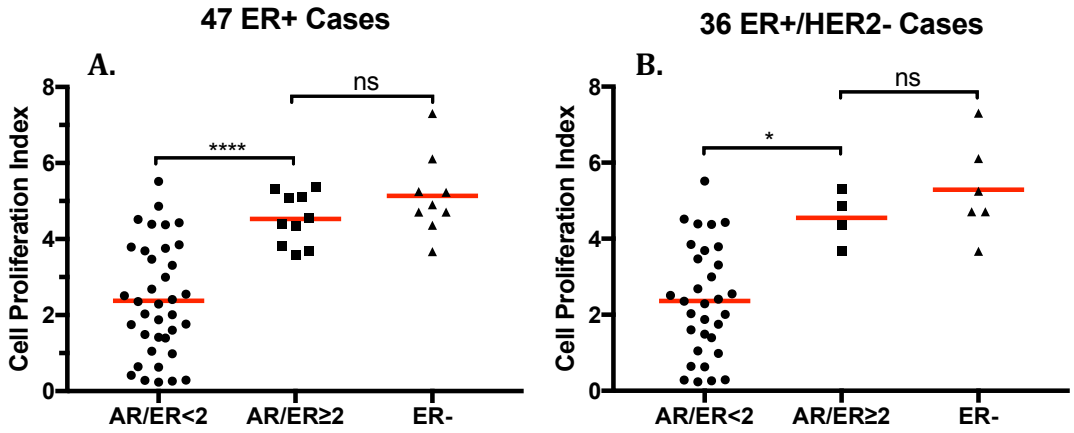


Figure 6. AR/ER ratio Vs. Cell Proliferation Index (CPI). CPI, evaluated as the mean expression of 5 proliferation genes, showed that patients with AR/ER≥2 have higher proliferation levels. **A.** All 47 ER+ BC patients. **B.** 36 ER+/HER2- BC patients.
**** $p<0.0001$; * $p<0.05$; ns – Non significant.

Moreover, although the expression of ER and PgR decrease during BC progression (from DCIS to invasive and from G1 to G3), AR expression is highly conserved during BC progression, as it is detected in a high percentage of metastatic tumours (66, 67). In addition, Gonzalez et al. (68) found that AR+ tumours are frequently positive for matrix metalloproteinases (MMPs), which have been involved in breast tumour dissemination. Finally, a recent study indicated that AR expression can induce the epithelial-to-mesenchymal transition in ER+ BC cells, conferring them with metastatic potential (69). Panet-Raymond et al. (70) reported that co-expression of both ER and AR reduces the trans-activation function of AR and Takagi et al. (71) suggested that AR signalling is suppressed in BC by high ER signalling activity.

All these results indicate that the interaction between the ER and AR levels may influence the AR activity. In line with this hypothesis, here it was found that BCs with a high AR/ER ratio are associated with aggressive biological features and worse prognosis.

To the best of our knowledge, only Cochrane et al. reported an association between the AR/ER ratio and the outcome (56). In agreement with our results, they showed that BCs with an AR/ER ratio ≥2 had a worse survival. However, they

reported a higher percentage of cases with an AR/ER ratio ≥2 than in our series (11.4% vs 6% respectively), which is probably related to differences in case selection since we excluded tumours with HER2 positivity. The molecular analyses with Prosigna®-PAM50 confirmed that most cases with AR/ER≥2 had a high to intermediate risk of recurrence. In addition, Prosigna®-PAM50 assay assigned 47.4% of the IHC-luminal cases to the non-luminal intrinsic molecular subtypes. As seen in non-luminal BCs, our TaqMan-qPCR results also showed that tumours with AR/ER≥2 have higher levels of cell proliferation and confirmed the use of AR/ER ratio as a marker of poor prognosis in ER+ BCs.

These results could suggest that tumours with a high AR/ER ratio could be resistant to hormone therapy. In fact, in vitro studies have demonstrated that hormone therapy-resistant tumours express higher levels of AR and lower ER levels than hormone therapy-sensitive tumours (72-75). To confirm these experimental data, Cochrane et al. demonstrated that AR/ER≥2 was associated with an increased risk of tamoxifen therapy failure in BC patients (56). Taken together, these data may suggest that BCs with an AR/ER≥2 could represent tumours that are changing or evolving from ER-dependence (luminal subtype) to AR-dependence, with the progressive loss of ER expression (non-luminal subtype).

This study has some limitations due to its retrospective design. We included in the analyses, patients with different treatment (hormone therapy and chemotherapy) and we do not have validation setting of patients to confirm our data. To address these limitations and validate these data, future studies need to include larger cohort of patients, who possibly underwent the same therapeutic approach. Notwithstanding, results presented here suggest that tumours with AR/ER≥2 should be carefully evaluated and reinforce the idea of targeting AR for BC treatment.

3. CHAPTER 2

"FOXA1 AND AR IN INVASIVE BREAST CANCER: NEW FINDINGS ON THEIR CO-EXPRESSION AND IMPACT ON PROGNOSIS IN ER-POSITIVE PATIENTS"

3.1 INTRODUCTION

An urgent need in BC care is to move a step forward from the standard histopathological and immunophenotypical diagnosis to better stratify patients by risk of recurrence and evaluate their eligibility to specific treatments. In BC, ER and AR regulate cell proliferation and differentiation. They are frequently co-expressed; up to 80% of BCs express AR, but, its prognostic role has not been satisfactory understood. It is well established that AR is linked to the expression and function of ER and other regulatory molecules (76). However AR may be expressed in ER- BC, where it modulates gene transcription by using regulatory molecules and pathways normally activated by ER (77). As a result, in ER- BC cells, androgens activate cell proliferation (78), whereas in ER+ cells, androgens inhibit cell proliferation (51, 79). In line with these data, previous studies demonstrated that patients with AR+/ER+ BC have a better prognosis compared to those affected by AR-/ER+ BC (42, 64).

FOXA1, a member of the forkhead family protein (36), which have the ability of creates an open chromatin configuration (pioneer factor) to recruit other transcriptional regulators, have been recognized as the major regulator of ER DNA binding and transcription of its target genes (80). Several studies (37, 81-88) evaluated the prognostic role of FOXA1 in BC, and demonstrated that in ER+ carcinomas the expression of FOXA1 is positively correlated with a better prognosis (Longer DFI and DSS). A recent study performed in luminal A BCs (ER+) shown recurrent mutations and high activity of FOXA1 (89), which suggest that evaluate it's gene and protein status may help explain heterogeneous features of hormone receptor-positive tumours.

In addition, it have been reported that FOXA1 promotes AR DNA binding too, in both ER+ and ER- BC cells (77, 90, 91). Interestingly, a study of AR and FOXA1 in a group of triple negative breast cancer (TNBC – ER-, PgR-, HER2-) suggested that co-expression of both markers seems to be associated with distinct clinicopathological features of luminal tumours compared to other TNBCs (38). These results provided insights about the close molecular connection between AR and FOXA1; however, clear implications of these biomarkers on tumour biology and patient prognosis have not been fully explained, mainly in ER+ tumours. Indeed, the role of FOXA1/AR co-expression in ER+ BC has not been investigated, although it has been suggested that the relative ratio among FOXA1, ER and AR could influence growth and aggressiveness of cancer cells (92).

Accordingly, the aim of this part of the research was first to assess the co-expression, at both protein and mRNA levels, of FOXA1 and AR in BC, and then to evaluate the prognostic impact of their co-expression in ER+ BC patients.

3.2 PATIENTS AND METHODS

Case series

We collected a series of 479 patients that underwent surgery for BC from June 1994 to December 2012 at the Breast Unit of the Città della Salute e della Scienza Hospital of Turin, Italy. All patients were treated with surgery, either mastectomy or wide local excision, followed by radiotherapy (Figure 7).

Clinical-pathological data such as age at time of diagnosis, surgery (conserving surgery vs radical mastectomy), type of therapy (hormonal therapy, chemotherapy), type and site of recurrences, histological types, tumour size (<15 mm vs ≥15 mm), nodal involvement, histologic grade and vascular invasion were collected. Medical charts of all patients were reviewed to confirm accuracy of previously recorded data. Tumour slides were re-evaluated to select representative blocks that were used to construct multicore tissue microarrays (TMAs, tissue arrayer Galileo TMA CK 3500, Integrated Systems Engineering Srl, Milan, Italy), as previously described (59).

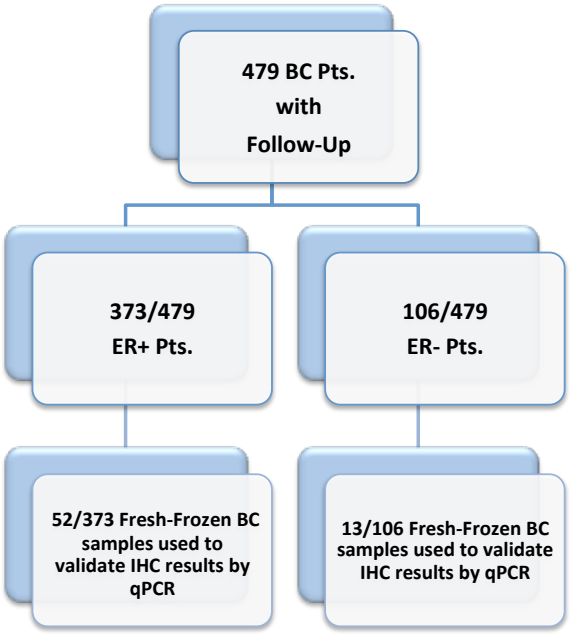


Figure 7. Flow chart for the study of FOXA1 and AR expression in 479 BC patients.

Immunohistochemistry (IHC)

To confirm the results of the diagnostic reports, IHC was performed on TMA sections using an automated slide processing platform (Ventana BenchMark AutoStainer, Ventana Medical Systems, Tucson, AZ, USA) and the following primary antibodies were used: prediluted anti-ER rabbit monoclonal antibody (SP1, Ventana-Roche, Tucson, AZ, USA); prediluted anti-Progesterone receptor (PgR) rabbit monoclonal antibody (1E2, Ventana-Roche); anti-Ki67 monoclonal antibody (MIB1, diluted 1:100 Dako); anti-human c-erbB2 oncoprotein (Ventana Pathway HER-2/Neu-4B5). In addition, AR and FOXA1 expression were tested using anti-AR mouse monoclonal antibody (AR441, diluted 1:50, Dako, Glostrup, Denmark) and prediluted anti-FOXA1 mouse monoclonal antibody (2F83, Ventana-Roche). Positive and negative controls (omission of the primary antibody and IgG-matched serum) were included for each IHC run.

The cut-off value for ER and PgR expression was set at 1%, as suggested by St Gallen recommendations (16), and the same cut-off was also adopted for AR and FOXA1 expression (42). The percentage of Ki67-positive cells was recorded and the cut-off for dichotomizing tumours with low and high proliferative fraction

was established at 20% according to 2013 St Gallen recommendations (93) and also on the basis of the median Ki67 value of our local laboratory (57, 61). HER2 status was classified as negative (score 0, 1+ and 2+ not amplified) or positive (when scored 3+ by IHC or HER2 amplified by FISH) according to the recommended guidelines for invasive carcinoma (23).

Real-time PCR (qPCR) analysis

To determine the specificity of AR and FOXA1 antibodies, gene expression levels (using qPCR) were compared with IHC results. The relationship between AR and FOXA1 was validated using relative quantification mRNA analyses.

qPCR for AR and FOXA1 mRNA was performed on 65 fresh-frozen BC samples (Figure 7). Total RNA was extracted from tissues using TRIzol Reagent (Invitrogen Ltd, Paisley, UK) following manufacturer's instructions. DNase I was added to remove remaining genomic DNA. 1 μg of total RNA was reverse-transcribed with iScript cDNA Synthesis Kit (Bio-Rad Laboratories Inc., Hercules, CA, USA), following manufacturer protocol. Primers (Annex 4) were designed using Beacon Designer 5.0 software according to parameters outlined in the Bio-Rad iCycler Manual. Specificity of primers was confirmed by BLAST analysis. qPCR was performed using a BioRad iQ iCycler Detection System (Bio-Rad Laboratories Inc., Hercules, CA, USA) with SYBR green fluorophore. Reactions were performed in a total volume of 25 ml containing 12.5 ml IQ SYBR Green Supermix (Bio-Rad Laboratories Inc., Hercules, CA, USA), 1ml of each primer at 10mM concentration, and 5ml of the previously reverse-transcribed cDNA template. The protocol used was as follows: denaturation (95°C for 5 min) and amplification repeated 40 times (95°C for 15 sec, 60°C for 30 sec). At each run, a melting curve analysis was performed to ensure a single specific amplified product for every reaction. Results were normalized using the Delta-Ct (Δct) method, using β-actin (ACTB) and Glyceraldehyde-3- Phosphate Dehydrogenase (GAPDH) as housekeeping genes. Nine fresh-frozen breast normal tissues were used as controls.

Statistical and Survival Analyses

Pearson's Chi square test and Student's t-test were preliminary performed to compare respectively categorical and continuous variables, and to evaluate potential differences in the variable distribution among groups. Disease-Free Interval (DFI) was calculated from the date of surgical excision of the primary tumour to the date of first disease relapse or last check-up. Disease-specific survival (DSS) was calculated from the surgical excision date of the primary tumour to the date of BC death or last check-up. Survival distribution curves were plotted using the Kaplan-Meyer method and the statistical comparisons were performed using the log-rank test. Cox regression analyses were carried out on DFI and DSS to calculate crude and adjusted HRs and 95% confidence intervals (CIs) for the different study group. Cases lost to follow up and cases with a non-BC related cause of death were censored at the last follow up control. A model was created to evaluate the prognostic role of different variables. The proportional hazard assumption was assessed with the Schoenfeld residuals. This did not give reasons to suspect violation of this assumption. The nature of variables (continue/categorical) included in the model was evaluated considering literature reports and the results of the log-likelihood ratio test. Akaike information criterion (AIC) was used for model selection. All statistical tests were two sided. P-values < 0.05 were considered significant. Statistical analyses were performed using Stata/SE12.0 Statistical Software (STATA, College Station, TX).

3.3 RESULTS

Correlation of FOXA1 and AR IHC expression with clinical-pathological features

Clinical and histopathological features of the whole population are reported in Table 7. The median follow up was 10.1 years. The majority of patients was over 50 years (>80%) of age and underwent conservative surgery. Positive expression of ER, AR and FOXA1 was observed in 78%, 60% and 85% of cases respectively. As previously reported (37, 84), also in this cohort FOXA1 positivity was associated with small tumour size (<15 mm), absence of lymph node metastases, low

histological grade, no special type (NST) histotype, low level of Ki67, as well as, with ER+ and PgR+ tumours (Annex 5). In the consecutive series of patients, 58% of cases showed AR+/FOXA1+ (Table 8), while 14% presented AR-/FOXA1- immunophenotype and only 1.7% of cases were AR+/FOXA1-. This latter subgroup, did not show specific features (Annex 6).

qPCR analysis: correlation between mRNA and protein levels of FOXA1 and AR in BC

A strict correlation of FOXA1 and AR mRNA with protein expression was found (Figure 8a and 8b). To correlate the expression of ER, AR and FOXA1, qPCR results were used, since this procedure allows quantifying more precisely the level of expression of each molecule. As shown in Figure 9, there was a linear correlation of the level of FOXA1 mRNA with the level of AR ($r=0.8975$; $P<0.001$) (Figure 9a) and ER ($r=0.7326$; $P<0.001$) mRNA (Figure 9b).

Furthermore, FOXA1 mRNA was closely related to AR mRNA expression, regardless of ER status. Indeed, FOXA1 mRNA was expressed in all samples with ER+/AR+ (27 cases) and ER-/AR+ (3 cases) (Low delta-Ct. Figure 10b and 10d), in 8/25 ER+/AR- cases and in only 1/10 ER-/AR- cases (High Delta-Ct. Figure 10c and 10e).

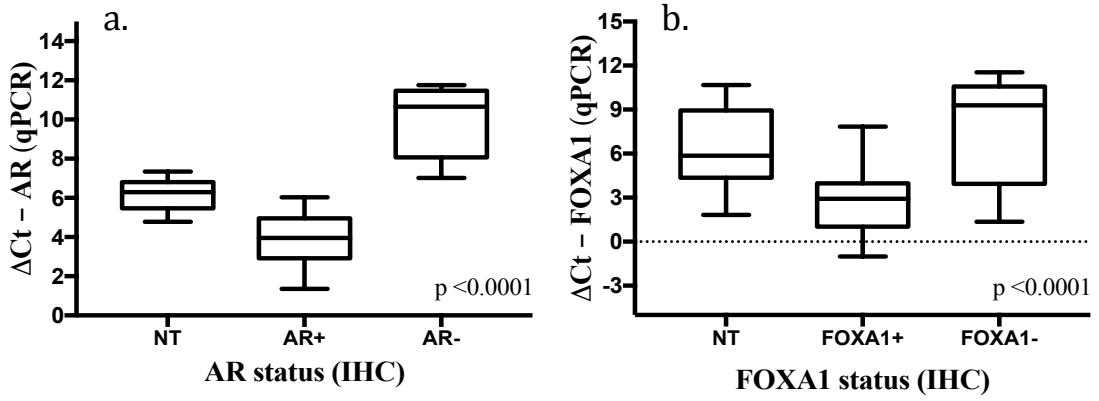


Figure 8. Protein (IHC) and mRNA (qPCR) expression for (**a)** Androgen receptor (AR) and (**b)** Forkhead box protein A1 (FOXA1). Normal Tissue (NT)**.** P value from ANOVA analysis

Table 8. Clinical and histopathological characteristics of 479 BC patients.

Characteristics		N (%)
Age	≤50	86 (18)
	>50	393 (82)
Type of surgery (missing 8 cases)	Conservative	282 (59.9)
	Mastectomy	189 (40.1)
Tumor Size (missing 7 cases)	<15 mm	176 (36.7)
	≥15 mm	296 (63.3)
Metastatic lymph nodes (missing 7 cases)	pN0	277 (58.7)
	pN1-3	195 (41.3)
Grade (missing 9 cases)	1	125 (26.6)
	2	187 (39.8)
	3	158 (33.6)
Histotype	CDI	305 (63.7)
	CLI	95 (19.8)
	others	79 (16.5)
Vascular invasion (missing 113 cases)	No	200 (54.6)
	Yes	166 (45.4)
ER%	0	106 (22.1)
	≥1%	373 (77.9)
PgR% (missing 48 cases)	0	122 (28.3)
	≥1%	309 (71.7)
Ki67% (missing 9 cases)	<20%	205 (43.6)
	≥20%	265 (56.4)
HER2 Status (missing 43 cases)	Negative	398 (91.3)
	Positive	38 (8.7)
FOXA1%	0	74 (15.4)
	≥1%	405 (84.6)
AR%	0	193 (40%)
	≥1%	286 (60%)
Therapy (missing 15 cases)	Only RT	18 (3.9%)
	HT	229 (49.4%)
	HT + CT	125 (26.9%)
	CT	81 (17.4%)
	No therapy	11 (2.4%)

RT: Radiotherapy. HT: Hormonal therapy. CT: Chemotherapy

Table 9. Correlations between FOXA1 expression and AR status according to immunohistochemistry test.

	FOXA1 Positive	*FOXA1 Negative*	P Value*
AR Positive	278	8	**<0.001**
AR Negative	127	66	

**Chi-Square (X^2)*

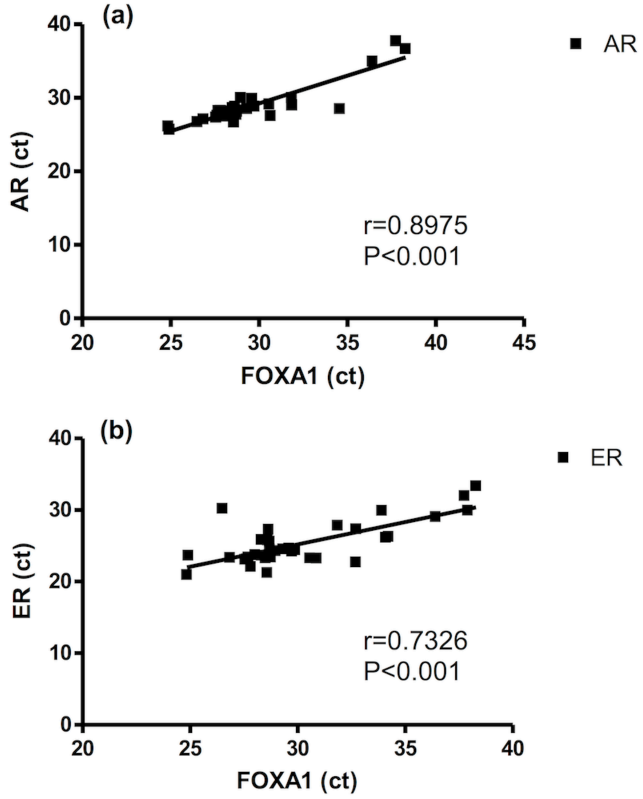


Figure 9. Spearman's correlation test show that FOXA1 mRNA level, positively correlated with mRNA levels of **(a)** Androgen receptor (AR) and **(b)** Estrogen receptor (ER).

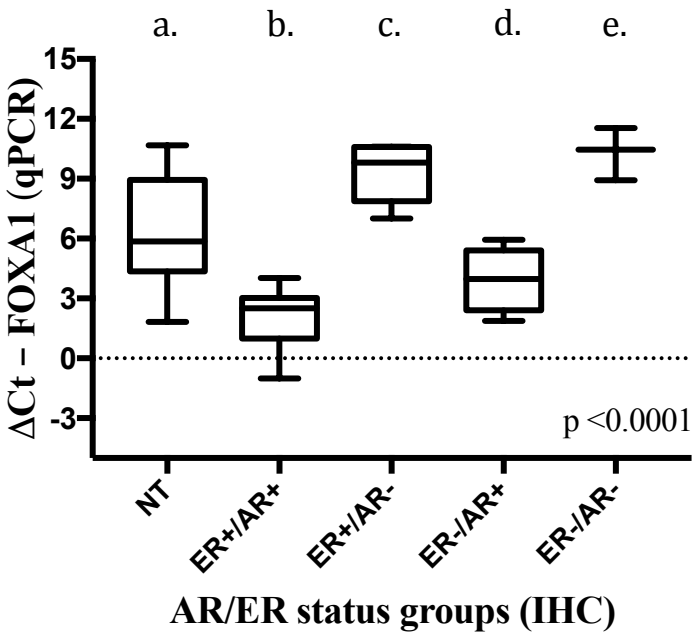


Figure 10. FOXA1 mRNA expression in tumours classified according to ER and AR status. **(a)** Normal Tissue (NT); (**b)** ER+/AR+; (**c)** ER+/AR-; (**d)** ER-/AR+; (**e)** ER-/AR-.

Impact of FOXA1 and AR IHC co-expression on prognosis

At univariate analysis performed on whole cohort, metastatic lymph nodes, histological grade, vascular invasion, ER and PR positivity, high Ki67 and HER2 overexpression were confirmed as significant prognostic factors. Additionally, the expression of AR and FOXA1 were associated with a better DFI and DSS. (Table 9. Annex 7)

To analyze the impact of FOXA1 and AR in patients with BC (ER+ or ER-), three BC subgroups were created (FOXA1+/AR+; FOXA1+/AR-; FOXA1-/AR-). We were unable to perform any analyses on the FOXA1-/AR+ BC since only 8 patients carried this phenotype (Table 8). As shown in Figure 11, in the consecutive series of patients, the lack of expression of both, FOXA1 and AR (FOXA1-/AR-), was related to a worse DFI and DSS compared to the other groups.

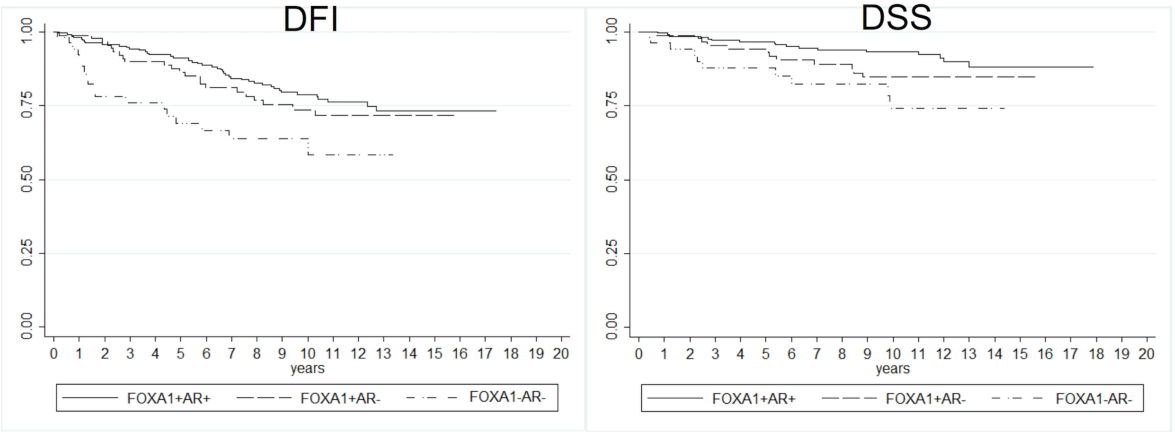
Finally, the relationship between FOXA1, AR and prognosis was investigated in BC patients stratified for ER expression. As shown in Figure 12, in

ER+ BC, FOXA1 expression was closely related to good prognosis independently of AR expression.

Multivariate analyses (Table 10) performed on ER+ BC confirmed that FOXA1 may provide more information than AR on DFI, but not on DSS. In the subset of patients with ER- BC, FOXA1, alone or in association with AR, did not show any relationship with outcome (data not shown).

Table 10. Univariate analysis of clinical and pathological data correlated with disease free interval (DFI) and disease specific survival (DSS).

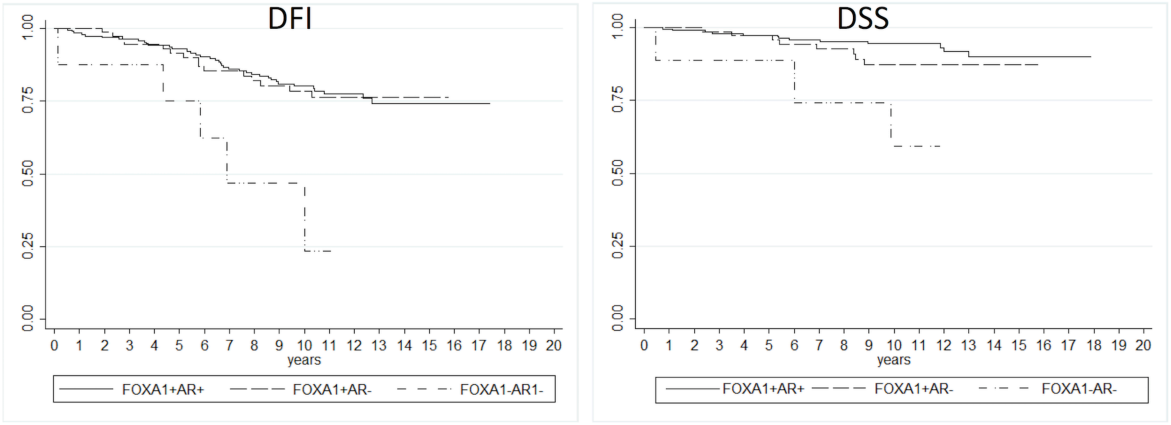
Characteristics		DFI			DSS		
		HR	CI	P	HR	CI	P
Age		0.98	0.96-1.00	0.110	1.00	0.98-1.03	0.727
Conservative vs Mastectomy		3.36	2.22-5.10	0.000	2.56	1.42-4.63	0.002
Metastatic lymph nodes	0	1			1		
	1	2.01	1.19-3.42	0.009	1.27	0.54-2.97	0.581
	2	5.63	3.22-9.86	0.000	5.26	2.44-11.3	0.000
	3	12.1	6.74-21.7	0.000	12.5	5.61-27.7	0.000
Histotype	CDI	1			1		
	CLI	0.75	0.44-1.26	0.275	1.03	0.52-2.06	0.925
	Other	0.59	0.30-1.15	0.122	0.40	0.12-1.32	0.132
Grade	1	1			1		
	2	2.53	1.35-4.74	0.004	3.34	1.12-9.94	0.030
	3	4.31	2.30-8.05	0.000	7.52	2.61-21.7	<0.001
Tumor Size >15 mm		4.98	2.46-10.1	0.000	5.42	1.90-15.4	0.002
Vascular invasion		5.16	2.99-8.90	0.000	3.84	1.86-7.93	0.000
ER Positive		0.44	0.28-0.69	0.000	0.34	0.18-0.64	0.001
PgR>20%		0.63	0.41-0.97	0.034	0.37	0.19-0.69	0.002
Ki67≥20%		3.10	1.96-4.92	0.000	4.15	1.99-8.64	0.000
FOXA1 Positive		0.54	0.32-0.90	0.019	0.43	0.21-0.88	0.022
AR positive		0.60	0.40-0.91	0.015	0.38	0.21-0.70	0.002
HER2 Positive		2.50	1.25-5.00	0.010	1.98	0.70-5.58	0.195



DFI	FOXA1+ /AR+	FOXA1+ /AR-	FOXA1- /AR-	Log-rank P
5-Years	88.4	80.5	64.6	0.0053
10-Years	76.1	71.1	56.3	

DFI	FOXA1+ /AR+	FOXA1+ /AR-	FOXA1- /AR-	Log-rank P
5-Years	95.1	90.6	85.3	0.0061
10-Years	93.4	84.7	73.6	

Figure 11. Kaplan–Meier estimates of DFI and DSS according to AR and FOXA1 in all breast tumours.



DFI	FOXA1+ /AR+	FOXA1+ /AR-	FOXA1- /AR-	Log-rank P
5-Years	89.9	84.5	57.4	0.0027
10-Years	77.2	75.3	21.5	

DFI	FOXA1+ /AR+	FOXA1+ /AR-	FOXA1- /AR-	Log-rank P
5-Years	95.7	97.2	90.9	0.0032
10-Years	94.4	87.3	58.9	

Figure 12. Kaplan–Meier estimates of DFI and DSS according to AR and FOXA1 in ER+ BC patients.

Table 11. Multivariate analysis. Association of patients and tumour characteristics with DFI and DSS among ER+ cases with complete data for all covariates.

Characteristics		DFI (global test p=0.5497)*			DSS (global test p=0.7496)*		
		HR	**CI**	**P**	**HR**	**CI**	**P**
Age		0.99	0.97-1.02	0.703	0.99	0.96-1.04	0.844
Tumor size≥15 mm		2.40	1.00-5.78	0.050	5.40	0.65-44.6	0.117
Metastatic lymph nodes	0	1					
	1	2.01	0.89-4.52	0.090	1.23	0.27-5.54	0.790
	2	4.24	1.68-10.7	0.002	4.27	1.07-17.0	0.040
	3	6.34	2.24-17.9	0.001	6.58	1.45-29.9	0.015
KI67≥20%		2.58	1.19-5.58	0.016	4.27	0.88-20.7	0.071
AR		0.87	0.45-1.70	0.701	0.57	0.22-1.50	0.258
FOXA1		0.24	0.08-0.74	0.013	0.34	0.04-3.08	0.340

3.4 DISCUSSION

Here, for the first time, was assessed the joint expression of FOXA1 and AR in BC, evaluating their prognostic impact according to ER status. It was confirmed that (i) the expression (protein and mRNA) of FOXA1 and AR is closely related: the majority of cases expressing AR showed FOXA1 positivity, conversely, negative expression of FOXA1 correlates with very low level of AR; (ii) the expression of FOXA1 is strictly related to good outcome, and in the subgroup of patients with ER+ BC FOXA1 may provide more information on DFI than AR.

FOXA1 is a “winged helix” transcription factor. It was demonstrated that, by interacting with histones H3 and H4, FOXA1 is responsible for opening compacted chromatin (94), permitting efficient interaction of ER with its response elements. For this reason, FOXA1 indicates the presence of a functional ER complex, which will respond to endocrine therapy (81, 95, 96). Moreover, FOXA1 may have a repressor effect on BC growth by promoting transcription of E-cadherin and cell cycle-dependent kinase inhibitor p27 (Kip1), thus reducing the motility and invasion of BC cells (97, 98).

These findings suggest that FOXA1 expression in BC may be associated with a better clinical outcome. In this study literature data was confirmed, demonstrating that FOXA1 is mainly expressed in low grade, lymph node negative BC tumours, with size <15 mm and low Ki67 index (84, 99, 100).

In addition, FOXA1 has been strongly associated with recruitment of AR (36) and, it has been suggested that in prostate epithelium FOXA1 acts with AR in promoting differentiation (101). ChIP-seq analysis of AR, ER, and FOXA1 in BC cell lines revealed a high level of co-occupancy between these markers, presumably due to the presence of forkhead motif found at AR and ER binding sites (80, 90, 102, 103). Furthermore, evidences of the relationship between AR and FOXA1 were supported by experiments demonstrating the co-localization of the two proteins on chromatin (77, 91, 103). Results reported here confirms these data, showing that BC tumours with high mRNA level of FOXA1 are generally ER and AR enriched. On the contrary, tissues with low FOXA1 mRNA level present low level of hormonal receptors, especially of AR.

In several studies has been demonstrated that AR expression is a favorable prognostic marker of disease outcome in ER+ BC (42, 64). This result has recently been confirmed in a meta-analysis conducted on 17,000 women with early-stage BC (30). The present work confirms the prognostic role of AR. However, the concurrent evaluation of the expression of both AR and FOXA1, shows that FOXA1 is superior to AR as prognostic marker in patients with BC, especially in ER+ cases. In fact, FOXA1 expression was always related to a better outcome even if AR was not detectable. Similar results were recently obtained in prostate cancer, where AR expression is the most important marker of prognosis (104) and in which it has been demonstrated that FOXA1 expression is closely related to prognosis independently of AR level.

In line with these data, studies on BC cell cultures suggested that AR functionality depends on role of FOXA1, which is required for AR to bind chromatin and for its transcriptional activity (77).

Sahu, B et al. suggested that in prostate cancers FOXA1 level may contribute to select specific AR binding sites on DNA, activating different gene

expression signatures (104). Data from this study indicate that FOXA1 may control the level of AR expression in ER+ BC, due to the very low number of AR positive and FOXA1 negative cases.

Finally, results presented here suggest that in BC the expression of FOXA1 is directly proportional to the expression of AR. Despite that, FOXA1 is found as a superior predicting marker of recurrences compared to AR in ER+ BC patients. Therefore, FOXA1 expression evaluated by IHC on ER+ BC specimens could be considered in routine diagnosis as an additional support to oncologists in the stratification and prognosis definition of luminal (ER+) BC patients.

4. CHAPTER 3

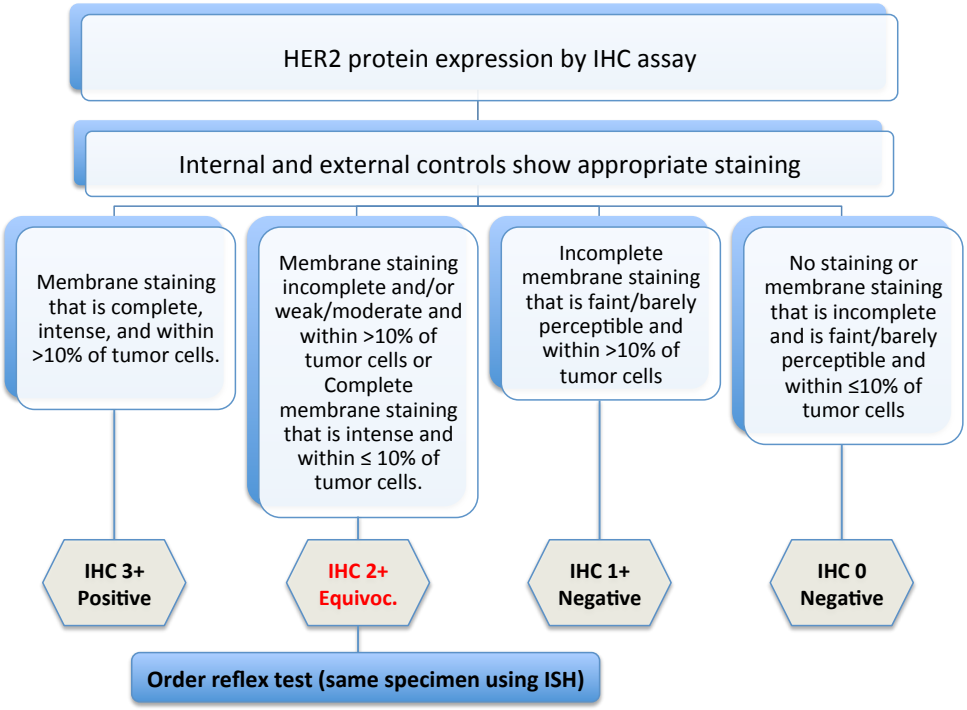
"ER-POSITIVE/HER2-DOUBLE-EQUIVOCAL BREAST CANCER: GENOMIC PROFILING, RELATIONSHIP WITH *HER2* GENETIC HETEROGENEITY, AND IMPLICATIONS FOR TREATMENT"

4.1 INTRODUCTION

HER2 status assessment is a fundamental prognostic and predictive step, since its protein overexpression or gene amplification have been recognized as marker of greater aggressiveness in approximately 15% of BC patients (21). In nearly 50% of these HER2 positive (HER2+) BC there is the coexistence of both, expression of ER and overexpression/amplification of HER2 (105, 106); however, several pre-analytical, analytical, and interpretational factors affect the precision and accuracy of HER2 evaluation, which also affects treatment decision-making.

Accordingly, the ASCO/CAP published recommendations for HER2 testing in BC (23). These guidelines indicate that HER2 status must first evaluate protein levels with IHC analysis, and when cases are classified as HER2 2+ by IHC (Figure 13a), recommendations suggests to perform a reflex tests like "In situ hybridization" (ISH) to identify *HER2* gene copy number. Furthermore, the guidelines provide a more detailed definition of intratumoral HER2 genetic heterogeneity (adoption of a single cut-off for both IHC and ISH >10%), besides that panelists acknowledged the presence of different heterogeneity patterns, namely (i) discrete populations of amplified and non-amplified cells, (ii) diffuse intermingling of amplified and non-amplified cells, or (iii) scattered/isolated amplified cells in a predominantly non-amplified tumour. Importantly, there was a consensus that discrete aggregated cells represented the only significant type of amplification (23), since there may be interobserver reproducibility between molecular pathologists and clinical data are lacking on the impact of scattered cell heterogeneity.

a.



b.

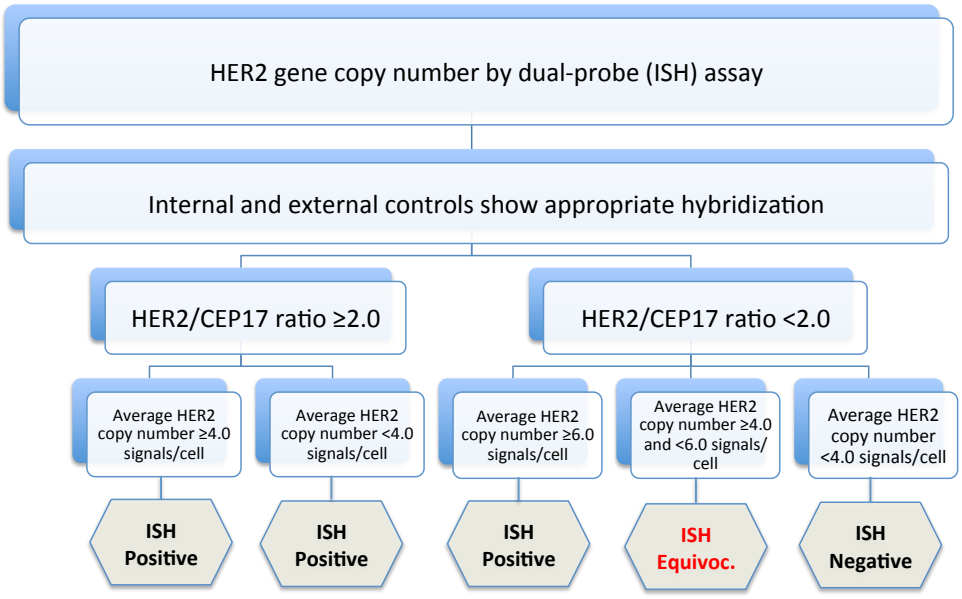


Figure 13. HER2 status assessment following ASCO/CAP guideline recommendations. **a.** Algorithm for HER2 protein level evaluation. **b.** Algorithm for *HER2* gene copy number evaluation. ISH equivocal cases are defined as "HER2 double-equivocal" carcinomas. Figure adapted from Wolff et al. 2013 (23).

Different authors (24, 25, 107-111) have highlighted that the guidelines have increased the number of cases with an equivocal result following ISH testing. If reflex tests are not effective, these cases can be defined as “double-equivocal” in terms of HER2 status, i.e., equivocal protein expression and gene copy number. It has been suggested that the use of alternative chromosome 17 probes might help reclassify these carcinomas as positive based on the HER2/CHR17 probe ratio (111-113). (Figure 13b). However, caution is advised, as chromosome 17 is well known to harbor complex rearrangements (like deletions on the small arm of chromosome 17, where probes typically target), leading to a higher ratio exclusively on the basis of lower mean chromosome 17 reference probe counts (60, 114). Regardless, the clinical relevance of equivocal HER2 copy number remains to be established.

HER2 gene levels have been previously examined in double-equivocal carcinomas using PCR-based methods and copy gains in 25% of cases, as well as, no copy number alterations in the remaining cases have been observed (75%) (110). When tested for HER2 protein levels using a quantitative proximity ligation assay, HER2 levels in double-equivocal carcinomas ranged from those similar to IHC-0/FISH-negative to those observed in IHC-2+/FISH-positive carcinomas (110). These data suggest that, rather than simply exhaustively exploring alternative methods to evaluate HER2 status, a complementary functional approach might be beneficial. On the other hand HER2 genetic heterogeneity is a frequent event in IHC-2+ (HER2 protein equivocal) carcinomas (up to 40%) (110, 115), more often featuring the diffuse intermingling pattern rather than discrete populations of amplified and non-amplified tumour cells (24, 110). Therefore, it’s important to note that the definitions of genetic heterogeneity may be affected by subjective evaluation and poor inter-observer reproducibility.

Hence, in this part of the thesis project we sought to stratify ER+/HER2- double-equivocal carcinomas using transcriptomics and to ascertain whether the prevalence and pattern of intratumoral *HER2* heterogeneity may affect the definition of the HER2 - equivocal category in the diagnostic setting.

4.2 PATIENTS AND METHODS

Cohort and FISH review

Forty-eight BC (n=32, Turin cohort; n=16, Milan cohort) scored as 2+ by IHC and harboring a HER2/CEP17 ratio <2 and HER2 copy numbers ≥4 and <6 by FISH were collected from the Pathology Division, Azienda Ospedaliera Citta' della Salute e della Scienza di Torino, University of Turin and the Pathology Division, European Institute of Oncology (IEO), Milan (23). A dedicated, anonymized database was created and clinicopathologic data (patient age, tumour size, histologic type, histologic grade, immunophenotype) and details of FISH output collected and recorded.

In addition to the original FISH scoring, two independent observers with expertise in *HER2* FISH testing reviewed the entire cohort and recorded mean *HER2* and CEP17 copy numbers, *HER2*/CEP17 ratios, and the prevalence and type of heterogeneity. Whenever *HER2* heterogeneity was detected, FISH results were reported either as whole (mean of *HER2* and CEP17 copy numbers of both amplified and non-amplified cells) or separate populations (mean *HER2* and CEP17 copy numbers and *HER2*/CEP17 ratios calculated within distinct populations).

Molecular subtyping by Prosigna

The formalin-fixed paraffin-embedded (FFPE) diagnostic blocks of the entire cohort of double-equivocal carcinomas (n=48) were cut for the Prosigna assay (NanoString Technologies, Seattle, WA) following the manufacturer's instructions. Briefly, a 4 μm thick section was H&E stained to identify the lesion and assess tumour cellularity. Depending on the tumour surface area, a series of 10 μm sections were mounted onto Superfrost glass slides (Thermo Fisher Scientific, Waltham, MA). RNA extraction and nCounter analysis were performed according to the Prosigna® instructions. Briefly, tissues obtained by macrodissection of 10 μm sections were processed with the Roche FFPET RNA Isolation Kit (Roche, Mannheim, Germany). Paraffin was removed with D-limonene, and tissue

specimens were digested with proteinase K overnight. Digested samples were bound to a silica column followed by an on-column DNase treatment to remove genomic DNA. Isolated RNA was eluted and tested using a spectrophotometer to ensure that it met Prosigna concentration and purity specifications. Isolated RNA was analyzed on the NanoString nCounter Dx Analysis System (NanoString Technologies, Seattle, WA), which delivers direct multiplexed gene expression measurements through digital readouts of mRNA transcript abundance. RNA was hybridized to 58 gene-specific probe pairs (50 target genes plus eight housekeeping genes) overnight at 65ºC in a single hybridization reaction. Each assay also included six positive quality controls and eight negative quality controls. Removal of excess probe followed by binding of the probe-target complexes to the surface of a specific nCounter cartridge was performed on the nCounter Prep Station (NanoString Technologies, Seattle, WA). Finally, the nCounter cartridge with immobilized probe/target complexes was read on the nCounter Digital Analyzer, thus providing risk of recurrence (ROR) scores, risk category, and molecular subtype information. Gene expression measurements were converted into intrinsic molecular subtypes, ROR scores, and risk categories using a fully pre-specified algorithm as previously described (62).

Global transcriptomics by microarray analysis

Two control groups of ER+ carcinomas, n=22 HER2 IHC score 0/HER2-non-amplified/negative (HER2-) and n=22 HER2 IHC score 3+/HER2-amplified/positive (HER2+), were subjected to global transcriptomics using the Whole-Genome DASL (cDNA-mediated Annealing, Selection, extension and Ligation; Illumina Inc., San Diego, CA) assay.

Tissue macrodissection and RNA extraction were performed as described above. RNA quality and quantity were assessed using the Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA). The Whole-Genome DASL assay was performed according to the manufacturer's instructions (WGDASL-HT Assay Guide 15018210 D). In brief, 500 ng total RNA of each sample was used. Fluorescent cDNA was obtained using the Human Whole-Genome DASL HT Assay kit (Illumina

Inc.). All the fluorescent cDNA was hybridized on HumanHT-12_V4 BeadChips for 18h. Following array washes with wash buffer, hybridized BeadChips were scanned using the Illumina HiScan SQ. Raw data were analyzed using Illumina Genome Studio software.

Genes with differential expression in HER2+ vs. HER2- BCs were identified based on Students't-test significance $p<0.01$ and on mean gene expression variations greater than ± 2-fold. A 24-gene classifier was derived. To best characterize the signature, we performed cluster analysis using GEDAS software and the "Fuzzy Self-organizing Maps" algorithm with cosenic distance to generate clusters (116).

Stratification of double-equivocal carcinomas by NanoString

Double-equivocal carcinomas confirmed by at least two out of the three FISH observers and control cohorts (ER+/HER2+, ER+/HER2-) were subjected to mRNA analysis using a customized nCounter GX CodeSet (Nanostring Technologies) including the 24-gene signature obtained by DASL, four housekeeping genes, six positive quality controls, and eight negative quality controls.

Reactions were performed according to the manufacturer's instructions. Briefly, RNA was isolated using the Roche FFPET RNA Isolation Kit (Roche). Total RNA concentration was assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific). Capture and reporter probes were hybridized to 300 ng of total RNA for 21 h at 65°C. Removal of excess probe followed by probe-target complex binding on the surface of an nCounter cartridge was performed on the nCounter Prep Station and data collected on the nCounter Digital Analyzer. Raw counts were normalized using nSolver Analysis Software v3.0. Background subtraction was performed for each sample by subtracting the mean of eight negative controls from all data points. Raw counts were further normalized to the six positive controls included in each CodeSet and to the four housekeeping genes (ACTB, B2M, GAPDH, and TBP).

Before proceeding with analyses, we assessed the relatedness of the samples included in the HER2+ and HER2- cohorts to exclude unrelated samples

with respect to expression of the genes included in the signature. Unrelated cases according to Pearson correlation coefficients were excluded from subsequent analyses. Analyses of genes significantly differentially expressed between subgroups was performed in MeV 4.8 software (117) using the between-subjects *t*-test (critical p-value 0.05). Unsupervised clustering was performed using nSolver Analysis software 3.0 (NanoString Technologies).

Cohort of BCs treated with neoadjuvant anti-HER2 therapy

Pathologic response data of a series of 37 HER2+ BC patients subjected to chemotherapeutic regimens including anti-HER2 therapy in the neoadjuvant setting were collected. The cohort comprised 10 HER2 double-equivocal invasive carcinomas of no special type and 27 invasive carcinomas of no special type matched for ER status and with comparable Ki67 range showing HER2 overexpression (score 3+) and HER2 amplification in >50% of tumour cells. All cases had been assessed by FISH in the same institution and scored according to the ASCO/CAP guidelines (23). The 10 double-equivocal carcinomas showed HER2 genetic heterogeneity (range 11-44%; mean 19%) in the form of diffuse intermingling of amplified and non-amplified cells (23). Pathologic response to neoadjuvant therapy (complete, partial, and no response, i.e., pCR, pPR, pNR, respectively) was recorded using the Pinder classification system (118). Pathologic response rates between the two cohorts were compared using the chi-squared test.

4.3 RESULTS

Clinicopathologic characteristics of double-equivocal carcinomas

The clinicopathologic details of the cohort are reported in annex 8. Most cases (42/48, 88%) were invasive carcinomas of no special type, four were invasive lobular carcinomas (8%), one had mixed ductal and lobular features (2%), and one was an inverted micropapillary carcinoma (2%). All except three were primary BC, the remaining cases either being metastatic lesions (a lung metastasis and a lymph

node metastasis) or local relapse (Annex 8). Most cases (57.7%, 26/45 cases with grading information) were G2, 40% were G3, and a single case was G1.

All cases were ER+ with over 50% positive cells and 62.5% (30/48) showed progesterone receptor (PR) expression in over 20% of tumour cells. Range of proliferation indices was comprised between 5% and 60% (mean 28%); 18 out of 48 (37%) cases had proliferation indices ≥30%. Based on the IHC surrogate proposed by the St. Gallen recommendations (57) using the 20% cut off for Ki67, 39/48 (81%) were defined as luminal B and 9/48 (19%) as luminal A.

FISH patterns

Following review, 25 out of 48 cases (52%) were confirmed to be equivocal by all observers (Figure 14); five additional cases (10.5%) were labeled as equivocal by 2 out of the 3 observers. Six cases (12.5%) were scored as HER2- by 2/3 observers, and in 12 cases (25%) at least one observer scored the FISH as positive because of the presence of a population of cells featuring *HER2* amplification in a discrete population of cells. In this population, *HER2*-amplified cells harbored a *HER2* copy number mean of 8 (range 6.2-13), CEP17 mean values of 4.48 (range 2.1-6.2), and all but three samples had a *HER2*/CEP17 ratio <2 (Annex 8). Of note, the other observer(s) also identified a tumour cell population featuring *HER2* amplification, but the pattern was interpreted as “scattered” and the merged counts led to a *HER2* copy number mean ≥4 and <6 (i.e., equivocal).

Molecular subtyping of double-equivocal carcinomas

The large majority (37/48, 77.1%) of double-equivocal BCs were classified by Prosigna as luminal B, nine cases (18.7%) were classified as luminal A, and two cases (4.2%) as HER2-enriched (Figure 15a). Considering only the 25 equivocal carcinomas confirmed by all observers, the frequency of HER2-enriched carcinomas was 8% (2/25). These two HER2-enriched carcinomas had mean *HER2* copy numbers of 4.3 and 4.1, respectively. One of the two cases featured scattered *HER2*-amplified cells accounting for 9% of the entire tumour cell population.

IHC and Prosigna molecular subtyping was concordant in the majority of cases (41/48, 85.4%) (Annex 8, Figure 15a). Three IHC-defined luminal A carcinomas were re-classified as luminal B (2 cases) and HER2-enriched by Prosigna®; four IHC-defined luminal B carcinomas were re-classified as luminal A (3 cases) or HER2-enriched by Prosigna (Annex 8). The three luminal B carcinomas by IHC re-classified as luminal A by Prosigna had tumour cellularities between 30% and <50%.

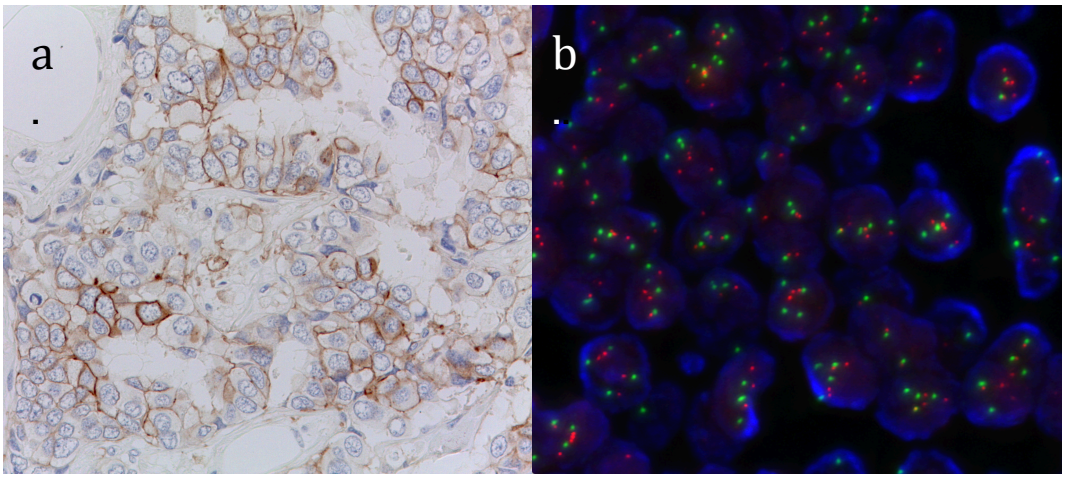


Figure 14. HER2 expression in double-equivocal carcinomas. Representative images of breast carcinomas scored as equivocal by all of the three independent observers. **a.** IHC assay (2+). **b.** Fluorescence In Situ Hybridization (FISH) pattern.

Double-equivocal carcinomas frequently (33/45, 73.3%) showed a high risk of recurrence (ROR), with ROR mean values of 73 even when the analysis was restricted to small (<2 cm) node-negative/micrometastatic BC (15/24, 62.5%; ROR mean: 61) (Annex 8, Figure 15b). When considering only the 19 G2 carcinomas with a tumour size <2 cm and node negative/micrometastatic, ten (53%), five (26%), and four (21%) showed high, intermediate, and low ROR, respectively. Finally, within the restricted subgroup of 9 luminal A carcinomas, 3 (33.3%) showed a high risk of recurrence with a mean ROR of 49. The two cases belonging to the HER2-enriched subtype were predicted to be at high risk of recurrence (ROR of 62 and 66, respectively) (Annex 8, Figure 15b).

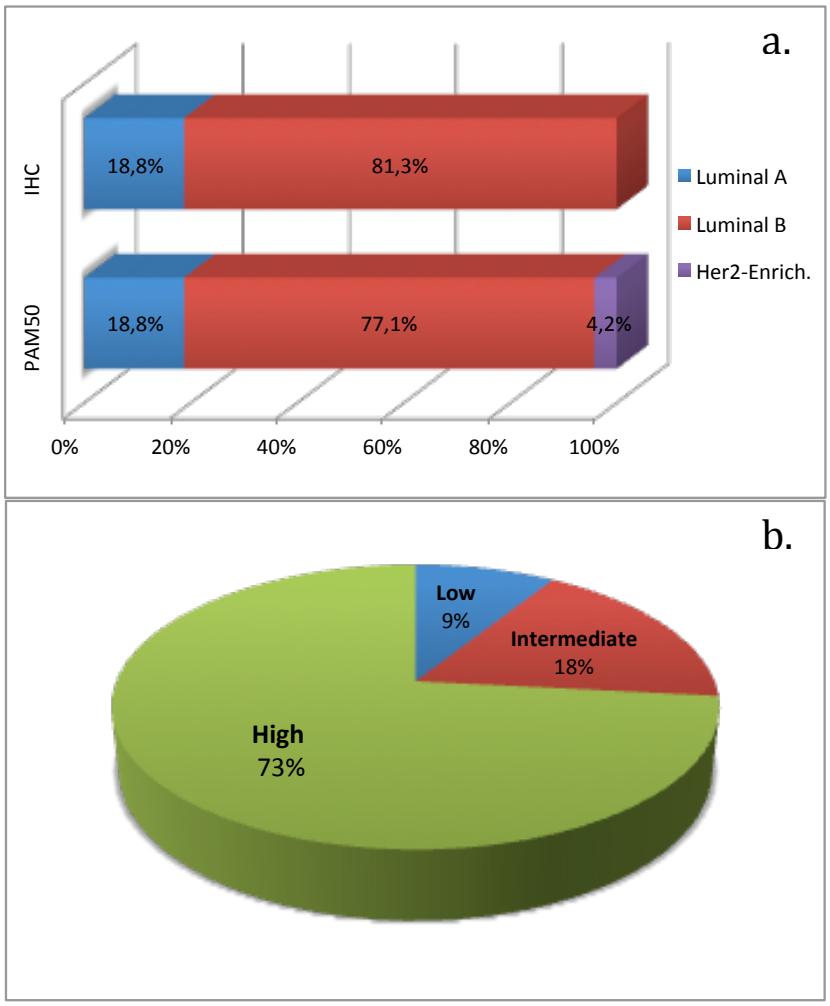


Figure 15. Molecular subtyping of the cohort of 48 double-equivocal carcinomas. a. Bar-plot comparing IHC and Prosigna-PAM50 defined molecular subtype. **b.** Percentage of double-equivocal cases with high, intermediate and low risk of recurrences (ROR) according to Prosigna assay.

HER2 mRNA levels

HER2 mRNA levels extrapolated by the Nanostring custom assay were significantly different between ER+/HER2+, ER+/HER2-, and ER+/HER2 - equivocal carcinomas ($p<0.0001$, ANOVA test, Figure 16). A greater overlap in terms of *HER2* mRNA levels was observed between HER2- and HER2 - equivocal carcinomas; however, outlier HER2 - equivocal tumours showed *HER2* mRNA expression comparable to the range of HER2+ tumours and were labeled as

luminal B by Prosigna. The two cases classified as “HER2-enriched” showed relatively low *mRNA* HER2 levels (4053 and 2808, respectively).

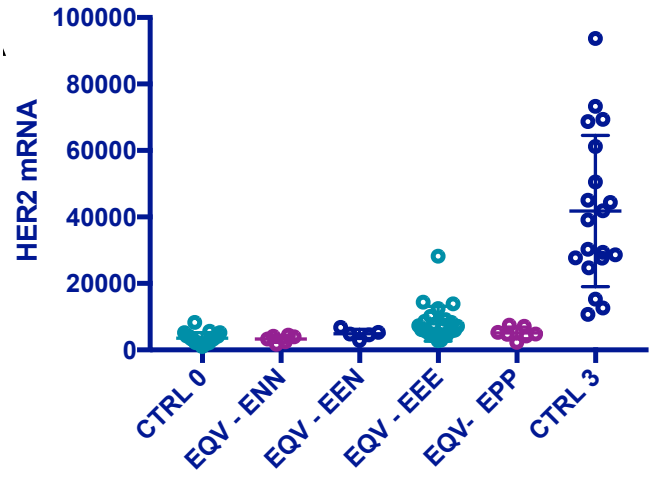


Figure 16. HER2 mRNA levels in double-equivocal breast carcinomas in comparison with HER2+ (CTRL 3) and HER2- (CTRL 0) carcinomas. The HER2 mRNA levels were extrapolated from the NanoString custom assay. Cases are subdivided according to results of the FISH review. E: equivocal; N: negative; P: positive. For instance EEP would stand for a case that was confirmed as equivocal by two observers and positive by the other. CTRL: control groups; EQV: HER2 double-equivocal carcinomas.

Transcriptomic stratification of double-equivocal carcinomas

According to clustering analyses, a 24-gene signature was derived from global transcriptomic analysis of differentially expressed genes between ER+/HER2- and ER+/HER2+ tumours (Annex 9). Of the 24 genes, 14 were confirmed to show differential expression between the distinct categories using the NanoString custom assay. In particular, eight genes including *HER2* were significantly overexpressed in HER2+ vs. HER2- and HER2+ vs. HER2 - equivocal tumours, but they also showed significantly higher expression in HER2 - equivocal compared to HER2- carcinomas. All genes mapped to the smallest region of amplification of the *HER2* locus (119, 120). Moreover, three genes (*TPRG1, NOVA1,* and *AGTR1*) were significantly more expressed in HER2- compared to HER2+ tumours. Analyses of these genes in The Cancer Genome Atlas (TCGA) BC dataset (121) showed a striking mutually exclusive pattern with *HER2*

expression. Finally, three other genes (*SORCS1, MAPT,* and *DSCR6*) were significantly more expressed in HER2- compared to HER2+ and in HER2 - equivocal compared to HER2+ tumours.

Unsupervised clustering of HER2+, HER2-, and HER2 - equivocal carcinomas based on these 14 genes produced two main clusters, one composed of cases with high expression of *HER2* amplicon-related genes and the other with cases displaying lower levels of such genes. The latter cluster could be further classified into two subgroups: one composed of cases with non-homogeneous expression of *HER2* amplicon-related genes together with high levels of *HER2* anticorrelated genes, the other with cases showing low levels of *HER2* amplicon-related genes as well as *HER2* anticorrelated genes (Figure 17).

Cases with distinct HER2 statuses showed significantly different distributions between the three clusters ($p<0.0001$, chi-squared test; Figure 17). All ER+/HER2+ carcinomas except one grouped within the cluster enriched for HER2 amplicon-related genes together with two double-equivocal carcinomas, the latter confirmed by at least 2/3 observers and classified as luminal B by Prosigna. The large majority of HER2 - equivocal carcinomas preferentially clustered with HER2- carcinomas and one HER2+ carcinoma in the remaining two clusters (Figure 17). Retrospective evaluation of the HER2+ carcinoma that did not group with the others showed heterogeneity of *HER2* amplification (25%). The two Prosigna HER2-enriched carcinomas, classified in the cluster characterized by cases displaying non-homogeneous *HER2* amplicon-related gene expression as well as *HER2* anticorrelated genes.

<u>Analysis of pCR rates in HER2-positive carcinomas treated by neoadjuvant HER2-targeted therapy</u>

Since some so-called “double-equivocal” carcinomas may harbor a non-negligible degree of HER2 heterogeneity above the 10% cut-off, we explored responses to chemotherapy and trastuzumab in a series of cases treated with anti-HER2 therapy in the neoadjuvant setting. Cases had originally been scored as HER2

positive based on the presence of a tumour population showing HER2 copy numbers ≥6; however, the overall tumour cell population led to a result in the equivocal range.

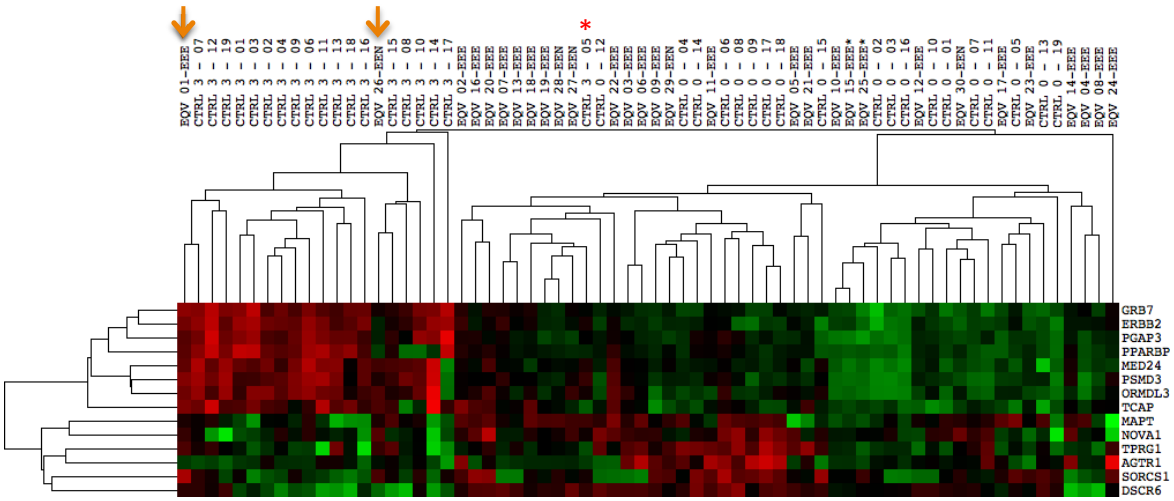


Figure 17. Hierarchical clustering of ER+/HER2 double-equivocal BCs, ER+/HER2+, and ER+/HER2- carcinomas. The unsupervised clustering was based on the signature of 14 genes found to be differentially expressed between two cohorts of ER+/HER2+ and ER+/HER2- carcinomas. Cases are represented in columns; genes are depicted in rows. The arrows indicates HER2 double-equivocal carcinomas clustering together with ER+/HER2+ carcinomas. The asterisk indicates a score 3+ carcinomas clustering with HER2 - equivocal and HER2- cases.

The pCR rate within this cohort was 10% and was significantly lower than ER and Ki67-matched 3+ carcinomas (59%) (p=0.01, Table 11). Of note, three cases showed an almost pCR, with <10% of residual tumour cellularity compared to core biopsy samples. When pCR and almost pCR categories were grouped, the difference in terms of response rate was not significantly different from score 3+ (Table 12). When examining the HER2 expression pattern in the nine cases with residual tumour, 5 out of 9 showed score 0, whereas 4/9 showed score 2+.

Table 12. Categorization of pathological response to chemotherapy and anti-HER2 therapy in the neoadjuvant setting in the two cohorts of HER2+ (score 3+) and HER2 - equivocal carcinomas showing a subclonal population of HER2 amplified cells accounting for >10%. Cases are categorized into pathological complete response (pCR) vs. partial response and

no response (collectively labelled as "no pCR"). The pCR rate is significantly higher in the group of HER2+ carcinomas (p= 0.007).

	pCR	No pCR	Total N
HER2 – equivocal. *HER2* heterogeneity in >10% of tumour cells	1	9	**10**
HER2+ (score 3+)	16	11	**27**
Total N	**17**	**20**	**37**

Table 13. Categorization of pathological response to chemotherapy and anti-HER2 therapy in the neoadjuvant setting in the two cohorts of HER2+ (score 3+) and HER2 - equivocal carcinomas showing a subclonal population of HER2 amplified cells accounting for >10%. Cases are categorized into pathological complete response (pCR), pathological partial response (pPR) and pathological no response (pNR). Pathological partial response is further categorized into three categories according to the classification proposed by Pinder et al. and pPRi (which corresponds to an almost pCR, i.e. <10% of tumour cells) is grouped. Althouh the pCR rate is higher in the subgroup of HER2+ carcinomas; the difference with HER2 - equivocal carcinomas harboring HER2 genetic heterogeneity is no longer significantly different (p= 0.2).

	pCR+pPRi	pPRii+pPRiii+pNR	Total N
HER2 – equivocal. *HER2* heterogeneity in >10% of tumour cells	4 (40%)	6 (60%)	**10**
HER2+ (score 3+)	17 (63%)	10 (37%)	**27**
Total N	**21**	**16**	**37**

4.4 DISCUSSION

Here it was observed that double-equivocal carcinomas represent a rather heterogeneous group of BCs, some of which harbor a variable degree of HER2 genetic heterogeneity while others display a more homogeneous population of tumour cells constantly harboring >4 but <6 HER2 copies. At the transcriptomic level, double-equivocal carcinomas are preferentially luminal B, and HER2 mRNA levels appear to be intermediate between ER+/HER2- and ER+/HER2+ carcinomas with a greater overlap with HER2- carcinomas. When exploiting a signature derived from differentially expressed genes between ER+/HER2+ and ER+/HER2- carcinomas, at least three groups were evident, one of which showed overexpression of genes negatively correlated with HER2 amplification.

Tumours with an equivocal HER2 status account for about 10% of all invasive BCs subjected to ISH following a score 2+ by IHC (25, 110). Whether or not patients with double-equivocal BC may be candidates for anti-HER2 therapies is controversial. The 2013 ASCO/CAP guidelines acknowledge that whenever the HER2 test result is ultimately deemed to be equivocal, the oncologist may consider HER2-targeted therapy, mainly based on clinical parameters (23). This stems from the intrinsic limitations of the current companion diagnostic tests, i.e., IHC and ISH, which can detect some degree of HER2 expression and HER2 gene gain, respectively, while not fulfilling the thresholds for HER2 positivity, i.e., HER2 copy number ≥6, HER2/CEP17 ratio >2.

In line with recent reports (24), we show that HER2 amplification heterogeneity can be appreciated in double-equivocal BC; besides that the interpretation of heterogeneity patterns can significantly affect the definition of these carcinomas. In about a third of the cases studied, a population of HER2-amplified cells accounting for >10% of the tumour was detected but differently interpreted by independent observers, i.e., scattered/isolated amplified cells in a predominantly non-amplified tumour and/or diffuse intermingling of amplified and non-amplified cells vs. aggregated cells leading to distinct tumour populations. This discordance stemmed from the fact that these carcinomas typically showed a pattern of genetic heterogeneity featuring predominantly diffuse intermingling of amplified and non-amplified cells that in some cases was interpreted as discrete aggregated cell clusters. This led to observers identifying HER2 amplification in a subclonal population of tumour cells. The relevance of subpopulations of HER2-amplified cells within otherwise non-amplified tumours remains a topic of debate with respect to both prognostic significance and potential benefit from trastuzumab therapy (122). Although the ASCO/CAP 2013 experts agreed that the only significant type of amplified population is from discrete aggregated cells, one may argue that this feature is not a requisite for IHC scoring. As a matter of fact, genetically heterogeneous tumours harbor a significant population of HER2-amplified cells and may therefore be sensitive to anti-HER2 therapy (122, 123). It is important to note that in our series these cases typically showed low levels of

HER2 amplification, i.e., low HER2 copy numbers and always harboring HER2/CEP17 ratios <2 due to co-occurrence of CEP17 gains, as also observed by others (24, 124). It could be speculated that these features are suggestive of complex rearrangements in chromosome 17, as in fact has been previously reported (60, 119, 125, 126), which indicate that study of HER2 double-equivocal BC merit further investigation.

From a clinical standpoint, the key question is whether these genetically heterogeneous tumours respond to trastuzumab. We explored this question by examining a cohort of patients treated with neoadjuvant trastuzumab and observed that the pCR rates of equivocal carcinomas with HER2 heterogeneity were significantly lower than 3+ carcinomas. Although only representing a limited number of cases, these results are in line with other recent preliminary data (97, 127). Furthermore, in our case series, when pCR and almost-pCR were considered together, the response rates reached about 40%. We cannot rule out that this was due to the beneficial effect of chemotherapy, but it is interesting to note that in the score 3+ subgroup only one patient displayed an almost-pCR response. Further studies in larger cohorts are warranted to ascertain the real impact of anti-HER2 therapy in this specific subset of BCs, taking into account patients who did not receive trastuzumab.

As well as containing carcinomas with HER2 genetic heterogeneity leading to equivocal ISH counts, our series was substantially composed of tumours featuring homogeneously HER2 - equivocal tumour cell populations. Whether the HER2 gain/HER2 protein expression showed by these tumour cells is capable of driving significant HER2 pathway activation that may be sensitive to anti-HER2 agents is unknown. At present, only comparative data between double-equivocal and HER2-negative carcinomas treated with chemotherapy regimens are available; for instance, Press et al. (128) observed comparable outcomes between the two categories. Whether patients affected by double-equivocal carcinomas do better with the addition of anti-HER2 agents has yet to be determined. Importantly, there are controversial results on the prognostic impact of double-equivocal HER2 status. Biserni et al. (129) reported an increased risk of death from BC during the

first five years of follow-up in patients with HER2 counts in the equivocal range compared to HER2- carcinomas when adjusted for stage. Conversely, Sneige et al. (130) and Criscitiello et al. (131) did not identify a significant association between risk of recurrence and HER2 - equivocal testing in patients with early BC.

The Prosigna analysis contributed a risk-based stratification of the cohort, since double-equivocal carcinomas preferentially harbored a high risk of recurrence and a mean ROR of about 73, a value similar to those reported for HER2+ disease (132). High-risk categorization was observed even within the subgroup of node-negative G2 carcinomas with tumour size <2 cm, thus highlighting the possible use of these assays in treatment decision-making. Furthermore, the molecular subtyping provided by the Prosigna assay might also be clinically useful. There was a predominance of luminal B carcinomas, which is not surprising as these carcinomas are consistently reported as ER+ by IHC (108-110), and the cohort evaluated here was entirely composed of ER+ tumours. Even if only a minority of cases fit the HER2-enriched category, the possibility to detect a HER2-enriched molecular subgroup in a BC otherwise classified as equivocal opens up the possibility to explore the beneficial effect of anti-HER2 agents in ad hoc window of opportunity trials. Interestingly, the two HER2-enriched tumours were not predictable either based on HER2 copy number/HER2 mRNA levels or by HER2 heterogeneity. Both displayed a high risk of recurrence.

As a further level of complexity, stratification of double-equivocal carcinomas by means of a gene signature derived from ER+/HER2+ and ER+/HER2- carcinomas showed that double-equivocal carcinomas preferentially clustered with ER+/HER2- cases and a subgroup expressed high levels of genes that negatively correlate with HER2 in the TCGA dataset. Notably, of these genes, AGTR1 has been described as a potential therapeutic target for ER+/HER2- BC (133, 134) and linked to resistance to neoadjuvant chemotherapy in ER+/HER2- BC (135). In addition, there is preclinical evidence to support a role for clinically available AGTR1 receptor blockers in reverting endocrine resistance in ER+/HER2- BC cells (136).

This study has several limitations, including the small sample size. It should be noted that these cases needed ISH testing to be recognized and the carcinomas analyzed were selected from two institutions with a high ISH testing workload per year. In addition, the samples underwent detailed review to dissect the exact details of the FISH pattern. Second, our main focus was transcriptomic characterization, so it cannot be excluded that mutational analysis, may reveal key driver alterations in this subgroup that could be used to support treatment decision-making and/or offer novel therapeutic avenues. Finally, no follow-up information for this cohort was available. Nevertheless, a surrogate of prognostic information stemmed from the multigene prognostic signature analysis.

Despite the limitations, this study highlighted the possibility to biologically and prognostically stratify ER+/HER2 double-equivocal carcinomas. In addition, further details on *HER2* genetic heterogeneity are provided, which represents a source of interobserver error and whose clinical significance remains unresolved. Only larger studies exploring the effect of treatment with or without anti-HER2 agents in these cases can provide a definitive answer to this matter of debate. On the one hand, our transcriptomic analysis seemed to suggest that only a small fraction of double-equivocal carcinomas could be classified as “HER2-driven/addicted”, while on the other one could argue that the analyses on the whole tumour population may have overlooked a potentially deleterious subclonal population with clinical implications.

5. GENERAL CONCLUSIONS

Here, the study of new (AR & FOXA1) and clasical (ER & HER2) BC markers shown that it's joint assessment, through different strategies, allow a better stratification of the ER+ BC subgroup according to its clinical, pathological and biological characteristics. Furthermore, the evaluation of the aforementioned markers provided additional prognostic information, complementary to the clasical prediction models; which could be useful to help clinicians in the selection of the optimal therapeutic approaches for ER+ BC patients. In particular, it was observed that:

- AR/ER≥2 was found a significant marker of poor prognosis in ER+/HER2- BCs, since patients with this characteristic have worse DFI and DSS than patients with ratio AR/ER<2. From a molecular point of view, our results suggest that cases characterized by AR/ER≥2 could be non-luminal tumours by genomic intrinsic subtyping (Prosigna-PAM50). Furthermore, as seen in non-luminal BCs, our TaqMan-qPCR assays on fresh-frozen tissue also showed that tumours with AR/ER≥2 have higher levels of cell proliferation, which confirm the use of AR/ER ratio as a marker of poor prognosis in ER+ BCs. Taken together, these findings provide insights into the importance of joint assessment of AR and ER expression in clinical routine to better classify ER+ BC patients according to their expected outcome and reinforce the idea of targeting AR for treatment of ER+/HER2- BC in which high AR levels are present.

- In BC tumours, analyses of protein and mRNA expression of FOXA1 was directly proportional to the expression of AR. Despite that, FOXA1 was found as a superior predicting marker of recurrences compared to AR in ER+ BC patients; this since prognosis of the patients with AR- BC was not different from that of those with AR+ tumours in the FOXA1+ cohort (FOXA1+/AR+ Vs. FOXA1+/AR-). Therefore, FOXA1 expression evaluated by IHC on ER+ BC

specimens could be considered in routine diagnosis as an additional support to oncologists in the stratification and the prognosis definition of this BC subgroup.

- Heterogeneity evaluation of ER+/double-equivocal HER2 BCs, indicated that presence of >10% *HER2*-amplified cells (HER2 ≥6) in a tumour population can lead to equivocal ISH results and if interpreted as discrete aggregated cells would be diagnosed as positive for *HER2* amplification in a subclonal tumour cell population. Regardless of the evidence of *HER2* genetic heterogeneity, double-equivocal BCs are preferentially luminal B rather than HER2-enriched, and when stratified based on a 14-gene signature preferentially clustered with HER2- rather than HER2+ carcinomas. Nevertheless, a subset of these tumours might be suitable for HER2-targeted therapy based on transcriptomic profiling. We also show that these luminal B carcinomas, even when small and node negative, preferentially have a high risk of recurrence. Taken together, these data suggest that transcriptomic analysis may help further dissect ER+/double-equivocal HER2 BCs and support better treatment decision-making for patients with these controversial and heterogeneous disease.

6. RECOMMENDATIONS AND PROSPECTS

Our data confirmed that BC is a heterogeneous disease accompanied by differences not only in clinical, but also in molecular and biological features. However the results obtained in the present study are very interesting, since they are indicative of the high heterogeneity, even within the specific BC subgroup of ER+ tumours.

This heterogeneity, which allowed us better stratify ER+ patients, could be considered not only in the prognosis classification but also in the selection and treatment decision-making for patients with ER+ BC disease. The evaluation of markers and strategies proposed here could be useful to establish more personalized treatments and monitoring its response (mainly to hormonal and HER2 targated therapies), in order to overcome the resistance generated by any of them.

To address these insights its neccesary to validate results presented above, so additional studies, evaluating AR, FOXA1 and HER2 equivocal expression need to be performed; first trying to evaluate these parameters in public databases (like TCGA) and then through collection of larger cohort of patients not only with a retrospective, but also, with a prospective design. Furthermore, future analyses must be conducted in patient's cohorts who possibly underwent the same therapeutic approach, mainly for AR analyses in ER+ cases, since AR expression have been related with TAM resistance.

From a molecular and biological point of view, seems evident that prognosis effects of AR expression related to ER expression could be due to its effects on proliferation of BC cells. Neverthless, these preliminar results must be confirmed in "in vitro" but more importantly in "in vivo" models, which allow to evaluate the tumoral growth rate in presence of both different AR and ER expression levels.

Finally, transcriptomic analysis highlighted the possibility to biologically and prognostically stratify ER+/double-equivocal HER2 BC. In addition, it was shown that *HER2* genetic heterogeneity, represents a source of interobserver error, whose clinical significance remains unresolved. Since it is crucial to understand

whether these cells harbor HER2 pathway activation, one possibility might be to perform mutational analysis of *HER2* gene, as well as, exploit single cell sequencing to ascertain the presence of clear activation of HER2 signaling in distinct subpopulations.

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