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How to identify patients with high-risk HR-positive/HER2-negative breast cancer in the absence of gene expression platforms

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A las mujeres de mi vida

A mi madre, por sus insaciables ganas de vivir

A mi abuela, por su fuerza

A mi hija, para que el cáncer de mama nunca esté presente en su vida

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ABSTRACT

HR-positive/HER2-negative (HR+/HER2-) is the most common breast cancer type, and it is a molecular heterogenic disease. This heterogeneity has direct prognostic and predictive implications in both early and advanced settings. Thus, identifying high-risk HR+/HER2- breast cancer patients in the clinical practice has become a necessity, even when genomic platforms are not available. In this project, we compared the intrinsic subtype classification defined by the PAM50/Prosigna® test with 4 immunohistochemistry-based biomarkers (estrogen receptor [ER], progesterone receptor [PR], Human epidermal growth factor receptor 2 [HER2], and Ki67) in two different cohorts of 517 and 1,417 patients with HR+/HER2- breast tumors, respectively. In a first study, we evaluated the performance of Ki67 as a continuous biomarker to identify Luminal A and Risk of Relapse (ROR)-low tumors. Moreover, we explored the optimal KI67 cutoff for selecting low-risk patients in the clinic. In a second study, we built and tested an IHC-based predictor to identify PAM50 non-luminal subtypes in HR+/HER2- breast cancer. Both projects should allow a more comprehensive understanding of the biological heterogeneity within HR+/HER2- early breast cancer and provide tools to identify patients with different relapsing risks. In the first study, we evaluated a cohort of 517 patients with ER+/HER2- and node-negative breast cancer. Although most patients had Luminal A (65.6%) and ROR-low tumors (70.9%), a substantial proportion (34-43%) of tumors with Ki67 0-10% had either ROR-medium or ROR-high disease; conversely, a substantial proportion (24-29%) of tumors with Ki67 10-20% had ROR-low disease. Also, we found that the optimal Ki67 cutoff for identifying Luminal A or ROR-low tumors was 14%, concordant with previous findings reported in the literature. In the second study, we created an IHC-based predictive biomarker using ER, PR, and Ki67 data, the NOLUS score, to identify PAM50 non-luminal disease, using a training dataset of 903 patients with HR+/HER2- breast tumors. When applied to the test set, the NOLUS score was statistically significantly associated with non-luminal disease ($p<0.01$) with an AUC of 0.902. The proportion of non-luminal tumors in NOLUS-positive and NOLUS-negative groups was 76.9% (56.4–91.0%) and 2.6% (1.4–4.5%), and the sensitivity and specificity of the pre-specified cutoffs were 59.3% and 98.7%, respectively. Based on these results, we conclude that Ki67 as a continuous variable is an unreliable biomarker to identify patients with Luminal A and/or ROR-low HR+/HER2- breast cancer. However, in the absence of gene expression platforms, the best Ki67 cutoff for determining ROR-low or Luminal A disease is 14%. The NOLUS score can help identify patients with non-luminal disease within HR+/HER2- breast cancer.

LIST OF ABBREVIATIONS

ADCs: antibody-drug conjugates

AI: aromatase inhibitor

AIMS: absolute assignment of breast cancer intrinsic molecular subtype

AKT: protein kinase B

ANOVA: analysis of variance

AR: androgen receptor

ASCO: American Society of Clinical Oncology

ATAC: Arimidex, Tamoxifen, Alone or in Combination

ATM: ataxia-telangiectasia mutated gene

BIG: Breast International Group

BRCA1: breast cancer gene 1

BRCA2: breast cancer gene 2

CAP: College of American Pathologists

CCNE1: cyclin E1 gene

CDK4/6i: cyclin-dependent kinase 4/6 inhibitors

CEP17: increased chromosome enumeration probe 17

CES: chemo-endocrine sensitivity score

CNAs: copy number alterations

DDFS: distant disease-free survival

DFS: disease-free survival

DRFS: distant recurrence-free survival

EBCTCG: Early Breast Cancer Trialists

ER: estrogen receptor

FDA: Food and drug administration

FGFR: fibroblast growing factor receptor

HER2: Human epidermal growth factor receptor 2

HER3: Human epidermal growth factor receptor 3

HR: hazard ratio

HR: hormone receptor

IHC: immunohistochemistry

ISH: in situ hybridization

mTOR: mammalian target of rapamycin

NCI: National Cancer Institute

OR: odds ratio

OS: overall survival

PAM50: Prediction Analysis of Microarray 50

PARPi: poly (ADP-ribose) polymerase inhibitors

pCR: pathologic complete response

PEPI: preoperative endocrine prognostic index

PFS: progression-free survival

PR: progesterone receptor

PI3K: phosphatidylinositol-4,5-bisphosphate 3-kinase

PIK3CA: phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha

PS: performance status

qRT-PCR: real-time quantitative reverse transcription-polymerase chain reaction

RB1: retinoblastoma 1 gene

RCB: residual cancer burden

RFS: relapse-free survival

RNAseq: RNA sequencing

ROC: receiver operating characteristic

ROR: risk of recurrence

RS: risk score

RTKs: receptor tyrosine kinases

SEER: Surveillance, Epidemiology, and End Results

SERDs: selective estrogen receptor down regulators

TCGA: Cancer Genome Atlas Project

TILs: tumor-infiltrating lymphocytes

TNBC: triple-negative breast cancer

TRK: tyrosine kinase

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INCLUDED ARTICLES AND IMPACT FACTOR

Reference	Impact Factor*	Rank in the category**
Limitations in predicting PAM50 intrinsic subtype and risk of relapse score with Ki67 in estrogen receptor-positive HER2-negative breast cancer. **Fernandez-Martinez A,** Pascual T, Perrone G, Morales S, de la Haba J, Gonzalez-Rivera M, Galvan P, Zalfa F, Amato M, Gonzalez L, Prats M, Rojo F, Manso L, Pare L, Alonso I, Albanell J, Vivancos A, Gonzalez A, Matito J, Gonzalez S, Fernandez P, Adamo B, Munoz M, Viladot M, Font C, Aya F, Vidal M, Caballero R, Carrasco E, Altomare V, Tonini G, Prat A, Martin M. Oncotarget 2017 Mar 28;8(13):21930-21937 PMID: 28423537 PMCID: PMC5400635 DOI: 10.18632/oncotarget.15748	5.168	**Oncology** Q1 (44/217) **Cell biology** Q2 (48/190)
A Pathology-Based Combined Model to Identify PAM50 Non-luminal Intrinsic Disease in Hormone Receptor-Positive HER2-Negative Breast Cancer. Pascual T, Martin M, **Fernandez-Martinez A**, Pare L, Alba E, Rodriguez-Lescure A, Perrone G, Cortes J, Morales S, Lluch A, Urruticoechea A, Gonzalez-Farre B, Galvan P, Jares P, Rodriguez A, Chic N, Righi D, Cejalvo JM, Tonini G, Adamo B, Vidal M, Villagrasa P, Munoz M, Prat A. Front Oncol. 2019; 9: 303 PMID: 31106144 PMCID: PMC6498671 DOI: 10.3389/fonc.2019.00303	4.848	**Oncology** Q2 (69/244)

Q: quartile. * Journal Citation Reports®, ISI Web of Knowledge; Thompson Reuters. ** At publication date.

GENERAL INTRODUCTION

Breast cancer is the most common cancer among women by far, with a worldwide estimated incidence of 2,088,849 new cases in 2018. It is also the leading cause of cancer death in females, with a mortality rate of 14.9 per 100,000 (1). However, over the past 30 years, the number of women who have died of breast cancer has steadily decreased thanks to early detection and treatment improvements. In particular, understanding the biological heterogeneity of breast cancer tumors has been essential for the development of targeted therapies and personalized treatment strategies.

In the clinical practice, three pathology-based biomarkers are routinely used to guide therapy decisions in breast cancer patients: the immunohistochemistry (IHC) evaluation of estrogen receptor (ER), progesterone receptor (PR), and the human epidermal growth factor receptor 2 (HER2) expression or HER2 gene amplification. Based on the hormone receptors (HR) and HER2 status, breast cancer can be classified into four main groups: HR-positive/HER2-negative (HR+/HER2-), HR-positive/HER2-positive (HR+/HER2+), HR-negative/HER2-positive (HR-/HER2+), and triple-negative breast cancer (TNBC).

HR+/HER2- is the most common cancer type, accounting for approximately 70% of all early-stage breast cancer cases (2). Following the guidelines, this breast cancer type has been traditionally treated with adjuvant endocrine therapy for 5 to 10 years and, in intermediate/high-risk patients, with a chemotherapy regimen generally containing taxanes and anthracyclines (3, 4). However, despite treatment with adjuvant endocrine and multi-agent chemotherapy, some patients still have a substantial risk of relapsing (5, 6). In the metastatic setting, endocrine therapy has traditionally been the backbone of treatment for this breast cancer type, together with an ovarian ablation or suppression in premenopausal women. In patients with symptomatic visceral disease or after progression to endocrine therapy, chemotherapy was indicated (3, 7). However, even with these treatments, patients diagnosed with metastatic HR+/HER2- breast cancer had a median overall survival of 2 to 3 years (8). Thankfully this scenario has changed in the last years mainly due to the incorporation of novel targeted drugs (i.e., mTOR inhibitors, cyclin-dependent kinase 4/6 inhibitors [CDK4/6i], and PIK3CA inhibitors) in combination with endocrine therapy (9-17).

HR+/HER2- breast cancer is a molecular heterogenic disease, and all the intrinsic molecular subtypes of breast cancer can be found in different proportions within this breast cancer group (18). This heterogeneity has direct prognostic and predictive implications in clinical practice in

both early and advanced settings. For example, in HR+/HER2- early breast cancer patients, PAM50/Prosigna® assay estimates a 10-year risk of recurrence (ROR) based on the prognostic differences among the 4 main breast cancer intrinsic subtypes, guiding adjuvant treatment decisions about the need for chemotherapy (19-22). In the metastatic setting, non-luminal HR+/HER2- tumors (HER2-Enriched and Basal-like) have been associated with estrogen independence, chemosensitivity, resistance to CDK4/6i, and a worse prognosis (18). Moreover, based on the integration of clinical parameters and PAM50, a new and promising prognostic tool called PAM50MET has been recently designed for HR+/HER2- metastatic breast cancer patients (23). Thus, identifying high-risk HR+/HER2- breast cancer patients in the clinical practice has become a necessity, even when genomic platforms are not available.

1. EPIDEMIOLOGY, GENETIC PREDISPOSITION, AND RISK FACTORS OF HR+/HER2- BREAST CANCER

In a study from the Surveillance, Epidemiology, and End Results (SEER) program of the National Cancer Institute (NCI) involving 57,483 breast cancer patients with known HR and HER2 status, HR+/HER2- tumors were the most frequent (72.7%) regardless of race, ethnicity, and age (2, 16) (**Figure 1**).

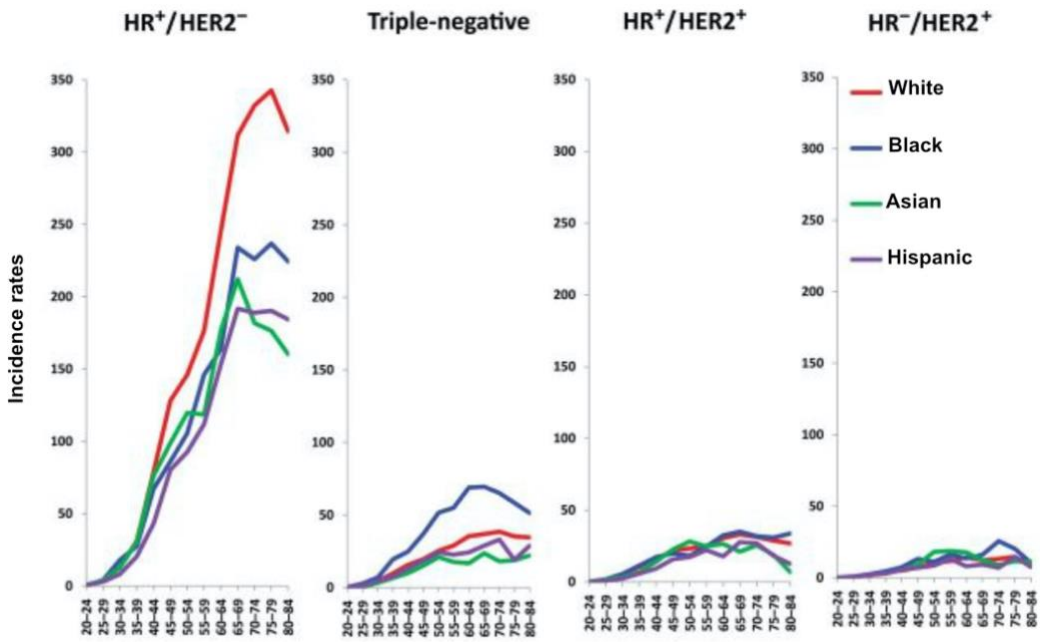

Figure 1. Age-specific incidence rates of breast cancer subtypes by race/ethnicity in the US.

This tumor type has a higher incidence in older patients, white women, and higher socio-economic regions (2). They usually carry a good prognosis, having a higher proportion of early stages at diagnosis and a lower proliferation rate than HER2+ and TNBC (2).

From an etiopathogenic perspective, HR+/HER2- breast tumors have a similar hereditary component than the overall breast cancer population (5 to 10%) (24), but with a slightly different germinal mutation pattern, having a higher prevalence of *BRCA2*, *CHEK2*, and *ATM* mutations (>1%) and a lower prevalence of *BRCA1* mutations compared to other breast cancer types (0.9%) (24).

In terms of lifestyle and environmental risk factors, HR+/HER2- breast cancer has been associated with menstruation-related factors (early age at menarche, later age at menopause), reproduction-related factors (nulliparity, late age at first birth, fewer children), exogenous hormone intakes (hormone replacement therapies, oral contraceptives) and also modifiable risk factors including obesity, physical inactivity, and alcohol intake. On the contrary, breastfeeding and physical activity are known as protective factors of this cancer type (25, 26).

2. IMMUNOHISTOCHEMISTRY-BASED DEFINITION OF HR+/HER2- BREAST CANCER

HR+/HER2- breast cancer is defined by a positive IHC determination of ER and/or PR and a negative HER2 expression or HER2 gene amplification. However, the definition of a positive vs. negative ER/PR and HER2 has changed over the years. Also, proliferation markers like Ki67 may supply additional prognostic information within this breast cancer type.

2.1 ESTROGEN AND PROGESTERONE RECEPTOR TESTING

The pathological report of invasive breast cancer should include the IHC evaluation of ER and PR using a standardized assessment methodology, like the Allred score or H-score (4). ER and PR are nuclear proteins. Following the last American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) recommendations (27), an IHC staining of 1% to 100% of tumor nuclei will be interpreted as positive. For reporting ER (not PR), if 1% to 10% of tumor nuclei are immunoreactive, the samples should be reported as ER-low positive (27). This new category is relevant in the clinical practice, as there is limited data on endocrine therapy benefit in this subset of patients.

HR are well-known prognostic factors in breast cancer. Traditionally, ER and PR have been associated with a more prolonged overall survival (OS) and disease-free survival (DFS) (28,

29). However, more recent clinical trial data with a longer follow-up suggest that after 5 years, the risk of recurrence of patients with ER-positive tumors is higher than the risk of other breast cancer tumor types (30). After 5 years of adjuvant endocrine therapy, these late relapses seem to be correlated with the tumor size, the nodal status, and the tumor grade, with risks ranging from 10 to 41% from 5 to 10 years of follow-up (31). PR appears to have an independent prognostic value of ER (32), being the absence of PR expression in ER-positive tumors, a biomarker for poor prognosis. This fact is also supported by the finding that patients with ER-positive, PR-negative disease usually show a more aggressive tumor intrinsic subtype composition (33).

In addition to its prognostic value, ER and PR are well-recognized predictive factors of response to endocrine therapy. In a patient-level meta-analysis conducted by the Early Breast Cancer Trialists (EBCTCG), 5 years of adjuvant tamoxifen was associated with a 41% annual risk-of-relapse reduction in ER-positive tumors, compared to a small benefit in ER-negative tumors. This predictive value was independent of PR expression, age, nodal status, or the use of adjuvant chemotherapy (34). A relationship between ER expression and response to endocrine therapy has also been observed in neoadjuvant endocrine studies with aromatase inhibitors (AI) (35). The role of PR as a predictive biomarker of endocrine therapy benefits has been a subject of controversy. The absence of PR in ER-positive tumors has been associated with more aggressive tumor biology (33). Thus, it has been hypothesized that ER-positive/PR-negative tumors are associated with endocrine resistance. Consistent with the hypothesis, these tumors have shown a lower benefit from endocrine therapy than those with ER-positive/PR-positive in the metastatic setting (36). However, studies in early-stage breast cancer have failed to demonstrate a relationship between PR expression end the response to endocrine therapy (37). In the ATAC trial (Arimidex®, Tamoxifen, Alone, or in Combination), PR's quantitative expression did not identify patients with a different benefit for anastrozole over tamoxifen (38). In the same line, in the Breast International Group (BIG) 1-98 trial, letrozole was superior to tamoxifen regardless of PR status (39).

2.2 HER2 RECEPTOR TESTING

HER2 testing should be carried out according to the ASCO/CAP guidelines (40) and should include HER2 IHC protein expression or HER2 gene amplification. The algorithm for IHC HER2 testing is summarized in **Figure 2**.

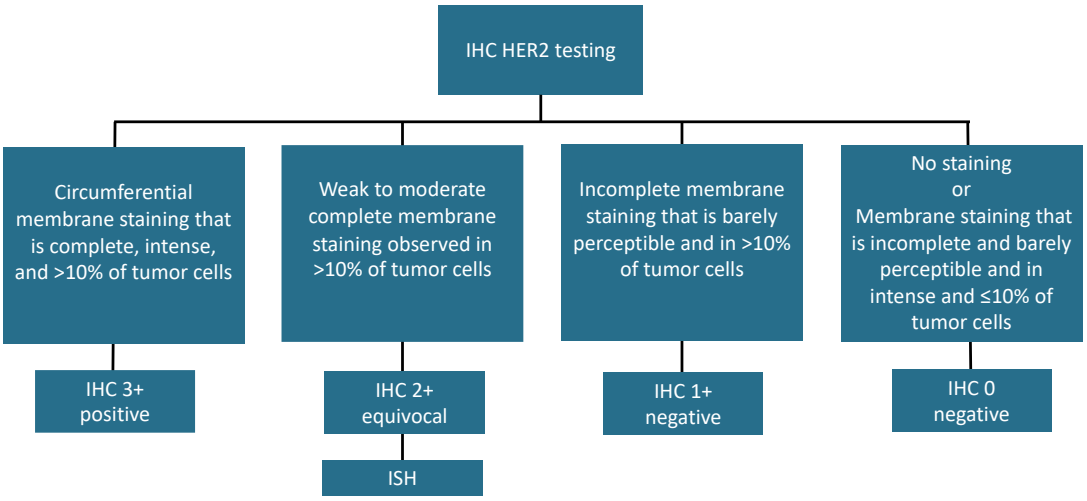

Figure 2. Algorithm for IHC HER2 testing.
Adapted from 2018 ASCO/CAP guidelines. IHC: immunohistochemistry; ISH: in situ hybridization.

HER2 gene amplification status may be determined directly from all invasive tumors using in situ hybridization (ISH) (fluorescent, chromogenic, or silver), replacing IHC or only for tumors with an ambiguous IHC score. A sample will be defined as HER2 positive by ISH if the number of HER2 copies is ≥6, or the HER2/ centromeric region of chromosome 17 (CEP17) ratio is ≥2 and HER2 copies ≥4, or HER2/CEP17 <2 and HER2 copies ≥6.

Traditionally, HER2 overexpression and/or amplification has been associated with an unfavorable prognosis in patients that were not treated with chemotherapy and HER2-targeted therapies (41, 42). However, the main benefit of HER2 testing in breast cancer patients is its predictive value, selecting the candidates to receive HER2-targeted agents in the adjuvant and the metastatic setting (3, 40).

An emerging HER2 category is HER2-low status, defined as IHC 1+ or IHC 2+ with a negative ISH. There is a high proportion of ER+/HER2- tumors that would be considered as HER2-low using this definition (up to 60% in some retrospective series) (43), and these tumors could now get a benefit from new therapeutic strategies involving antibody-drug conjugates (ADCs) (44).

2.3 KI67 RECEPTOR TESTING

Proliferation markers such as the Ki67 labeling index also help prognostic stratification of ER+/HER2- breast cancer. Ki67 is a nuclear marker expressed in all phases of the cell cycle except for the G0 phase. The IHC assessment of Ki67 has become the most widely used

method for comparing proliferation between different breast tumor samples (45). However, this biomarker has shown low lab-to-lab analytical validity as multiple methods and cutoffs have been applied to define high versus low Ki67 (46). In the last years, numerous efforts have been made by the international Ki67 Breast Cancer Working Group to standardize the Ki67 IHC methodology (45). Nevertheless, the ASCO Tumor Marker Guidelines Committee does not recommend the routine use of Ki67 to assess prognosis in patients with newly diagnosed breast cancer (47).

Despite heterogeneity in the methodological procedures and different scoring systems, the relationship between Ki67 and prognosis has been extensively studied. In two large metanalysis, Ki67 levels were correlated with a high risk of recurrence and worse survival in early breast cancer (48, 49). In the metastatic setting, several studies have shown a correlation between Ki67 levels and a bad prognosis (50). In HR+/HER2- breast cancer, Ki67 has an essential prognostic role after neoadjuvant treatment with endocrine therapy, being part of the preoperative endocrine prognostic index (PEPI) score together with other pathology biomarkers like the tumor size, the nodal status, and the ER level (51). Patients with a PEPI score of 0 at surgery had such a low relapse rate that further adjuvant systemic therapy beyond an endocrine agent's continuation appears unnecessary. In contrast, patients with a high PEPI score had a statistically significant higher risk of early relapse, and they should be offered all appropriate adjuvant treatments, including chemotherapy (51). Ki67 has also shown a clinical utility as a prognostic biomarker when performed after a short-term induction (2 weeks) of presurgical endocrine therapy in HR+/HER2- breast cancer (52). In this study, the prognostic value of Ki67 improved when the biomarker was assessed in breast samples taken after a short-term endocrine therapy treatment compared to the prognostic value at baseline (52).

In patients treated with preoperative endocrine therapy and CDK4/6i, Ki67 is also emerging as an endpoint of treatment efficacy. Thus, a complete cell cycle arrest defined as a Ki67 level lower than 2.7% has been used as an efficacy endpoint in the NeoPalAna clinical trial (NCT01723774) (53). Moreover, the Ki67 percentage of change from baseline to 2 weeks of treatment has been used as an efficacy endpoint in the NeoMONARCH trial (NCT02441946) (54). Finally, tumors whit a natural logarithm of Ki67 IHC staining lower than 1 after 15 days of treatment were defined as responders in the POP window opportunity study (NCT02008734) (55). However, the relationship between the dynamic changes of Ki67 in response to CDK4/6i and survival has not been extensively studied.

3. MOLECULAR HETEROGENEITY OF HR+/HER2- BREAST TUMORS: BREAST CANCER INTRINSIC SUBTYPES

3.1 INTRODUCTION TO THE BREAST CANCER INTRINSIC SUBTYPES

In 2000, based on the gene expression pattern of 65 surgical specimens of human breast tumors from 42 different individuals, Charles M. Perou described the intrinsic molecular subtypes of breast cancer (56). Based on hierarchical clustering, he identified a list of genes with more significant variation in expression between different tumors than between paired samples from the same tumor, the Intrinsic Gene list. A supervised cluster using this list of genes showed the presence of three central molecular portraits of breast cancer:

- A group of ER-positive tumors characterized by a relatively high expression of genes expressed by breast luminal cells: Luminal subtype.
- A group of ER-negative tumors with a positive IHC staining for either keratins 5/6 or 17 with a high relative expression of breast basal epithelial genes: Basal-like subtype.
- A cluster of tumors with high expression of ERBB2 oncogene-related genes: HER2-Enriched subtype.

In 2001, using a more extensive set of breast cancer samples, Therese Sørlie described two different luminal groups for the first time: a lower proliferation group or Luminal A and a higher proliferation group or Luminal B. She also showed a significant difference in survival between the four different intrinsic subtypes. The Basal-like and HER2-Enriched subtypes were associated with the shortest survival times, and the Luminal A subtype was associated with the longest survival times (57). Finally, in 2010, Aleix Prat described the Claudin-Low intrinsic subtype, a new entity characterized by a low expression of luminal differentiation markers, high enrichment for epithelial-to-mesenchymal transition markers, immune-related genes, and cancer stem cell-like features. These five intrinsic subtypes of breast cancer have been intensively studied during the last years, showing significant differences in their incidence, risk factors, prognosis, and treatment sensitivity (58).

The clinical implementation of the breast cancer intrinsic subtypes has been possible thanks to the PAM50 predictor development. In 2009, Joel S. Parker developed a supervised risk predictor of breast cancer relapse by integrating the tumor size, the nodal status, and the tumor intrinsic subtype defined by a 50-gene predictor (PAM50) (19). In 2013 the US Food and Drug Administration (FDA) approved Prosigna® (NanoString Technologies, Seattle, WA, USA). This

diagnostic test uses nCounter technology to quantify the mRNA expression of the PAM50 genes and tumor size and node involvement to calculate a risk of recurrence score (ROR) at 10 years. Since then, multiple correlative studies have included intrinsic subtype analyses.

3.2 MOLECULAR CHARACTERIZATION OF BREAST CANCER INTRINSIC SUBTYPES

A complete molecular characterization of breast tumors led by the Cancer Genome Atlas Project (TCGA) highlights the importance of breast cancer intrinsic subtypes (59). In this study, more than 500 primary breast tumors were extensively characterized at a DNA (copy number changes, somatic and germline mutations, methylation), RNA (miRNA and mRNA gene expression), and protein level (protein and phosphor-protein expression). The integration of all this information showed 4 main breast cancer groups perfectly recapitulated by the four main intrinsic subtypes (Luminal A, Luminal B, Her2-Enriched, and Basal-like) obtained by the PAM50 predictor (19). The main genomic and proteomic features of the tumor intrinsic subtypes are summarized in **Table 1** (59).

Table 1. Genomic and proteomic features of tumor intrinsic subtypes.

Subtype	Luminal A	Luminal B	Basal-like	HER2-enriched
DNA mutations	*PIK3CA* (49%) *TP53* (12%) *GATA3* (14%) *MAP3K1* (14%)	*TP53* (32%) *PIK3CA* (32%) *MAP3K1* (5%)	*TP53* (84%) *PIK3CA* (7%)	*TP53* (75%) *PIK3CA* (42%) *PIK3R1* (8%)
Copy number alterations (CNAs)	Most diploid; many with quiet genomes; 1q, 8q, 8p11 gain; 8p, 16q loss; 11q13.3 amp (24%)	Most aneuploid; many with focal amp; 1q, 8q, 8p11 gain; 8p, 16q loss; 11q13.3 amp (51%); 8p11.23 amp (28%)	Most aneuploid; high genomic instability; 1q, 10p gain; 8p, 5q loss; *MYC* focal gain (40%)	Most aneuploid; high genomic instability; 1q, 8q gain; 8p loss; 17q12 focal *ERRB2* amp (71%)
mRNA expression	High luminal expression signature Low proliferation	Lower luminal expression signature High proliferation	Basal signature High proliferation	HER2 amplicon signature High proliferation
DNA methylation		Hypermethylated phenotype	Hypomethylated phenotype	
Protein expression	High estrogen signaling High MYB	Less estrogen signaling High FOXM1 and MYC	High expression of DNA repair proteins, PTEN, and INPP4B loss signature (pAKT)	High protein and phospho-protein expression of EGFR and HER2

3.2.1 LUMINAL A

Luminal A is the most frequent breast cancer intrinsic subtype in both the overall population and within HR+/HER2- breast cancer (59). These tumors have a heterogeneous spectrum of copy number alterations (CNAs) and somatic mutations (60). Luminal A tumors have the highest number of genes mutated more frequently than expected by chance, with the highest mutation rate of *PIK3CA* (45%), followed by *MAP3K1, GATA3, TP53, CDH1, and MAP2K4.* This subtype is characterized by an increased expression of the luminal signature, which contains genes also overexpressed in the mammary ducts' luminal epithelium (*ESR1*, *GATA3*, *FOXA1*, *XBP1,* and *MYB*). Compared to Luminal B, Luminal A tumors usually present lower proliferation rates.

3.2.2 LUMINAL B

Luminal B breast cancer is also unique about the profile of gene CNAs and somatic mutations. Compared to Luminal A, Luminal B tumors have a lower frequency of *PIK3CA* mutations and a higher frequency of *TP53* mutations. Luminal B tumors tend to have a lower expression of luminal signature and a more increased proliferation than Luminal A, and they usually show a hypermethylated phenotype.

3.2.3 HER2-ENRICHED

HER2-Enriched is the predominant subtype within HER2-positive tumors. However, not all clinically HER2-positive tumors are HER2-Enriched. Furthermore, HER2-positive is the most heterogeneous breast cancer type. In TCGA, only 50% of HER2-positive tumors are HER2-Enriched. This subtype is mainly characterized by DNA amplification of HER2. HER2-Enriched/HER2-positive tumors show a significantly higher expression of several tyrosine kinases receptors (RTKs), including FGFR4, EGFR, HER2 itself, and genes within the HER2 amplicon (including *GRB7*). HER2-Enriched tumors usually have a high frequency of *PIK3CA* mutations (39%) and a lower frequency of *PTEN* mutations. Finally, the HER2-Enriched mRNA subtype typically shows high aneuploidy and the highest somatic mutation rate.

3.2.4 BASAL-LIKE

Basal-like is the predominant breast cancer intrinsic subtype within the TNBC subgroup of patients (around 75% of all the TNBC are basal-like). These tumors harbor a high frequency of *TP53* mutations (80%), and, from a gene expression level, almost all the basal-like tumors have lost the *TP53* function. *PIK3CA* is the second most mutated gene in this subtype but at a lower frequency than luminal tumors (~9%). At a gene expression level, basal-like tumors are

characterized by a high expression of keratins 5, 6, and 17 and increased proliferation genes expression. A hyperactivation of *MYC* and *H1F1* pathways are also frequent. Finally, basal-like tumors usually have the lowest level of DNA methylation. A study of the DNA germline mutations confirmed the strong association between germline *BRCA1* mutations and the basal-like subtype.

3.3 ONCOGENIC PATHWAYS BY TUMOR INTRINSIC SUBTYPE

3.3.1 TP53 PATHWAY BY TUMOR INTRINSIC SUBTYPE

The *TP53* pathway is differentially inactivated between different tumor intrinsic subtypes (**Figure 3**) (59). Compared to Luminal A and B, HER2-Enriched and Basal-like tumors have a higher mutation rate of *TP53* (75% and 84%, respectively). Within luminal tumors, the frequency of the *TP53* mutations in Luminal A (12%) is significantly lower than in luminal B (29%). Intrinsic subtypes differ not only by mutation frequencies but also by mutation types. Thus, *TP53* mutations in Basal-like tumors are mostly nonsense and frameshift, whereas, in Luminal A and Luminal B tumors, missense mutations are the predominant ones. *TP53* mutation's timing also depends on the tumor subtype, being the first significant event in luminal tumors but occurring after *PTEN* loss in Basal-like tumors (61).

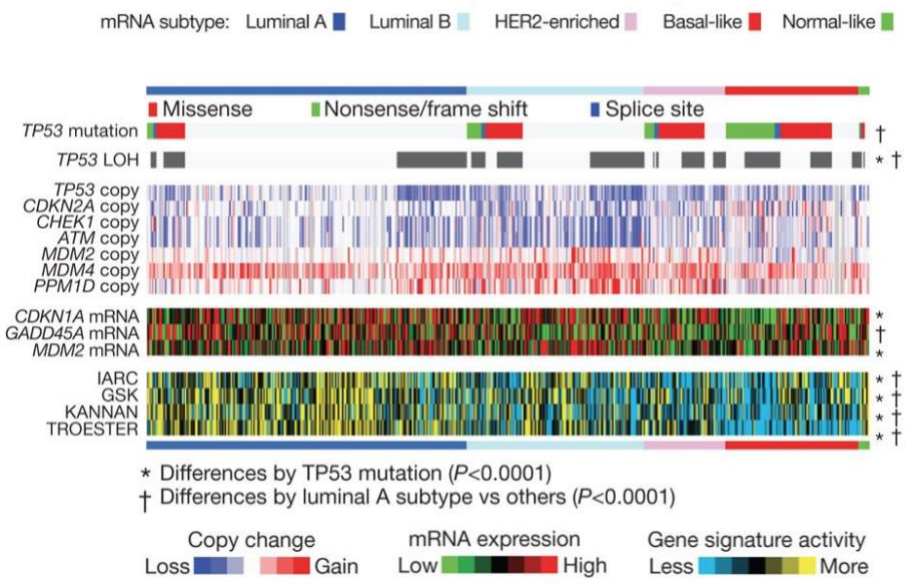

Figure 3. TP53 pathway by tumor intrinsic subtype.
Adapted from TCGA (59).

At a gene expression level, the *TP53* pathway activity shows a loss of *TP53* in almost all Basal-like tumors. Within luminal breast cancer, individual markers of *TP53* functionality and activity are highest in Luminal A cancers than Luminal B, indicating that the *TP53* pathway remains primarily intact in Luminal A versus Luminal B tumors.

3.3.2 PI3K PATHWAY BY TUMOR INTRINSIC SUBTYPE.

Mutations of *PIK3CA* are prevalent in luminal cancers (49% in Luminal A and 32% in Luminal B, respectively), whereas *PTEN* loss is more common in Basal-like tumors (**Figure 4**). HER2-Enriched subtype also has a high frequency of *PIK3CA* mutations (39%) with a lower frequency of *PTEN* and *PIK3R1* mutations and genomic losses of *PTEN* and *INPP4B*. With only a 9% mutation frequency, *PIK3CA* is the second most commonly mutated gene in Basal-like tumors. However, inferred PI3K pathway activity at gene expression and protein levels are highest in Basal-like cancers, probably due to an alternative activation involving loss of *PTEN* and *INPP4B* and/or amplification of *PIK3CA*.

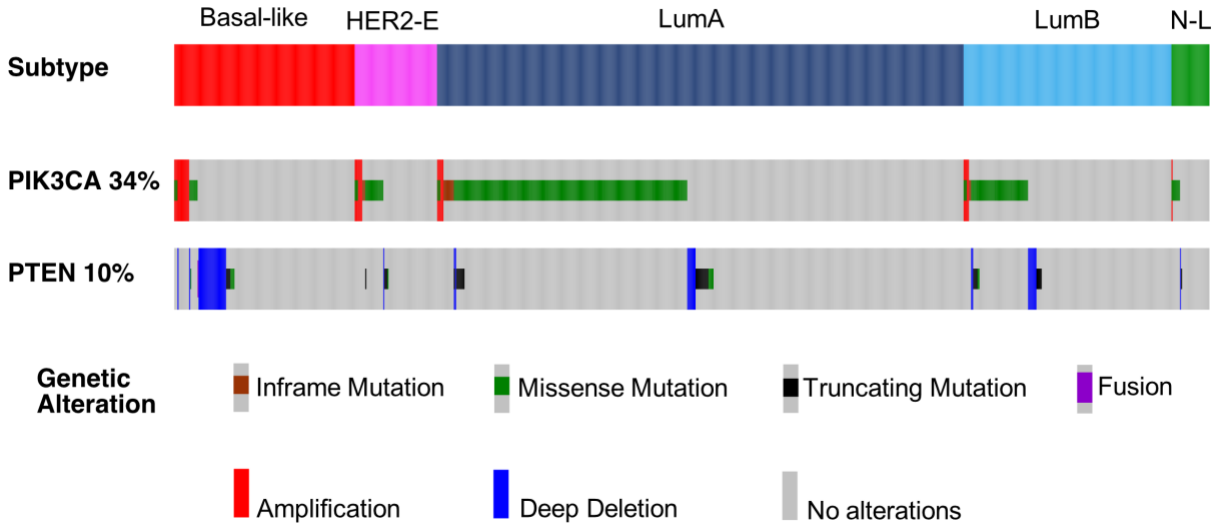

Figure 4. *PIK3CA* and *PTEN* somatic mutations and copy number alterations by tumor intrinsic subtype.

TCGA PanCancer Atlas. cBio Cancer Genomics Portal. HER2-E: HER2-Enriched; LumA: Luminal A; LumB: Luminal B; N-L: Normal-like.

3.3.3 CELL CYCLE AND RETINOBLASTOMA PATHWAY (RB1) BY TUMOR INTRINSIC SUBTYPE

At a gene expression level, the *RB1* pathway shows specific intrinsic subtype alterations. *RB1* itself, by mRNA and protein expression, is detectable in most luminal cancers, expressing the highest levels within luminal A tumors. Cyclin D1 amplification and increased gene expression preferentially occurred within luminal tumors, especially within Luminal B. In contrast, *CDKN2C* (also known as p18) is low in luminal A cancers (**Figure 5**). Finally, *RB1* activity signatures are also higher in luminal than Basal-like cancers, characterized by the *RB1* loss and the cyclin E1 amplification.

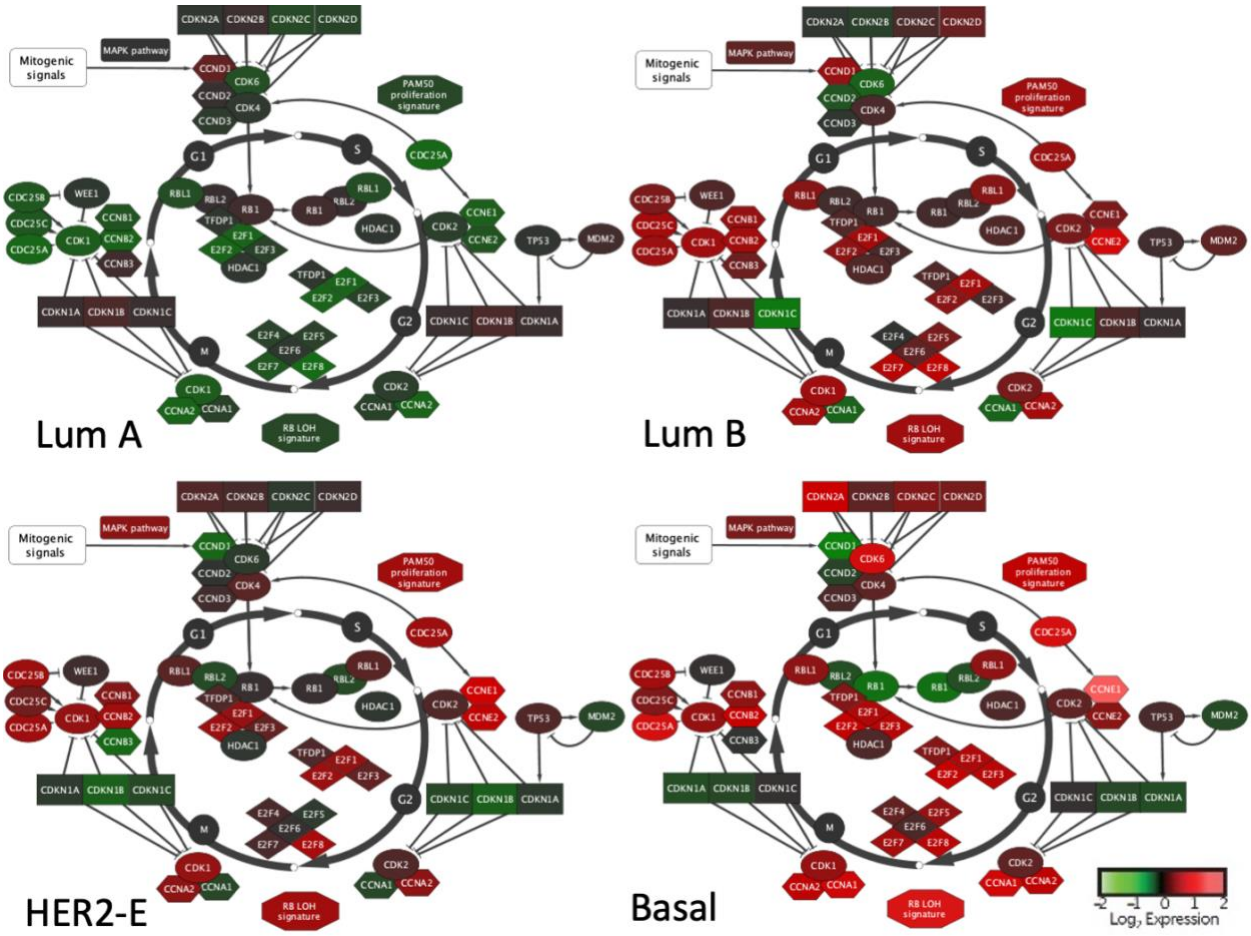

Figure 5. Cytoscape® plot of the cell cycle gene expression by tumor intrinsic subtype. TCGA data (n = 1,061). Arrows are based on protein-to-protein interactions. The nodes are genes, and their color (green to red) is based on the log2 gene expression values.

3.4 ST. GALLEN SURROGATE DEFINITION OF BREAST CANCER INTRINSIC SUBTYPES

In 2011, the St Gallen International Breast Cancer Expert Panel adopted a new pathological-based classification of breast tumors to guide treatment decisions based on recognizing tumor intrinsic subtypes (**Table 2**) (62).

Table 2. Definition of breast cancer IHC-based surrogate subtypes.

Surrogate Subtype	Clinicopathologic definition
Luminal A	• ER and/or PgR positive • HER2 negative • Ki67 low
Luminal B/HER2-negative	• ER and/or PgR positive • HER2 negative • Ki67 high
Luminal B/HER2-positive	• ER and/or PgR positive • HER2 positive
HER2 positive	• HER2 overexpressed or amplified • ER and PgR absent
TNBC	• ER and PgR absent • HER2 negative

In this classification, the Ki67 labeling index is crucial in distinguishing Luminal A vs. Luminal B like tumors. The cut point to define a Ki67 value as low or high has been controversial over the years. In the 2011 St. Gallen consensus, the Ki67 cutoff was established in 14% based on Maggie Cheang's work (62, 63). In this study, tumors from a cohort of 357 patients were subtyped by gene expression profiles using the real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) PAM50 classifier. Then, 144 tumors that were both Luminal A and Luminal B by gene expression profile and HR-positive/HER2-negative by IHC were selected. With a sensitivity of 72% and 77% specificity, the best Ki67 index cut point to distinguish Luminal B from luminal A tumors was 13.25% (**Figure 6**).

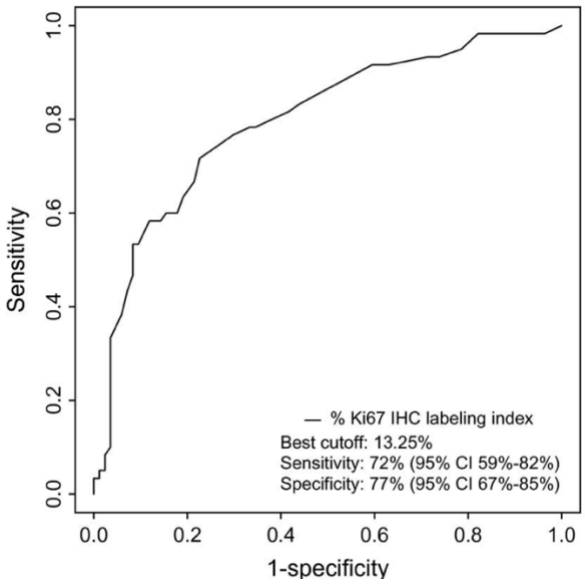

Figure 6. ROC analysis Ki67 as a continuous variable to identify Luminal B disease.
The relationship between Ki67 and subtype was analyzed in a cohort of 144 Luminal A and B tumors (63).

When applying this cutoff to another external dataset of 2,847 HR+ breast cancers, Luminal B tumors defined by Ki67 higher than 13.25% were statistically significantly associated with low breast cancer recurrence (**Figure 7**) (63).

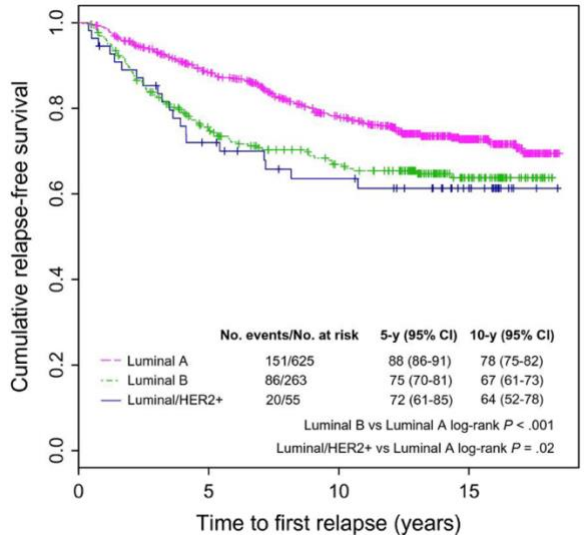

Figure 7. Relapse-free survival by breast cancer surrogate subtype.
A cohort of 934 lymph node-negative HR-positive patients who received no adjuvant systemic therapy was used for this analysis (63).

In the 2013 St. Gallen consensus, the cutoff value of 14% as defined by the 2011 St. Gallen consensus was questioned, and a new cutoff value of 20% was suggested instead (64). In 2015, most of the panel voted for a Ki67 of 20% to distinguish Luminal B disease. However, they also proposed that Ki67 scores should be interpreted in light of local laboratory values, using each lab's median gene expression to define Ki67 high vs. low (65). All these recommendations have led to confusion regarding how to interpret and use Ki67 scoring in the clinical setting. Moreover, in all these studies, a research version of the PAM50 predictor and not the gold standard Prosigna® was used to obtain the tumor intrinsic subtype.

3.4 INTRINSIC SUBTYPE DISTRIBUTION WITHIN HR+/HER2- BREAST TUMORS

The implementation of the PAM50 predictor in 2009 (19) allowed us to investigate the tumor intrinsic subtype distribution from different breast cancer cohorts, including several clinical trials. In 2018, our group did a PubMed search for research articles and scientific abstracts with intrinsic subtype and HR information. A total of 39 studies with data from 13,264 breast tumors were identified. In all the cases, intrinsic subtypes were obtained from different gene expression platforms by applying the PAM50 predictor. As expected, most tumors were HR+/HER2- (n = 10,755, 81.08%), and all the intrinsic subtypes were identified within this tumor type. In a cohort of 9,258 early HR+/HER2- breast tumors, the subtype distribution was: 54.5% of Luminal A, 34.94% Luminal B, 5.8% HER2-Enriched, 2.16% Basal-like, and 2.59% of Normal-like tumors (**Figure 8A**). Compared to early HR+/HER2- breast cancer, the proportion of HER2-Enriched tumors seems higher in patients with advanced or metastatic HR+/HER2-negative breast cancer (**Figure 8B**). The acquisition of a HER2-Enriched profile of HR+/HER2- metastatic patients, could be due to patient selection, a shift in tumor biology, or the combination of both and might reflect estrogen-independency. Comparative gene expression analysis from 123 paired primary and metastasis HR+/HER2- breast tumors have shown that 10–15% of prior Luminal A or B tumors acquire a HER2-Enriched profile in the metastatic setting (66), and FGFR4 could have an essential role as a HER2-Enriched driver in these cases (67).

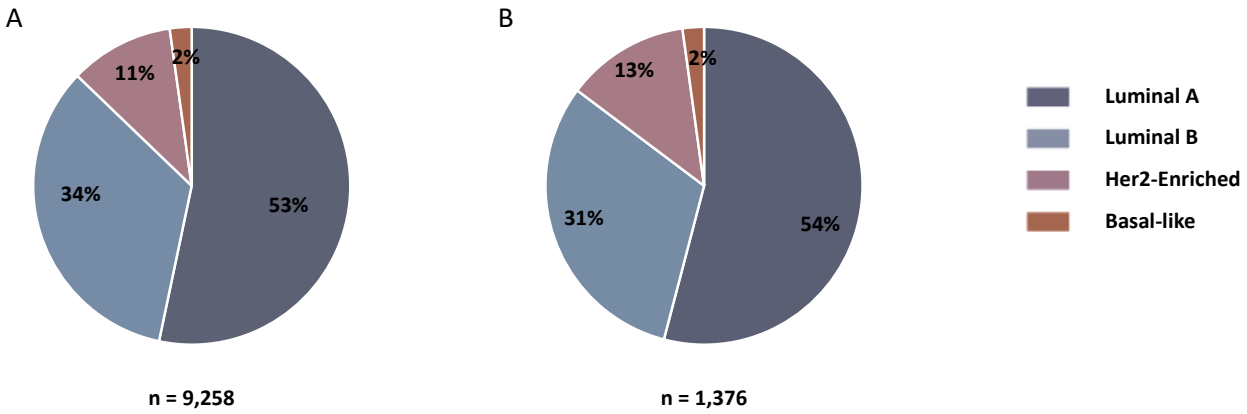

Figure 8. Molecular heterogeneity of HR+/HER2- breast cancer.

8A. Molecular heterogeneity of HR+/HER2- early breast cancer. 8B. Molecular heterogeneity of HR+/HER2- metastatic breast cancer.

From an IHC point of view, non-luminal tumors could be identified within all ER expression ranges, even in tumors with ER expression higher than 10%. However, these tumors had a significantly lower expression of PR and a substantially higher expression of Ki67 (**Figure 9**).

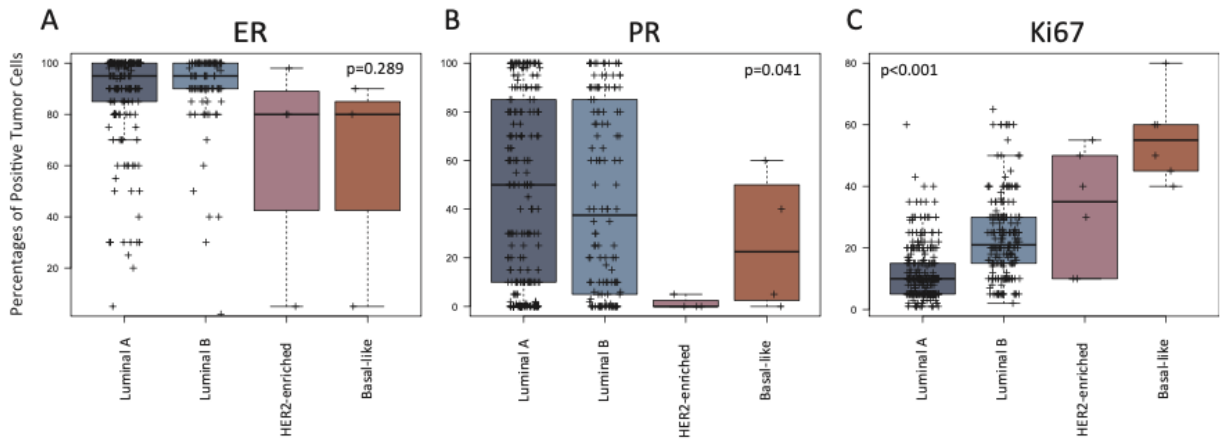

Figure 9. Estrogen receptor, progesterone receptor, and Ki 67 positive cells across intrinsic subtypes.

517 HR+/HER2- breast tumors were analyzed. The p-values were calculated by an ANOVA test, comparing medians across all the subtypes. ER: estrogen receptor; PR: progesterone receptor.

4. CLINICAL IMPLICATIONS OF THE MOLECULAR HETEROGENEITY OF HR+/HER2- BREAST CANCER

4.1 PROGNOSTIC IMPLICATIONS OF THE MOLECULAR HETEROGENEITY OF HR+/HER2- BREAST CANCER

The prognostic differences between the different tumor types within the HR+/HER2- breast cancer have led to the development of multiple gene expression-based tests such as Prosigna (19), OncotypeDX (68), MammaPrint (69), and EndoPredict (70). These platforms, already clinically implemented, help clinicians and patients make decisions about the necessity of (neo)adjuvant chemotherapy added to endocrine therapy to prevent relapses. Besides the fact that only Prosigna® was created based on the prognostic differences of tumor intrinsic subtypes, all these 4 prognostic scores have a significantly different distribution by intrinsic subtype (**Figure 10**), being Luminal A tumors the ones with lower scores compared to non-luminal subtypes (HER2-Enriched and Basal-like).

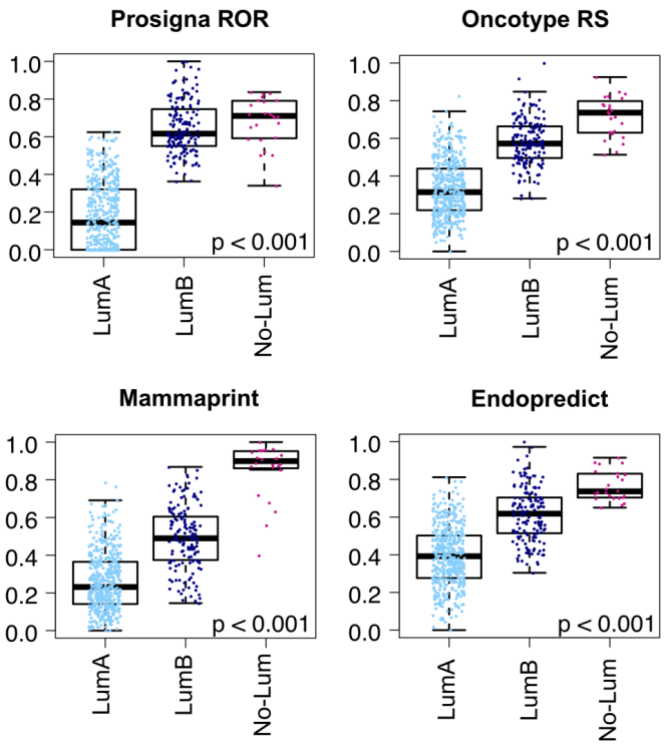

Figure 10. Distribution of Prosigna®, Oncotype®, Mammaprint®, and Endopredict® in TCGA ER+/HER2- tumors by tumor intrinsic subtype.
All the scores were calculated using an RNAseq research version and scaled for visualization. The p-values were obtained by an ANOVA test. LumA: Luminal A; LumB: Luminal B; No-Lum: no Luminal.

Multiple studies have consistently shown the prognostic differences between Luminal A and Luminal B tumors over the years. Simultaneously, the two non-luminal HR+/HER2- intrinsic subtypes (i.e., HER2-Enriched and Basal-like) have also shown a worse prognosis than luminal tumors in different scenarios.

In a retrospective cohort of patients with node-negative breast cancer who did not receive adjuvant therapy (19, 71), the intrinsic subtype showed a statistically significant prognostic ability to predict relapse-free survival (RFS) in both ER-positive (p-value 1.89e-10) and ER-negative patients (p-value = 0.012). In a second retrospective series (BC TAM), distance recurrence free-survival (DRFS) was significantly higher in Luminal A patients with no adjuvant treatment (log-rank p-value = 1.61e-05) and patients treated with five years of adjuvant tamoxifen (log-rank p-value 0.006) (22). In the TransATAC study (72), Dowsett et al. showed how the PAM50-ROR model based on the tumor intrinsic subtypes predicted a high risk of distance recurrence group of patients diagnosed with HR+/HER2- disease. In the ABSCG-8 study (20), the same score was able to add prognostic information beyond clinicopathologic characteristics, showing Luminal A the highest 10-years DRFS (HR 2.85, 95% CI 2.04-4.00, p-value < 0.001). In another retrospective study of 4 series with more than 1,380 patients with breast cancer treated during 5-years with adjuvant tamoxifen (73), DFRS at 8.5 years was 90.9% for Luminal A, 75.3% for Luminal B, 73.7% for HER2-Enriched, and 66.2% for Basal-like subtypes in patients without nodal involvement (n = 610), and 75.4% for Luminal A, 53.4% for Luminal B, 53.3% for HER2-Enriched and 62.2% for Basal-like patients with node-positive disease (n = 699), respectively.

The prognostic value of tumor intrinsic subtypes is also preserved when exploring combinations of endocrine therapy and different targeted drugs. In the EGF3008 phase III trial, 644 patients with metastatic HR+/HER2+ breast cancer were treated with first-line letrozole +/- lapatinib. Luminal B, HER2-Enriched, and Basal-like subtypes showed 1.46, 2.87, and 2.26 times more risk of PFS than Luminal A tumors. The tumor intrinsic subtype added more prognostic information than any other clinical variable introduced in the multivariate model (74). Similar results were also observed at an OS level. PAM50 has also been applied to 261 tumor samples from the BOLERO-2 phase III trial samples (75, 76). In this study, 724 patients with HR+/HER2- disease previously treated with a non-steroidal aromatase inhibitor were randomized (3:1) to receive exemestane +/− everolimus. Tumor specimens were obtained from baseline or metastatic tissue. Interestingly, the proportion of HER2-Enriched patients was higher in metastatic group than in primary tumors (32% versus 19%, respectively). In this trial, the non-

luminal subtype was independently associated with inferior PFS (6.67 months in luminal vs. 5.16 months in non-luminal, adjusted HR: 0.66) and OS (33.08 months in luminal vs. 19.65 months in non-luminal, adjusted HR: 0.52). The prognostic implications of intrinsic subtypes have also been studied in different trials investigating endocrine therapy's efficacy combined with CDK4/6i. This is the case of the PALOMA-2 and PALOMA-3 phase III trials that led to palbociclib's approval in the first and second lines for HR+/HER2- advanced or metastatic breast cancer treatment in combination with letrozole and fulvestrant, respectively. In a correlative gene expression analysis of these two trials, a single sample predictor algorithm (AIMS predictor), was applied to obtain the tumor intrinsic subtype (77, 78). In the PALOMA-2 trial, intrinsic subtypes were obtained for 155 out of 666 tumors. The subtype distribution was: 50% Luminal A, 30% Luminal B, 19% HER2-Enriched, 1% Normal-like and <1% Basal-like. In the letrozole + placebo arm, the median PFS was 17 months for patients with Luminal A, 11 months for Luminal B, 11 months for HER2-Enriched, and 6.4 for Basal-like breast tumors. Due to the efficacy of palbociclib mostly in luminal tumors, in the letrozole + palbociclib arm, the prognostic differences by subtypes were even higher, with a median PFS of 30.4 months for patients with Luminal A, 19.6 months for Luminal B, 13.8 months for HER2-Enriched, and 5.6 for Basal-likes tumors, respectively (79). In the PALOMA 3 trial, intrinsic subtypes were obtained from 302 out of 521 tumor samples. The subtype distribution was 44% Luminal A, 30.8% Luminal B, 20.9 HER2-Enriched, 2.6% Normal-like, and 1.7% Basal-likes. In the palbociclib + fulvestrant arm, the median PFS was 16.6 months for patients with Luminal A, 9.2 months for Luminal B, and 9.5 for non-luminal tumors (80). These findings should be considered carefully, as the AIMS subtype predictor differs from the PAM50 subtype predictor, and discrepancies between both methods have been already described. Finally, in a pooled analysis of the MONALEESA phase III trials, intrinsic subtype was significantly associated with survival in both the placebo and the ribociclib treatment arms, even after adjusting the models for clinical factors (81).

From a prognostic point of view in the metastatic setting, a new and promising prognostic tool called PAM50MET has been recently designed for HR+/HER2- breast cancer patients (23). This biomarker based on elastic net modelling included a total of 21 features. Some variables like ECOG Performance Status (PS) of 0 vs. 1, older age, Luminal A subtype, and a high gene expression of *SCL39A6*, *MAPT*, *PGR*, *NAT1*, *ESR1,* and *TMEM45B*, were associated with longer PFS. On the contrary, variables like having more than 3 metastatic sites, having a HER2-Enriched subtype, the correlation to the Basal-like centroid, and a high expression of *CCNB1*, *PHGDH*, *FGFR4*, *GRB7*, *FOXA1*, *NUF2*, *GPR160,* and *UBET2C*, were significantly associated

with a worse survival outcome. The performance of the PAM50MET in the train (EGF30008) and the external validation (BOLERO-2) as measured by the C-index were significantly higher compared to models that only included clinical or PAM50 features. If prospective evaluated, this prognostic tool might help identify candidates for endocrine therapy only or novel combinations. Once again, it reassures PAM50 intrinsic subtypes' prognostic relevance in the HR+/HER2- metastatic setting.

All the previous data indicate that Luminal A have a better outcome than Luminal B tumors, and non-luminal have a worse outcome than luminal tumors. These prognostic implications are maintained in both early and metastatic HR+/HER2- breast cancer. They are preserved in different scenarios: in untreated patients, in the context of endocrine therapies, and in the company of endocrine and different targeted therapies combinations (lapatinib, everolimus, and palbociclib).

4.2 ENDOCRINE SENSITIVITY BASED ON THE MOLECULAR HETEROGENEITY OF HR+/HER2- BREAST CANCER

Within HR+/HER2- breast cancer, patients with HER2-Enriched and Basal-like tumors have a terrible prognosis even in the presence of endocrine therapy due to their estrogen independence. Several biomarker studies support this hypothesis. In the ACOSOG Z1031 trial, patients with HR+/HER2- breast cancer were randomized to receive 4 months of presurgical endocrine therapy with either letrozole, anastrozole, or exemestane. Using Ki67 as the surrogate biomarker of endocrine responsiveness, patients with Basal-like and HER2-Enriched breast tumors showed persistently high Ki67 values (>20%) after completing the treatment, consistent with an estrogen-independent growth pattern of non-luminal tumors besides their ER positivity by IHC (82). In a second neoadjuvant study, 112 postmenopausal patients with ER-positive early breast cancer were treated with neoadjuvant anastrozole (83). The benefit from anastrozole, defined by a proportional fall in Ki67 levels after two weeks of treatment, was similar in patients with Luminal A and Luminal B tumors. However, patients with Basal-like and HER2-Enriched disease showed low reductions in Ki67 upon treatment. Finally, in the NeoPalAna trial (53), 50 patients were treated with neoadjuvant anastrozole-only for 28 days, followed by palbociclib's addition for 4 months. Two of the 50 patients were identified as non-luminal (one Basal-like and one HER2-Enriched), and none of them showed a Ki67 suppression after 4 weeks of anastrozole. Moreover, in patients with ER-low expressing tumors (i.e., 1–9% positive tumor cells), the evidence of survival benefit of endocrine therapy is not Level 1 (27). Besides all this evidence, a phase III randomized clinical trial of endocrine therapy vs. placebo

in patients with HR+/HER2- /non-Luminal tumors is unlikely to be done. However, it is reasonable for oncologists to discuss the pros and cons of endocrine therapy with patients whose cancers contain low ER levels to make a decision based on the totality of information about the individual case (27).

4.3 CHEMOTHERAPY SENSITIVITY BASED ON THE MOLECULAR HETEROGENEITY OF HR+/HER2- BREAST CANCER

Non-luminal disease, especially Basal-like, has shown higher chemosensitivity than luminal disease in patients with HR+/HER2- breast cancer. In a retrospective study from a single institution, 180 core needle biopsies from patients with HR+/HER2- disease who underwent neoadjuvant chemotherapy, non-luminal tumors represented 11.6% of the samples (n = 7 HER2-Enriched and n = 14 Basal-like). More importantly, the residual cancer burden (RCB) 0/1 rates varied significantly based on intrinsic subtype and were 9.3%, 20.0%, 14.3%, and 50.0% for patients with Luminal A, Luminal B, HER2-Enriched, and Basal-like tumors, respectively (84). In the SOLTI-NEOERIBULIN phase II clinical trial, 101 patients with HR+/HER2- breast cancer were enrolled. PAM50 was performed before starting treatment with neoadjuvant eribulin for 4 cycles. The overall pathologic complete response (pCR) rate in the breast was low (6.27%). Interestingly, pCR rates differed by subtype: 33.3% in patients with HER2-Enriched, 12.5% in Luminal B, 0% in Basal-like, and 0% in Luminal A tumors. Besides, 100% of HER2-Enriched tumors converted to Normal-like in the residual specimen. Thus, patients with HR+/HER2-/HER2-Enriched breast cancer may benefit the most from eribulin treatment (85). In another retrospective study, PAM50 was evaluated on 451 patients with HR+/HER2- breast cancer treated with multi-agent chemotherapy across different neoadjuvant trials (86). pCR rates in breast and axilla of patients with Luminal A, Luminal B, HER2-Enriched, and Basal-like tumors were 5%, 15%, 16%, and 36%, respectively. This difference was found statistically significant and independent of known clinical-pathological variables. Patients with non-luminal tumors showed higher pCR rates than patients with luminal tumors (30.0% vs. 8.9%, adjusted OR: 4.20). Finally, recent data suggest that patients with HR+/HER2- Luminal B-like tumors (defined by Ki67 >20%) could benefit more from dose-dense chemotherapy vs. standard chemotherapy compared to patients with Luminal A-like disease. However, the interaction test for Luminal B-like vs. Luminal A-like biomarker was not significant (87).

Overall, these data suggest that non-luminal HR +/HER2- disease is more chemo-sensitive, concordant with their low endocrine sensitivity. This chemosensitivity vs. endocrine-sensitivity, depending on tumor intrinsic subtype, has been the starting point for developing the chemo-

endocrine sensitivity score or CES score (88). This biomarker, defined as the distance between the correlation to Luminal-A and the correlation to the Basal-like centroids, has proven to discriminate endocrine-sensitive vs. chemotherapy-sensitive tumors just based on the intrinsic subtype, showing a prognostic value not only in patients with HR+/HER2- disease but also in HR+/HER2+ (89).

4.4 CDK4/6I SENSITIVITY BASED ON THE MOLECULAR HETEROGENEITY OF HR+/HER2- BREAST CANCER

Over the last few years, CDK4/6is, combined with endocrine therapy, has changed the way we treat advanced HR+/HER2- breast cancer. Three CDK4/6i are currently FDA-approved for first- and second-line treatment of metastatic HR+/HER2- breast cancer: namely abemaciclib (LY835219), palbociclib (PD0332991), and ribociclib (LEE011). The addition of these drugs to ET has shown the most significant PFS benefit and some benefits for OS in several metastatic phase II-III clinical trials (10, 11, 13-16, 90). Moreover, multiple ongoing phase II/III trials will address the value of adding them to endocrine therapy in the adjuvant/neoadjuvant setting. However, both *de novo* and acquired resistance to CDK4/6i are frequent. Given the cost of these drugs, biomarkers' identification for patient selection beyond simple ER/PR/HER2 status is needed. Although there have been multiple studies concerning the role of several cell cycle-related genes and proteins as potential biomarkers for CDK4/6i response, none of these to date has been clinically adopted.

Two biomarkers have shown consistent results across more than one correlative analysis at an mRNA level. The gene expression level of cyclin E1 (*CCNE1)* have shown a promising predictive value, with a significant interaction test for palbociclib treatment in the PALOMA-3 trial (Low-CCNE1 HR 0.32, 95% CI 0.20-0.50; High-CCNE1 HR 0.85, 95% CI 0.58-1.26; interaction p-value 0.00238) (80). This finding has also been validated in an independent window opportunity study, the POP trial. In this study, *CCNE1* mRNA levels were also able to identify palbociclib-resistant tumors, defined as those tumors without a decrease in Ki67 after two weeks of preoperative palbociclib (55, 80). Interestingly, *CCNE1* gene expression levels are statistically different by tumor intrinsic subtype, as shown in **Figure 11**. Thus, future research will be required to assess the impact of *CCNE1* mRNA high expression on CDK4/6i resistance stratified by tumor subtype.

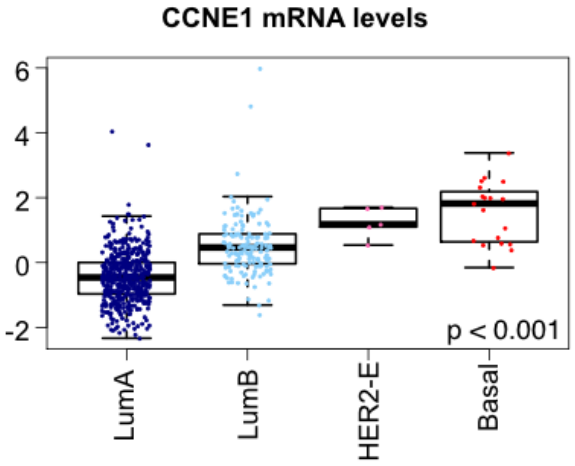

Figure 11. Distribution of cyclin E1 gene expression by tumor intrinsic subtype within ER+/HER2- tumors.

A subset of ER+/HER2- TCGA tumors (n = 650) was used for this plot. LumA: Luminal A; LumB: Luminal B; HER2-E: HER2-Enriched, Basal: Basal-like. The p-value was obtained by an ANOVA test.

The intrinsic subtype has also shown consistent results as a CDK4/6i biomarker across multiple preclinical studies and clinical trials. In preclinical research using 47 human breast cancer cell lines, luminal tumors were most sensitive to palbociclib growth inhibition (91). In the PALOMA-2 trial, patients with Luminal A and Luminal B tumors got the highest benefit of palbociclib addition compared to patients with non-luminal subtypes (79). In the PALOMA-3, there was an absolute PFS benefit with the addition of palbociclib of 11.8 months for patients with Luminal A, 5.7 months for Luminal B, and 4 months for non-luminal disease. However, no significant interaction was found between Luminal A vs. Luminal B tumors and palbociclib treatment effect. In a recent pooled analysis of both trials, both Luminal A and Luminal B subtypes significantly benefit from palbociclib addition (92). The predictive implications of intrinsic subtype have also been analyzed in a pooled analysis of the MONALEESA-2, -3, and -7 trials (81). In this study, a significant PFS benefit of ribociclib vs. placebo was observed in patients with Luminal A, Luminal B, and HER2-Enriched but not in patients with Basal-like tumors. The interaction between tumor intrinsic subtype and treatment was statistically significant (p-value 0.045). Also, in the metastatic setting, the PEARL trial, a study comparing CDK4/6i versus chemotherapy, only women with non-luminal tumors benefit from chemotherapy vs. palbociclib (93). Finally, in the neoadjuvant NeoPalAna trial (53), one patient was identified as Basal-like and another as HER2-enriched at baseline. Interestingly, these two patients didn't show a Ki67 suppression

after 4 weeks of anastrozole and 4 months of anastrozole and palbociclib combination, being considered as palbociclib-resistant patients. All this data together suggests a clear benefit of CDK4/6i in luminal tumors, with no benefit in Basal-like subtype. The sensitivity of HR+/HER2- HER2-Enriched tumors to CDK4/6i is still a controversy. These tumors seem to be resistant to palbociclib in the neoadjuvant setting using endpoints based on Ki67. On the contrary, they seem to get a high benefit from palbociclib and ribociclib in the metastatic setting when analyzing survival outcomes. These results indicate that the correlation of neoadjuvant Ki67-based endpoints with survival should be further studied. Finally, more correlative analyses from the major phase III studies are needed to clarify the intrinsic subtype's predictive role in CDK4/6i benefit.

4.5 PI3K INHIBITORS AND THE HETEROGENOUS SPECTRUM OF PIK3CA MUTATIONS IN HR+/HER2- BREAST CANCER

Based on the SOLAR-1 trial, the Therascreen® *PIK3CA* mutation assay and the alpha-specific PI3K inhibitor alpelisib received the FDA-approved to identify and treat patients with advanced *PIK3CA*-mutated HR+/HER2- breast cancer in combination with fulvestrant (17). The proportion of PIK3CA mutations is different by tumor intrinsic subtype, being more frequent in luminal tumors. However, the spectrum of *PIK3CA* mutations is similar in HR+/HER2- compared to HER2-positive and TNBC tumor types (94). In a retrospective study including 4,055 HR+/HER2- breast cancer patients, the of *PIK3CA* mutations were: H1047R (35%), E545K (18%), E542K (11%), N345K (6%), H1047L (4%) and other mutations (27%). Interestingly, some of these specific mutations, like N345K, are not captured by the FDA-approved Therascreen® panel, which could have therapeutic implications.

4.6 NEW TARGETED DRUGS FOR HR+/HER2- BREAST CANCER.

Due to HR+/HER2- breast cancer's heterogeneity, multiple targeted therapies are in clinical development or have been already approved to treat defined populations of HR+/HER2- breast cancer patients. Some of these treatments are summarized in **Table 3**.

Table 3. Summary of new targeted agents for HR+/HER2- breast cancer treatment.

Group	Mechanism of action	HR+/HER2- target population	Drugs	Development phase
PARP inhibitors	Poly (ADP-ribose) polymerase inhibitors. Blocking PARP helps keep BRCA1/2 mt cancer cells from repairing their damaged DNA, causing them to die	BRCA1/2 mt	Olaparib (95) Talazoparib (96)	FDA approved FDA approved
TRK inhibitors	Tyrosine Kinase inhibitors: block the TRK protein, which is involved in cell signaling and cell growth, in patients whose disease has the NTRK gene without a known acquired resistance mutation	NTRK gene fusion without a known acquired resistance mutation	Larotrectinib (97) Entrectinib (98)	FDA approved FDA approved
New SERDs	New Selective Estrogen Receptor Down regulators: block endocrine-dependent and independent ER signaling by ablation of ER	HR+/HER2-	Elacestrant GDC-9545 SAR439859 AZD9833	Phase III NCT03778931 Phase II NCT04436744 Phase II NCT04059484 Phase II NCT04214288
AR inhibitors	Androgen Receptor Inhibitors: block AR activation and AR-mediated cellular proliferation	AR+	Orteronel Enzalutamide (+ alpelisib) Enzalutamide (+ fulvestrant) Enzalutamide (+ exemestane) GTx024	Phase II NCT01990209 Phase I NCT03207529 Phase II NCT02955394 Phase II NCT02007512 Phase II NCT01616758
Anti-HER3	Block HER3	HER3+	U3-1402 MM-121 (+/- exemestane) MM-121 (+/- paclitaxel)	Phase I/II NCT02980341 Phase II NCT01151046 Phase II NCT01421472

FGFR inhibitors	Fibroblast Growing Factor Receptor inhibitors: binds to and inhibits FGFR, which may result in the inhibition of FGFR-related signal transduction pathways, and, so, the inhibition of tumor cell proliferation and tumor cell death	FGFR amplified	Erdafitinib (+ fulvestrant + palbociclib) TAS-120 (+/- fulvestrant) Lucitanib AZD 4547 (+ fulvestrant) AZD 4547 (+ AI) Infigratinib Rogaratinib Debio 1347	Phase Ib NCT03238196 Phase II NCT04024436 Phase II NCT02053636 Phase II NCT01202591 Phase II NCT01791985 Phase Ib NCT04504331 Phase I NCT04483505 Phase II NCT03344536
AKT inhibitors	Bind and block AKT (protein kinase B), which blocks the PI3K/AKT signaling pathway	HR+/HER2-	Ipatasertib (+ fulvestrant + palbociclib) Ipatasertib (+ paclitaxel) Ipatasertib (= Atezolizumab) AZD5363	Phase III NCT04060862 Phase III NCT03337724 Phase II NCT03280563 Phase II NCT02077569
ADC HER2	Antibody-drug conjugates of anti-HER2 and chemotherapy agents. Upon antibody/antigen binding and internalization, these drugs inhibit DNA replication and induce cell cycle arrest and tumor cell apoptosis.	HER2-low	Trastuzumab deruxtecan RC48-ADC	Phase III NCT04494425 Phase III NCT04400695

4.8 IMMUNE INFILTRATION IN NON-LUMINAL SUBTYPES

With the current interest in immune-oncology, it is becoming more evident that the interaction between tumor and microenvironment may provide useful prognostic and predictive information also in HR+/HER2- breast cancer. Moreover, evidence suggests that both chemotherapy and endocrine therapy may interact with the tumor-immune interplay (99). In general, HR+/HER2- breast tumors are less immune infiltrated than HER2-positive and TNBC, and the prognostic role of tumor-infiltrating lymphocytes (TILs) in HR+/HER2− breast cancer is debated. In a pooled analysis of 3,771 patients with breast cancer treated with neoadjuvant chemotherapy in the context of 6 different clinical trials conducted by the German Breast Cancer Group, the infiltration by TILs was higher in TNBC and HER2-positive breast tumors compared to HR+/HER2-, with a percentage of high-TILs (defined as > 60%) of 30%, 19% and 16% in each

group respectively. Interestingly, the pCR rates were higher for all the high-TILs groups, and TILs as a continuous variable showed a statistically significant association with pCR, both independently of the IHC-based tumor type. In a univariate Cox regression model for DFS prediction, TILs were significantly associated with a better outcome in both HER2-positive and TNBC, with no differences in HR+/HER2- breast cancer patients (100). This apparent paradoxical association between TILs and outcome in HR+/HER2- breast cancer may suggest a peculiar immune infiltrate biology of this tumor type. In another mono-institutional case-cohort series of 987 patients with early HR+/HER2− breast cancer patients who underwent surgery at the European Institute of Oncology, TILs were evaluated both as a continuous variable and dichotomized in low (< 5%) versus high (≥ 5%). The main outcome was distant disease-free survival (DDFS). In this study, higher TILs were associated with bad prognostic features, like higher node involvement ($p = 0.003$), higher tumor grade ($p < 0.0001$), higher peritumoral vascular invasion ($p = 0.003$), higher proliferation defined by Ki-67 ($p = 0.0001$), Luminal-B like tumors (HR+/HER2-/Ki67 >20%) ($p < 0.0001$), and more chemotherapy use ($p < 0.00019$). TILs were significantly associated with a better DDFS (HR 0.52, 95% CI 0.33–0.83, $p = 0.006$) only in the cohort of patients treated with adjuvant chemotherapy at a prognostic level. Still, there was not a significant association in the non-chemotherapy group (101). This data suggests that in high-risk HR+/HER2- breast tumors, TILs could have a prognostic impact.

Using the tumor intrinsic subtype gene expression definition, some descriptive analyses have shown how HER2-Enriched and Basal-like tumors have a higher immune infiltration than luminal specimens (102-104). However, there is limited data about this immune infiltration's role by PAM50 tumor intrinsic subtype within HR+/HER2- tumors and their prognostic value.

These differences in immune infiltration by intrinsic subtype within HR+/HER2- breast cancer have established the rationale for developing the TATEN SOLTI-1716 clinical trial (NCT04251169). This phase II study would test pembrolizumab's efficacy in combination with paclitaxel in patients with locally advanced or metastatic non-luminal (HER2-Enriched and Basal-like) HR+/HER2- breast tumors after recurrence or progression while receiving previous therapy with a CDK4/6i.

RATIONALE

Over the past decade, significant advances in outcomes prognostication within early-stage HR+/HER2- breast cancer have relied predominantly on multi-gene molecular assays. In particular, Prosigna® (NanoString Technologies, Seattle, WA, USA) provides information about the ROR at 10 years based on the prognostic differences between the breast cancer intrinsic subtypes (i.e., Luminal A, Luminal B, HER2-Enriched and Basal-like). Moreover, in the metastatic setting, non-luminal HR+/HER2- tumors (i.e., HER2-Enriched and Basal-like) have been associated with estrogen independence, chemosensitivity, resistance to CDK4/6i, and worse survival (18). However, gene expression platforms are costly and are not routinely performed on all clinical patients who could benefit from advanced molecular tests. Thus, there is a clinical need for identifying patients with high-risk HR+/HER2- tumors when molecular testing is not an option. This thesis will address some critical gaps in knowledge, providing scientific evidence for identifying this high-risk group of patients using the information provided by 4 widely used IHC biomarkers (i.e., ER, PR, HER2, and Ki67).

Distinguish between Luminal A and Luminal B tumors in the absence of genomic tests is relevant to select which group of patients could avoid the toxic effects of (neo)adjuvant chemotherapy due to an excellent long-term prognosis when treated with ET alone. Traditionally, the Ki67 labeling index has been used for this purpose. Scientific evidence supports a Ki67 cutoff of ~ 14% to differentiate these two entities (63) using an old version of qRT-PCR PAM50 classifier. However, during the last years, a Ki67 cutoff of 20% has been accepted by the medical community to discriminate between Luminal B and Luminal A disease (64). In this thesis, we will use a combined cohort of 517 patients diagnosed with HR+/HER2- node-negative breast cancer with available Ki67 and Prosigna® information to answer the following scientific questions:

1. How much risk of breast cancer recurrence at 10 years have these patients depending on the Ki67 levels?
2. How is the performance of Ki67 as a continuous variable to predict Luminal A and/or low-ROR disease within patients with HR+/HER2- breast cancer?
3. Which Ki67 cutoff can better discriminate Luminal A from Luminal B disease in this cohort of HR+/HER2- node-negative patients using the gold standard Prosigna® assay for subtype prediction?

On the other hand, within HR+/HER2- tumors, HER2-Enriched, and Basal-like subtypes are associated with poor outcome, low response to anti-estrogens, and a high response to chemotherapy. Still, no validated biomarker has been described to identify these two molecular entities in the absence of gene expression profiling. In this thesis, we will address the following questions:

1. Are the ER, PR, and Ki67 levels able to discriminate non-luminal (i.e., HER2-Enriched and Basal-like) from luminal disease?
2. Can we build an IHC-based combined classifier to discriminate non-luminal vs. luminal tumors?
3. If so, how is the accuracy of the IHC-based subtype classifier to identify high-risk molecular subtypes within HR+/HER2- breast tumors?

HYPOTHESIS

HR+/HER2- breast cancer is highly heterogeneous, and this heterogeneity has proven to have direct prognostic and predictive implications in both early and advanced scenarios. Consequently, identifying high-risk molecular subtypes within HR+/HER2- breast tumors has become necessary in clinical practice. We hypothesize that IHC-based predictive biomarkers can help identify patients with high-risk HR+/HER2- tumors when genomic platforms are not available.

OBJECTIVES

- To analyze the distribution of Prosigna®-based intrinsic subtype and ROR-group by Ki67 levels.
- To test the ability of Ki67 as a continuous variable to discriminate Luminal A and/or ROR-low disease defined by Prosigna®.
- To determine the best Ki67 cutoff to discriminate Luminal A and/or ROR-low breast tumors depending on the tumor size (> o < than 2cm).
- To analyze the distribution of ER, PR, and Ki67 by tumor intrinsic subtype defined by Prosigna®.
- To build an IHC-based combined classifier for identifying non-luminal disease within HR+/HER2- breast tumors.
- To test the accuracy of prediction of the IHC-based biomarker in an independent test set of patients diagnosed with HR+/HER2- breast cancer.

RESULTS AND METHODS

ARTICLE 1

Limitations in predicting PAM50 intrinsic subtype and risk of relapse score with Ki67 in estrogen receptor-positive HER2-negative breast cancer.

Fernandez-Martinez A, Pascual T, Perrone G, Morales S, de la Haba J, Gonzalez-Rivera M, Galvan P, Zalfa F, Amato M, Gonzalez L, Prats M, Rojo F, Manso L, Pare L, Alonso I, Albanell J, Vivancos A, Gonzalez A, Matito J, Gonzalez S, Fernandez P, Adamo B, Munoz M, Viladot M, Font C, Aya F, Vidal M, Caballero R, Carrasco E, Altomare V, Tonini G, Prat A, Martin M.

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Research Paper

Limitations in predicting PAM50 intrinsic subtype and risk of relapse score with Ki67 in estrogen receptor-positive HER2-negative breast cancer

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ABSTRACT

PAM50/Prosigna gene expression-based assay identifies three categorical risk of relapse groups (ROR-low, ROR-intermediate and ROR-high) in post-menopausal patients with estrogen receptor estrogen receptor-positive (ER+)/ HER2-negative (HER2-) early breast cancer. Low risk patients might not need adjuvant chemotherapy since their risk of distant relapse at 10-years is below 10% with endocrine therapy only. In this study, 517 consecutive patients with ER+/HER2- and node-negative disease were evaluated for Ki67 and Prosigna. Most of Luminal A tumors (65.6%) and ROR-low tumors (70.9%) had low Ki67 values (0-10%); however, the percentage of patients with ROR-medium or ROR-high disease within the Ki67 0-10% group was 42.7% (with tumor sizes ≤2 cm) and 33.9% (with tumor sizes > 2 cm). Finally, we found that the optimal Ki67 cutoff for identifying Luminal A or ROR-low tumors was 14%. Ki67 as a surrogate biomarker in identifying Prosigna low-risk outcome

patients or Luminal A disease in the clinical setting is unreliable. In the absence of a well-validated prognostic gene expression-based assay, the optimal Ki67 cutoff for identifying low-risk outcome patients or Luminal A disease remains at 14%.

INTRODUCTION

In the past 10 years, several commercialized multigene prognostic tests have been developed to help guide treatment decisions in patients with early breast cancer [1]. Among them, the PAM50/Prosigna assay (NanoString Technologies, Seattle, WA), identifies the intrinsic molecular subtype (Luminal A, Luminal B, HER2-enriched and Basal-like) and estimates the 10-year risk of relapse (ROR) [2–6] using formalin-fixed paraffin-embedded (FFPE) specimens.

Currently, due to a lack of reimbursement, multigene tests are not readily available for all patients in many countries. Consequently, the use of immunohistochemistry (IHC)-based biomarkers, such as Ki67, has been proposed instead, in order to identify patients with low-risk outcome who may be safely spared chemotherapy [7–9]. However, the 2015 St. Gallen panel proposed that Ki67 scores should be interpreted in light of local laboratory values, and recommended to use the median expression of each lab to define high and low values [9, 10]. In addition, a majority of the panel accepted a threshold value of Ki67 within the range of 20-29%, to distinguish Luminal A from Luminal B disease. These recommendations have led to confusion regarding how to interpret and use Ki67 scoring in the clinical setting.

Here, we aimed to compare the ability of IHC Ki67 to identify those patients at a low risk of recurrence as defined by the clinically and analytically validated commercial version of the PAM50 assay.

RESULTS

Cohort characteristics

Of the 697 patients, a total of 517 (74.2%) had ER+/HER2-, node-negative disease and Prosigna data available; this cohort was the focus of all further analyses (Figure 1). Prosigna subtype distribution was 56.9% Luminal A, 40.8% Luminal B, 1.2% HER2-enriched, and 1.2% Basal-like (Table 1, Supplementary Table 1). ROR risk group distribution was 38.5% ROR-low, 33.1% ROR-intermediate and 28.4% ROR-high (Supplementary Table 2). Statistically significant differences across the 3 cohorts were observed in ROR-groups but not in subtypes distribution. (Supplementary Tables 1 and 2).

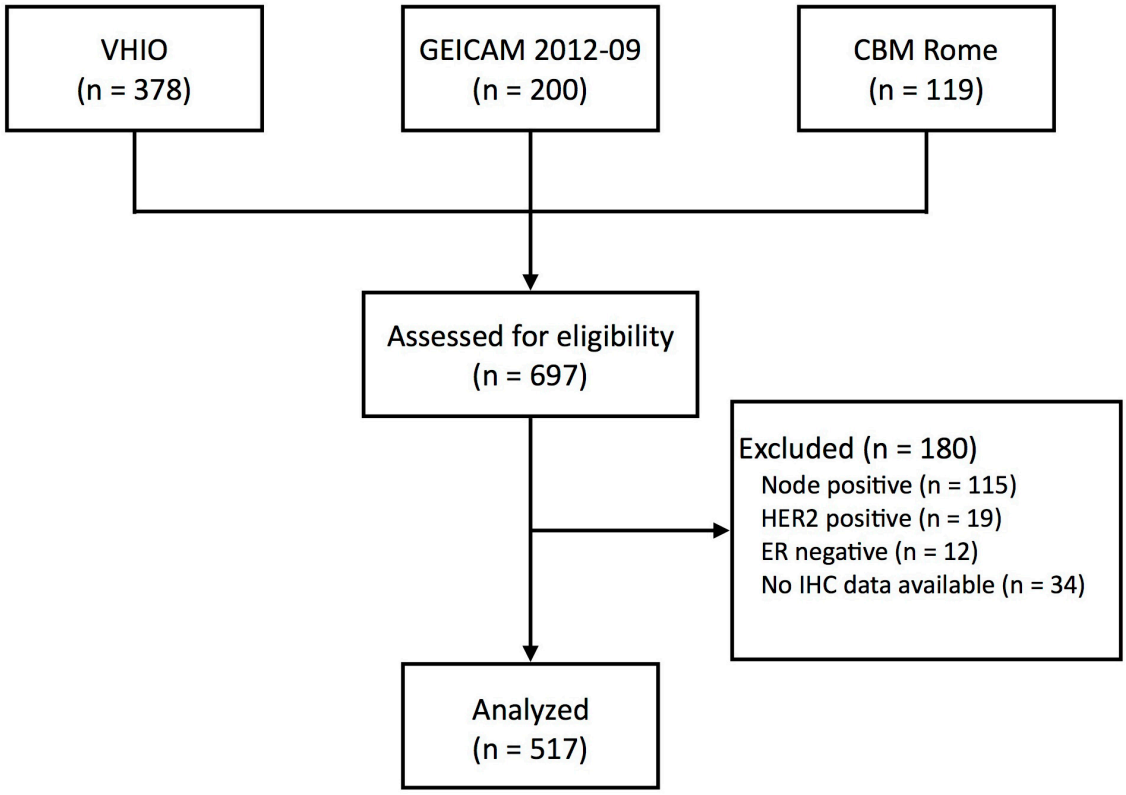

Figure 1: CONSORT diagram. VHIO, Vall d'Hebron Institute of Oncology; GEICAM, Spanish Breast Cancer Research Group; CBM Rome, Università Campus Bio-Medico di Roma.

Table 1: Distribution of subtypes and ROR within each Ki67 group in 517 patients with HR+/HER2- node-negative disease, ROR-med, ROR-medium

	Ki67 Group			
	0-10%	**11-20%**	**21-30%**	**>30%**
Intrinsic Subtypes				
Luminal A	193 (81.4%)	63 (51.6%)	29 (29.3%)	9 (15.3%)
Luminal B	42 (17.7%)	59 (48.4%)	69 (69.7%)	41 (69.5%)
HER2-enriched	2 (0.8%)	0	1 (1.0%)	3 (5.1%)
Basal-like	0	0	0	6 (10.2%)
Total	237	122	99	59
ROR and T≤2cm				
ROR-Low	102 (57.3%)	28 (28.9%)	12 (15.2%)	3 (6.5%)
ROR-Med	52 (29.2%)	44 (45.4%)	25 (31.6%)	8 (17.4%)
ROR-High	24 (13.5%)	25 (28.5%)	42 (53.2%)	35 (76.1%)
Total	178	97	79	46
ROR and T>2cm				
ROR-Low	39 (66.1%)	6 (24.0%)	5 (25.0%)	4 (30.8%)
ROR-Med	17 (28.8%)	13 (52.0%)	9 (45.0%)	3 (23.1%)
ROR-High	3 (5.1%)	6 (24.0%)	6 (30.0%)	6 (46.2%)
Total	59	25	20	13

Subtype and ROR concordance with Ki67

The concordance rates between Prosigna subtype (i.e. Luminal A vs. others) and IHC subtype (Luminal A-like vs. others) when Ki67 cutoffs of 14% and 20% were used were 70.8% (kappa score = 0.43; moderate agreement) and 69.1% (kappa score = 0.38; weak agreement), respectively. The percentages of Luminal A tumors within Ki67 0-10%, 10-20%, 20-30% and >30% groups were 81.4%, 51.6%, 29.3% and 15.3%, respectively (Table 1 and Supplementary Table 3). The distribution of ROR-low tumors within Ki67 0-10%, 10-20%, 20-30% and >30% groups were 59.5%, 29.7%, 17.2% and 11.9% respectively. (Table 1 and Supplementary Table 4). The percentage of ROR-med/high patients within the Ki67 0-10% group was 42.7% (within tumor size ≤2 cm) and 33.9% (within tumor size > 2 cm) (Table 1 and Supplementary Table 4). Although not all Luminal A tumors are included in the ROR-low group, the ROR-low group is a subset of the Luminal A group and consists of only Luminal A tumors.

Identification of Luminal A subtype using Ki67

We compared the distribution of Luminal A and non-Luminal A tumors as a function of Ki67 using a density plot (Figure 2A). As expected, Luminal A tumors were more represented within low Ki67 scores and non-Luminal A tumors were more represented within high Ki67 scores, although considerable overlap was observed. To try to identify an optimal Ki67 cutoff to discriminate Luminal A versus non-Luminal A, we estimated the performance of Ki67 (as a continuous variable). The result revealed an area under the receiver operating characteristic (auROC) curve of 0.79 and an optimal cutoff of 14% (Figure 3A). It is noteworthy to highlight that this is practically the same Ki67 cutoff reported by the original work by Cheang and colleagues [11], where PAM50 quantitative real time polymerase chain reaction (qRT-PCR) based subtyping was compared to Ki67 data for the first time.

Identification of ROR-low using Ki67

Similar to subtype identification, we compared the distribution of ROR-low, ROR-intermediate and ROR-high as a function of Ki67 using 2 density plots, one within tumor sizes ≤2 cm (Figure 2B) and the other one within tumor sizes above 2 cm (Figure 2C). As expected, ROR-low tumors were more represented within low Ki67 scores and ROR-intermediate/high tumors were more represented within high Ki67 scores, although considerable overlap was observed. To try to identify an optimal Ki67 cutoff to

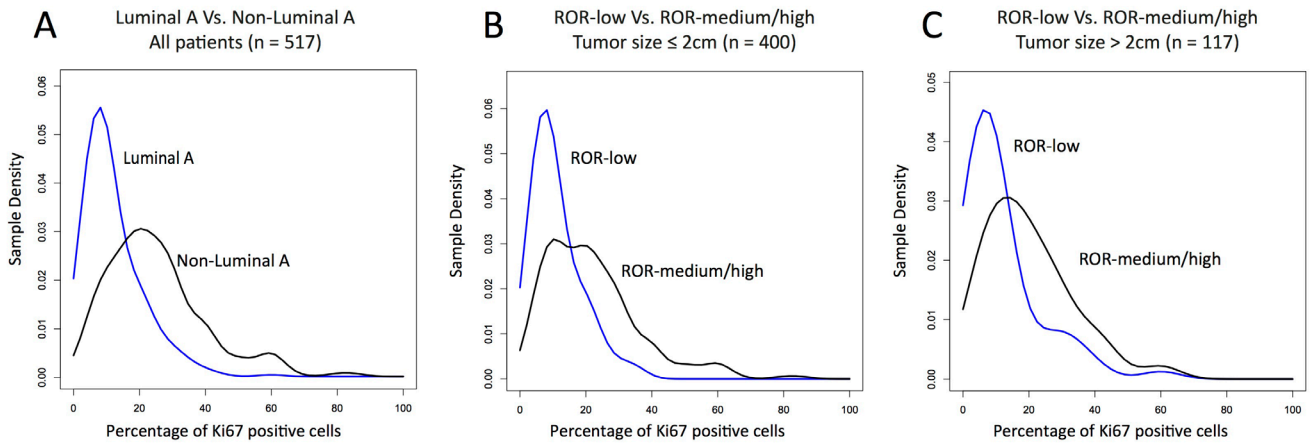

Figure 2: Density of the intrinsic subtypes and ROR-groups based on Ki67-positive cells. (A) Density plot in Luminal A and non-Luminal A tumors within all patients; **(B)** Density plot of the 3 ROR-groups within tumor sizes ≤2 cm; **(C)** Density plot of the 3 ROR-groups within tumor sizes > 2 cm.

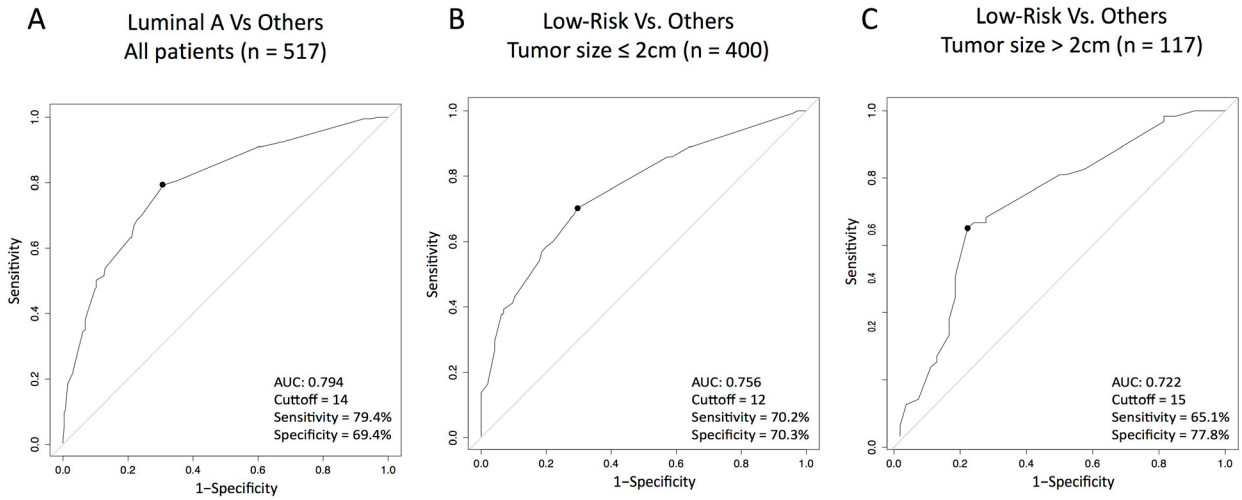

Figure 3: Performance of Ki67 (as a continuous variable) to predict Luminal A or ROR-low disease within HR+/HER2- node-negative disease. (A) Predicting Luminal A disease (vs. others); **(B)** Predicting ROR-low disease (vs. others) within tumor sizes ≤ 2 cm; **(C)** Predicting ROR-low disease (vs. others) within tumor sizes >2 cm tumors. AUC, area under the curve.

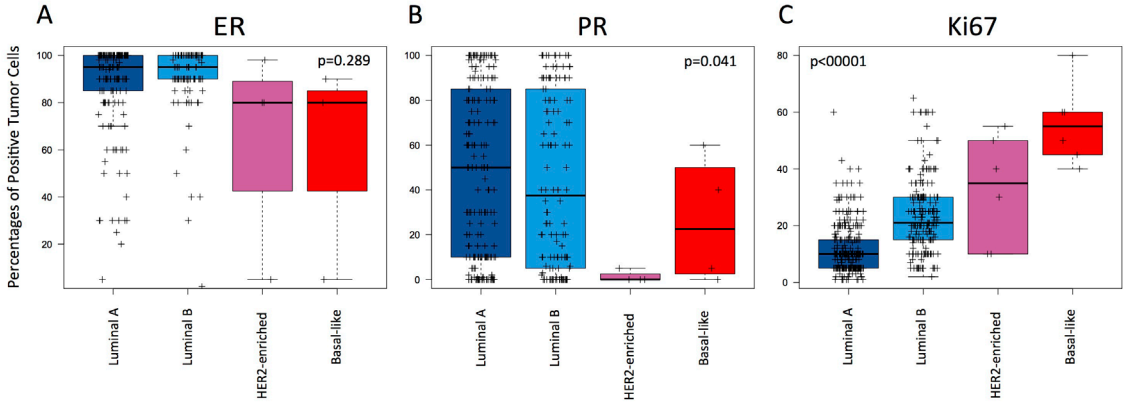

Figure 4: Levels of estrogen receptor (ER), progesterone receptor (PR) and Ki67-positive cells across the intrinsic subtypes within HR+/HER2-negative node-negative disease. (A) ER; **(B)** PR; **(C)** Ki67. P-values were calculated by comparing mean values across all groups.

discriminate ROR-low versus ROR-intermediate/high, we estimated the performance of Ki67 (as a continuous variable) to identify both groups. The results revealed auROC curves within tumor sizes of ≤2 cm and >2 cm of 0.76 and 0.72, respectively (Figure 3B-3C). The optimal Ki67 cutoffs for identifying ROR-low samples within tumor sizes of ≤2 cm and >2 cm were 12% and 15%, respectively.

Identification of Luminal A or ROR-low disease using ER and PR levels

Finally, we evaluated if the quantitative expression of ER and PR by IHC could help identify either Luminal A. None of the two IHC-based biomarkers was found useful (Figure 4). However, non-Luminal subtypes (i.e. HER2-enriched and Basal-like combined) showed statistically significant lower ER (62.6% vs 88.6%, p-value=0.003), lower PR (13.75% vs 46.5%, p-value=0.016) and higher Ki67 (43.1% vs 16.18%, p-value<0.001), respectively, compared to both luminal subtypes combined.

DISCUSSION

To our knowledge, this is the first report that compares ROR and subtype prediction using Prosigna and Ki67 in the same sample set. Our results highlight the important discrepancy between both biomarkers, and challenge the notion that gene expression-based assays are not needed in patients with HR+/HER2- disease with either low (i.e. <10%) or high (i.e. >20%) Ki67 scores.

The prognostic ability of Prosigna assay has been tested in samples from two phase III clinical trials, Arimidex, Tamoxifen, Alone or in Combination trial (ATAC) and Austrian Breast & Colorectal Cancer Study Group 08 (ABCSG08) [3, 12], involving a total of 2,485 post-menopausal patients treated with adjuvant endocrine therapy alone for 5 years. The results showed that Prosigna assay can identify a group of patients who do not need adjuvant chemotherapy due to their low risk (i.e. <10%) of distant recurrence at 10 years with endocrine therapy administered only [3, 4]. Moreover, Prosigna ROR score and intrinsic subtypes are predictors of late recurrence [5, 13] and response to multi-agent chemotherapy in the neoadjuvant setting [14]. In the recently reported American Society of Clinical Oncology (ASCO) Clinical Practice Guidelines, Prosigna was identified as an assay with the highest level of evidence to guide decisions on adjuvant systemic therapy in patients with ER+/HER2- and node-negative tumors [15].

In 2009, Cheang et al. [11] compared Ki67 and gene expression, using the qRT-PCR-based PAM50 version, and identified 13.25% as the optimal Ki67 cutoff to identify Luminal A versus Luminal B disease. The authors noted that despite this result, the sensitivity and specificity was around 75%, meaning that 1 out of 4 patients evaluated would not be classified correctly. With similar sensitivity and specificity (79.4% and 69.4% respectively), our study confirms that ~14% is an optimal cutoff for identifying low risk outcome patients who can be spared adjuvant chemotherapy when gene expression-based assays are not available.

In our view, our findings are important as much as it places the Ki67 cutoff at 14%; in 2013 St. Gallen International Expert Consensus proposed a Ki67 cutoff of 20% together with tumor size and nodal status to help identify low risk patients [8], and the 2015 St. Gallen panel recommended to use the median expression of Ki67 of each lab to define high and low Ki67 values [9, 10]. Although recommendations from the international Ki67 in breast cancer working group have led to improvements in reproducing of Ki67 [16], several studies have reported a high inter-laboratory variability in Ki67 scoring [17, 18].

Our study has several limitations. First, we do not have survival outcome data. Thus, we cannot compare the true prognostic value of the discrepant cases between the two assays. However, the level of analytical and clinical validation of the Prosigna assay to identify low-risk outcome patients, or Luminal A disease, is higher than the levels of validation of Ki67. According to Simon et al. criteria [19], Ki67 has not reached level 1 evidence mainly due to the suboptimal inter-laboratory reproducibility and the lack of a clinically useful cutoff [20]. Second, the IHC assessment of Ki67 was done using three different assays across the three cohorts of the study. However, the results regarding performance and the optimal Ki67 cutoff were not affected when adjusted for each type of cohort (Supplementary Figures 1, 2 and 3 and Supplementary Tables 5, 6 and 7). Third, the number of samples in the group of patients with tumors > 2 cm was low.

To conclude, although Ki67 has repeatedly shown to be prognostic [21, 22] and predictive of chemotherapy response [23, 24], the clinical value of Ki67 in identifying low risk outcome patients or Luminal A disease who might be safely spared chemotherapy remains uncertain. In absence of a well-validated prognostic gene expression-based assay, the optimal Ki67 cutoff in identifying low risk outcome patients (together with tumor size and nodal status) or Luminal A disease remains at 14%. However, it is worth highlighting that ~50% of patients with Luminal A-like disease (e.g. ER+/PR>20%/HER2- and Ki67<14%), node-negative and a tumor size above 2 cm, will not be classified as ROR-low.

MATERIALS AND METHODS

Cohorts of patients

Prosigna and IHC data were evaluated from 3 independent cohorts (Spanish Breast Cancer Research Group GEICAM/2012-09 prospective study [25], Vall d'Hebron Institute of Oncology [VHIO] Translational Genomics Lab and Campus Bio-Medico University of

Rome [CBM-Rome] Molecular Diagnostic Lab) with a total of 697 consecutive postmenopausal women with early breast cancer (Figure 1). The GEICAM/2012-09 was a prospective study of the Spanish Breast Cancer Research Group to characterize the impact of Prosigna assay in adjuvant treatment decision of 200 postmenopausal patients with ER+/HER2- breast cancer without nodal involvement [25]. VHIO and CBM-Rome tested 378 and 119 independent tumor samples (as of November 31st, 2016) coming from patients treated in clinical practice in Spain and Italy and whose medical oncologist decided to order a Prosigna® assay. Similar to GEICAM 2012-09 study, we selected patients with ER-positive/HER2-negative early breast cancer without nodal involvement. All procedures were performed in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration ethical standards. Informed consent was obtained from all individual participants included in the study.

Immunohistochemistry

IHC data was obtained from either central review (GEICAM/2012-09 and CBM-Rome) or from medical reports (VHIO) sent to the pathology laboratory. Ki67 was assessed by IHC using CONFIRM anti-Ki67 (30-9) Rabbit Monoclonal Primary Antibody (Ventana Medical System) in the GEICAM/2012-09 cohort. Anti-Ki67 MIB1 clone antibody (Dako, Glostrup, Denmark) was used in the CBM-Rome cohort. No data on Ki67 assessment is available for VHIO samples since Ki67 determinations were done in multiple local labs. In all samples from GEICAM/2012-09 and Campus Bio-Medico, Ki67 interpretation criteria were done according to the latest international recommendations [16].

We defined Luminal A-like or Luminal B-like tumors according to the IHC surrogate definitions of breast cancer subtypes proposed in the 13th St Gallen International Breast Cancer Conference [9]: Luminal A-like tumors were defined as HER2-negative, ER-positive with a low Ki67 assessment (<14%) and Luminal B-like tumors were defined as HER2-, ER-positive with a high Ki67 determination (≥14%). Tumors with a low-Ki67 determination (<14%) were considered as Luminal B-like tumors if PR was <20% (when PR was available) [16]. A cutoff of 20% of Ki67 was also explored.

Prosigna assay

FFPE tumors were analyzed using the commercialized and standardized PAM50/Prosigna assay (NanoString Technologies, Seattle, WA) [1–6]. We have followed the specifications of the package insert 2015-07 LBL-C0223-05.

Statistical analysis

All statistical analyses were performed using R version 3.2.2 (www.r-project.org). We used the Cohen's kappa coefficient to analyze the agreement between IHC-subtypes and Prosigna-subtypes. Quantitative data from visual assessment of Ki67 IHC determination (as a continuous variable) was compared against Luminal A and ROR-low groups as defined by Prosigna. The optimal cutoff value for Ki67 was selected by using the auROC method and maximizing the Youden index (the sum of sensitivity and specificity minus one).

Abbreviations

ROR: risk or recurrence; ER: estrogen receptor; HER2: human epidermal growth factor receptor 2; FFPE: formalin-fixed paraffin-embedded; IHC: immunohistochemistry; auROC: area under the receiver operating characteristic curve; qRT-PCR: quantitative real time polymerase chain reaction; PR: progesterone receptor; ATAC: Arimidex, Tamoxifen, Alone or in Combination Clinical Trial; ABCSG08: Austrian Breast & Colorectal Cancer Study Group 08 ROR; ASCO: American Society of Clinical Oncology; GEICAM: Spanish Breast Cancer Research Group; VHIO: Vall d'Hebron Institute of Oncology.

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CONFLICTS OF INTEREST

Uncompensated advisory role of AP for Nanostring Technologies.

Author contributions

AP, MM and AFM participated in the design of the study. All authors participated in data acquisition, analysis and interpretation. All authors were involved in drafting the article or revising it critically for important intellectual content, and approved the final version of the manuscript.

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ARTICLE 2

A Pathology-Based Combined Model to Identify PAM50 Non-luminal Intrinsic Disease in Hormone Receptor-Positive HER2-Negative Breast Cancer.

Pascual T, Martin M, **Fernandez-Martinez A**, Pare L, Alba E, Rodriguez-Lescure A, Perrone G, Cortes J, Morales S, Lluch A, Urruticoechea A, Gonzalez-Farre B, Galvan P, Jares P, Rodriguez A, Chic N, Righi D, Cejalvo JM, Tonini G, Adamo B, Vidal M, Villagrasa P, Munoz M, Prat A.

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A Pathology-Based Combined Model to Identify PAM50 Non-luminal Intrinsic Disease in Hormone Receptor-Positive HER2-Negative Breast Cancer

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Background: In hormone receptor-positive (HR+)/HER2-negative breast cancer, the HER2-enriched and Basal-like intrinsic subtypes are associated with poor outcome, low response to anti-estrogen therapy and high response to chemotherapy. To date, no validated biomarker exists to identify both molecular entities other than gene expression.

Methods: PAM50 subtyping and immunohistochemical data were obtained from 8 independent studies of 1,416 HR+/HER2-negative early breast tumors. A non-luminal disease score (NOLUS) from 0 to 100, based on percentage of estrogen receptor (ER), progesterone receptor (PR) and Ki67 tumor cells, was derived in a combined cohort of 5 studies (training dataset) and tested in a combined cohort of 3 studies. The performance of NOLUS was estimated using Area Under the ROC Curve (AUC).

Results: In the training dataset ($n = 903$) and compared to luminal disease, non-luminal disease had lower percentage of ER-positive cells (median 65.2 vs. 86.2%, $p < 0.01$) and PR-positive cells (33.2 vs. 56.4%, $p < 0.01$) and higher percentage of Ki67-positive cells (18.2 vs. 13.1%, $p = 0.01$). A NOLUS formula was derived: -0.45*ER -0.28*PR $+0.27$*Ki67 $+ 73.02$. The proportion of non-luminal tumors in NOLUS-positive ($\geq$51.38) and NOLUS-negative ($<$51.38) groups was 52.6 and 8.7%, respectively. In the testing dataset ($n = 514$), NOLUS was found significantly associated

with non-luminal disease ($p < 0.01$) with an AUC 0.902. The proportion of non-luminal tumors in NOLUS-positive and NOLUS-negative groups was 76.9% (56.4–91.0%) and 2.6% (1.4–4.5%), respectively. The sensitivity and specificity of the pre-specified cutoff was 59.3 and 98.7%, respectively.

Conclusions: In the absence of gene expression data, NOLUS can help identify non-luminal disease within HR+/HER2-negative breast cancer.

Keywords: intrinsic subtype, non-luminal, PAM50, breast cancer, gene expression

INTRODUCTION

Gene expression profiling has had a considerable impact on our understanding of hormone receptor-positive (HR+)/HER2-negative breast cancer biology (1, 2). During the last decade, two intrinsic molecular subtypes within HR+/HER2-negative disease (i.e., Luminal A and Luminal B) have been identified and intensively studied (3–5). These studies have led to well-validated prognostic gene expression-based tests such as Prosigna (6), OncotypeDX (7), MammaPrint (8), Breast Cancer Index (9),and EndoPredict (10). The implementation of these 4 platforms in the clinical practice has been essential in order to identify a subset of Luminal A tumors that can safely spare (neo)adjuvant chemotherapy treatments because of their good prognostic (11–13).

At the same time, cumulative evidence from recent studies suggests that 5–30% of HR+/HER2-negative tumors are not Luminal A or B by gene expression and fall into the HER2-enriched (HER2-E) and Basal-like categories (14). From a clinical perspective, these non-luminal tumors have been associated with low estrogen dependency (15–17), high chemo-sensitivity (18–20), potential lower activity of CDK4/6 inhibitors (21, 22) and poor outcome in both early and the advanced/metastatic breast cancer (22–24). Thus, clinical utility of the identification of the two non-luminal subtypes within HR+/HER2-negative disease is now being pursued.

In this study, we sought to validate a simple pathology-based model to help clinicians and researchers identify non-luminal disease within HR+/HER2-negative breast cancer in the absence of gene expression data.

MATERIALS AND METHODS

Study Design

PAM50 gene expression and pathology-based data from 1,416 HR+/HER2-negative early breast tumors were obtained from 8 independent studies that are summarized in **Table 1** (20, 25–30). The GEICAM/9906 is a phase III adjuvant trial in women with lymph node-positive disease that compared treatment with fluorouracil, epirubicin, and cyclophosphamide (FEC) or with FEC followed by weekly paclitaxel (FEC-P) (25). A total of 531 HR+/HER2-negative tumor samples were analyzed (26). SOLTI-1007 NeoEribulin trial is a neoadjuvant trial within HER2-negative breast cancer, where patients were treated with eribulin monotherapy for 4 cycles (20). A total of 93 HR+/HER2-negative baseline tumor samples were analyzed. Pre-operative endocrine treatment (PETx) cohort is a retrospective Spanish registry of 56 patients with HR+/HER2-negative disease treated with neoadjuvant endocrine therapy. From this study, baseline samples were analyzed (30). From GEICAM/2009-03_CONVERTHER, a study that aimed to compared pathology and gene expression data between primary and metastatic tumor samples, we obtained 50 HR+/HER2-negative primary tumor samples (28, 31). GEICAM/2012-09 is a prospective study of the Spanish Breast Cancer Research Group to characterize the impact of Prosigna assay in adjuvant treatment decision of postmenopausal patients with HR+/HER2-negative breast cancer without nodal involvement (27). A total of 174 primary tumor samples were included. Hospital Clinic of Barcelona (HCB) cohort is a consecutive series of 194 tumor samples where Prosigna has been performed as routine clinical care (29). Università Campus Bio-Medico di Roma (CBM) cohort is a consecutive series of 145 tumor samples where Prosigna has been performed as routine clinical care (29). Instituto de Investigación Biomédica de Málaga (IBIMA) cohort includes 180 HR+/HER2-negative baseline tumors treated with neoadjuvant chemotherapy as routine clinical practice (18).

Pathology-Based Data

The formalin-fixed paraffin-embedded tumor samples analyzed met the following criteria: (1) they were obtained from untreated primary tumors, (2) estrogen receptor (ER) and progesterone receptor (PR) positivity was defined as >1% positive tumor cells according to the ASCO/CAP guidelines (32), (3) HER2-negativity was defined according to the 2013 ASCO/CAP guidelines (33). Ki67 IHC was quantified according to the 2011 Guidelines developed by the International Ki67 in Breast Cancer working group (34).

PAM50 Intrinsic Subtyping

A research-based PAM50 subtyping assay was performed using the nCounter as previously described (24, 35, 36), except in GEICAM/9906, where a research-based PAM50 qRT-PCR-based assay was used, and GEICAM/2012-09, HCB, IBIMA, and CBM datasets, which used the standardized and commercial version of the PAM50 assay (i.e., Prosigna®). Original subtype calls obtained from each study were used. From the research-based PAM50 version, we eliminated any tumor samples identified as normal-like.

TABLE 1 | Main features of the cohorts analyzed in this study.

	GEICAM/ 9906	SOLTI-Neoeribulin	PETx	GEICAM/ 2009-03	GEICAM/ 2012-09	HCB	IBIMA	CBM
Dataset	Training	Training	Training	Training	Training	Testing	Testing	Testing
N	531	93	56	50	173	194	176	144
IHC	Centralized	Local	Local	Centralized	Centralized	Centralized	Centralized	Centralized
Platform	qRT-PCR	nCounter	nCounter	nCounter	nCounter	nCounter	nCounter	nCounter
PAM50 non-luminal disease (%)	77 (14.5)	12 (12.9)	3 (5.3)	7(14)	5 (2.9)	7 (3.6)	21 (11.9)	5 (3.5)
HER2-E (%)	71 (13.4)	1 (1.1)	3 (5.3)	6 (12)	4 (2.3)	4 (2.1)	7 (4.0)	3 (2.1)
Basal-like (%)	6 (1.3)	11 (11.8)	0	1 (2)	1 (0.6)	3 (1.5)	14 (7.9	2 (1.4)

Non-luminal Disease Score (NOLUS)

A combined score to identify non-luminal disease by PAM50 was derived from a combined dataset of 5 studies (i.e., training dataset) using ER, PR, and Ki67 levels (i.e., % of positive tumor cells). The optimal cutoff was defined as the point with the most significant (Fisher's exact test) split between Luminal and non-Luminal disease. Once NOLUS was developed, the final model and cutoff were tested in 513 HR+/HER2-negative tumors (i.e., testing set) from 3 independent databases: HCB, IBIMA, and CBM studies.

Statistical Analysis

Univariate and multivariable logistic regression analyses were done to investigate the association of each IHC biomarkers with non-luminal disease. Odds ratios (ORs) and 95% confidence intervals (CI) were calculated for each variable. The performance of NOLUS was estimated using Area Under the ROC Curve (AUC). 10-fold cross-validation was conducted (37). The significance level was set to a two-sided α of 0.05. We used R version 3.3.1 for all the statistical analyses.

RESULTS

Proportion of Non-luminal Disease Within HR+/HER2-Negative Breast Cancer

A total of 903 HR+/HER2-negative tumor samples from 5 studies were used as the training dataset (**Table 1**). In this cohort, non-luminal subtypes represented 11.6% (105/903) of the cases, ranging from 2.9% in GEICAM/2012-09 to 14.5% in GEICAM/9906. As expected, a relationship between chemotherapy cohorts and higher proportion of non-luminal disease was found. The 3 chemotherapy cohorts had proportions of non-luminal disease >10%, whereas the 2 hormonotherapy cohorts, the Spanish neoadjuvant endocrine therapy registry (PETx) and the GEICAM/2012-09 prospective study, had 2.9 and 5.4% of non-luminal tumors, respectively.

Expression of ER, PR, and Ki67 in Non-luminal Disease in the Training Dataset

ER, PR, and Ki67 were found differentially expressed ($p < 0.001$) between PAM50 luminal ($n = 798$) and non-luminal ($n = 105$) disease. Non-luminal disease had lower percentage of ER-positive cells (median 65.2 vs. 86.2%, $p < 0.01$) and PR-positive cells (33.2 vs. 56.4%, $p < 0.01$) and higher percentage of Ki67-positive cells (18.2 vs. 13.1%, $p = 0.01$) compared to luminal disease (**Figure 1**).

Predicting Non-luminal Disease Using ER, PR, and Ki67

To evaluate if ER, PR, and Ki67 (measured as continuous variables) provide independent information from each other regarding the identification of non-luminal disease, a multivariable logistic regression model was applied (**Table S1**). Interestingly, the expression of the 3 biomarkers was found independently associated with non-luminal disease. Using this multivariable result, we developed a combined score, called non-luminal disease score (NOLUS), that weights the value of each biomarker to identify non-luminal disease. The estimated coefficient of each variable in the logistic model was used to derive NOLUS (0–100) = −0.45*ER% −0.28*PR% + 0.27*Ki67% + 73, where ER, PR, and Ki67 are measured as continuous variables based on the percentage of positive tumor cells by immunohistochemistry.

Next, we identified a NOLUS cutoff to identify non-luminal disease based on the most significant split using a Fisher's exact test. Using this cutoff of 51.38, the proportion of NOLUS-positive (≥51.38) tumors and NOLUS-negative (<51.38) tumors was 6.3 and 93.7%, respectively. In addition, the proportion of non-luminal tumors in NOLUS-positive and NOLUS-negative groups was 52.6% (95% CI 38.9–66.0) and 8.7% (95 CI 6.97–10.77), respectively ($p < 0.001$) (**Figure 2**).

Validation of NOLUS in the Testing Dataset

The testing dataset was composed of 514 HR+/HER2-negative tumor samples from 3 independent studies (HCB, IBIMA and CBM). The proportion of non-luminal disease here was 6.2% (33/514). NOLUS as a continuous variable was found significantly associated with non-luminal disease ($p < 0.01$) with an AUC 0.902 (**Figure 2**). The proportion of non-luminal tumors in NOLUS-positive and NOLUS-negative groups was 76.9% (56.4–91.0) and 2.6% (1.4–4.5), respectively ($p < 0.01$). The sensitivity was 59.3 and the specificity was 98.7%. To identify only HER2-E, the sensitivity was 42.8 and the specificity was 96.0%. To identify only Basal-like, the sensitivity was 53.9 and the specificity was 99.0%.

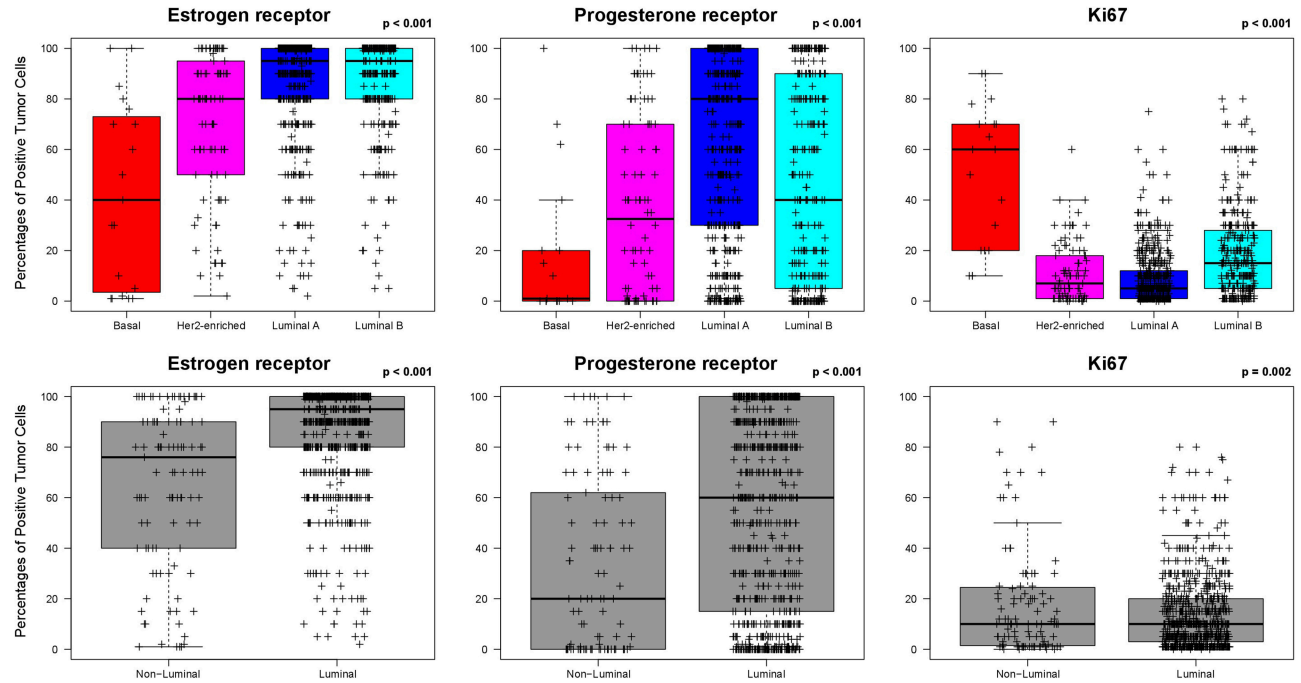

FIGURE 1 | Levels of estrogen receptor (ER), progesterone receptor (PR) and Ki67-positive cells across the PAM50 intrinsic subtypes in HR+/HER2-negative breast cancer. Data was obtained from the training dataset.

NOLUS in All Datasets

We explored NOLUS in all datasets combined. The odds of being non-luminal subtype increase 6.8% for every point increase (OR = 1.068, 95% CI 1.06–1.08, $p < 0.001$). The rates of non-luminal in NOLUS-negative and NOLUS-positive were 6.52 and 60.24%, respectively (Adjusted OR = 23.82, 95% CI 13.97–40.61, $p <$ 0.001) (**Figure 3**).

Finally, the model was validated using 10-fold cross validation. The data was separated into 10 sets, each set containing 10% of the data. For each validation round, 9 sets were used as training data, and the other set was used as testing data to validate the model using the linear discriminant analysis method. The accuracy of the model with 10-fold cross-validation was 0.97 (Cohen's kappa coefficient = 0.83).

DISCUSSION

In this study, we aimed to identify a pathology-based model that is easy, fast and with the potential to be widely implemented to identify non-luminal disease within HR+/HER2-negative breast cancer when gene expression data is not available. The main reasons are that there is accumulating evidence that non-luminal disease within HR+/HER2-negative disease represents a distinct biological and clinical entity (14) that deserves substantial attention and that gene expression-based assays are not always readily available in daily clinical practice. To our knowledge, this is the first report to attempt to derive a pathology-based predictive model to identify PAM50 non-luminal disease within HR+/HER2-negative disease.

The importance of intrinsic subtyping was highlighted in one of the most complete molecular characterization studies that has ever been performed in breast cancer (4). In this study, led by The Cancer Genome Atlas Project (TCGA), more than 500 primary breast cancer were extensively profiled at the DNA (i.e., methylation, chromosomal copy-number changes and somatic and germline mutations), RNA (i.e., miRNA and mRNA expression) and protein (i.e., protein and phosphor-protein expression) levels using the most recent technologies (4). In a particular analysis of over 300 primary tumors, 5 different data-types (i.e., all except DNA mutations) were combined together in a cluster of clusters in order to identify how many biological homogenous groups of tumors one can identify in breast cancer. The consensus clustering results showed the presence of 4 main entities of breast cancer but, more importantly, these 4 entities were found to be very-well recapitulated by the 4 main intrinsic subtypes (Luminal A, Luminal B, HER2-E, and Basal-like) as defined by mRNA expression only (3, 5, 6, 36, 38–40). Overall, these results suggest that intrinsic subtyping captures the vast majority of the biological diversity occurring in breast cancer.

Although the incidence of the Basal-like and HER2-E subtypes within HR+/HER2-negative tumors is below 10% in the primary disease setting (4), current evidence suggest that this frequency is much larger in the advanced/metastatic setting, specially following endocrine treatment (14). The increase proportion of the HER2-E subtype in the metastatic setting may be due to setting selection, a change in the biology of the tumor due to the

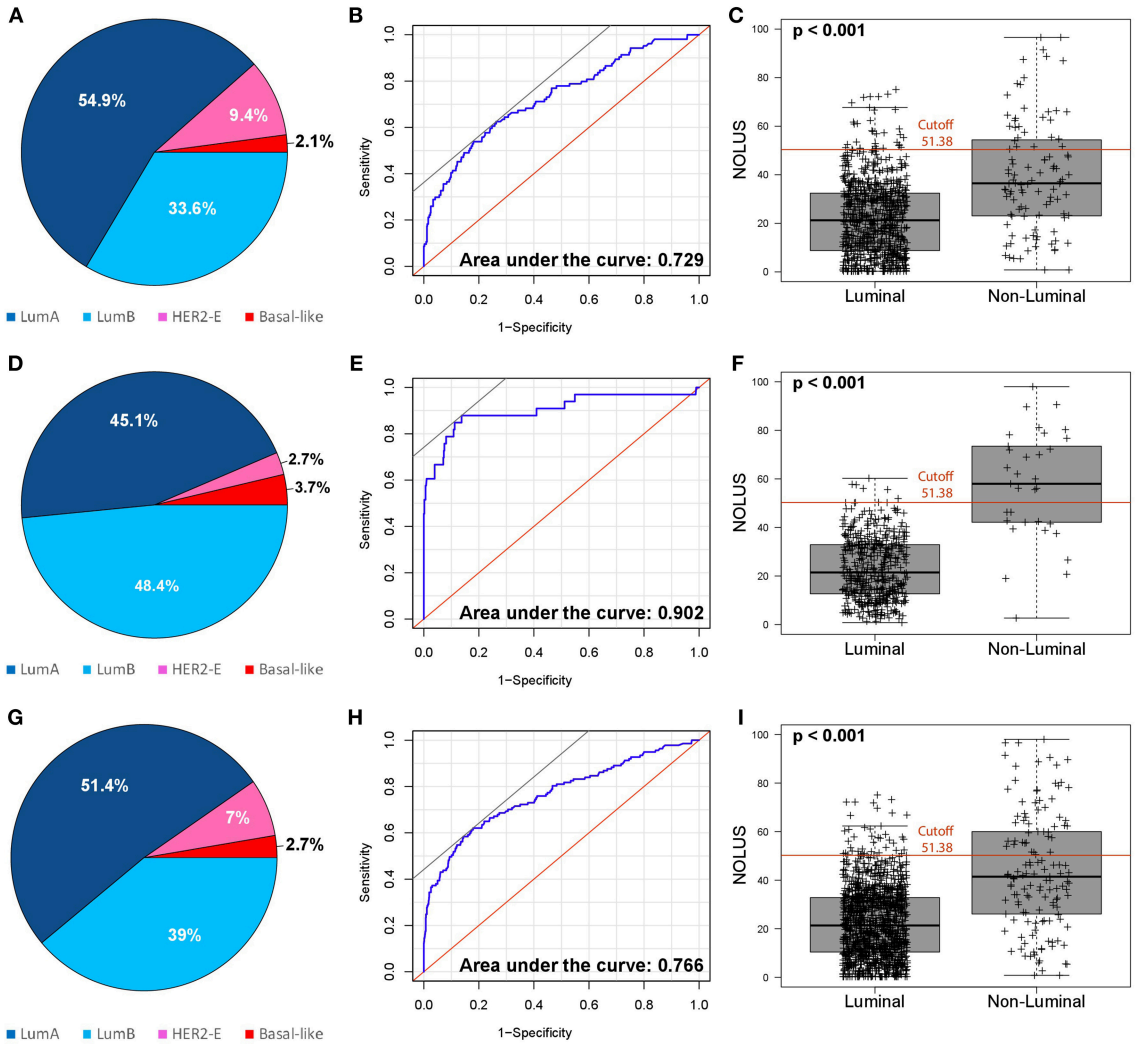

FIGURE 2 | Performance of NOLUS score to predict non-luminal subtype. **(A)** Distribution of the intrinsic subtypes in the training dataset; **(B)** NOLUS score to predict non-luminal disease in the training dataset; **(C)** Expression of NOLUS in luminal vs. non-luminal tumors with the pre-specified cutoff in the training dataset; **(D)** Distribution of the intrinsic subtypes in testing dataset; **(E)** NOLUS score to predict non-luminal disease in the testing dataset; **(F)** Expression of NOLUS in luminal vs. non-luminal tumors with the pre-specified cutoff in the testing dataset; **(G)** Distribution of the intrinsic subtypes in all patients; **(H)** NOLUS score to predict non-luminal subtype in all patients; **(I)** Expression of NOLUS in luminal vs. non-luminal tumors with the pre-specified cutoff in all patients.

inherent evolution of the tumor or the effects of the treatment, or a combination of both. Current evidence supports this latter possibility. Patients with early HR+/HER2-negative/HER2-E breast cancer have a higher probability of relapse than luminal disease. Therefore, it is likely that a given population of patients with metastatic disease is more enriched for the HER2-E subtype compared to patients with early breast cancer. Moreover, using 123 pairs of primary vs. metastatic tumor samples with a high proportion of HR+/HER2-negative tumors, Cejalvo et al. (28) showed that the HER2-E signature and HER2-E subtype are enriched in the metastatic samples compared to primary tumors. For example, 13% of primary Luminal A and B tumors were identified as HER2-E in the relapsed tumor sample. Overall, the proportion of HER2-E tumors in primary vs. metastatic was 11.4 vs. 22%, respectively. Moreover, in a retrospective analysis of tumor samples from the BOLERO-2 study, where patients with HR+/HER2-negative advanced disease resistant to an aromatase inhibitor, the proportion of HER2-E in primary vs. metastatic tumors was 19 vs. 32% (41). Recently, gene expression data from the PALOMA-2 clinical trial have been presented (21, 22). In this retrospective analysis, which included 68% (445/666) of the tumors of both primary and metastatic tumors within the clinical trial population, the HER2-E population represented 19 and the Basal-like population represented 1%.

The prognostic value of the Basal-like and HER2-E intrinsic subtypes in HR+/HER2-negative breast cancer has been evaluated in several studies (22–24). For example, intrinsic subtyping performed in a cohort of 1,380 patients with ER+

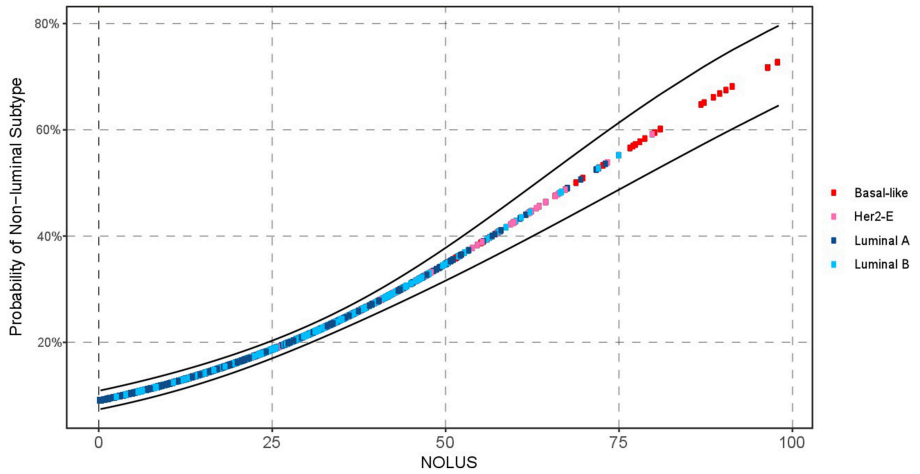

FIGURE 3 | Probability of non-luminal disease as a function of NOLUS in all patients.

early breast cancer treated with 5 years of adjuvant tamoxifen-only (23) demonstrated the presence of a 7% of non-Luminal disease. These patients showed a statistically significant worse outcome compared to Luminal A subpopulation. The prognostic value of the HER2-E intrinsic subtype has been evaluated also in 3 retrospective studies involving HR+/HER2-negative metastatic patients (22, 24, 41). In the EGF30008 Phase III clinical trial, intrinsic subtyping was performed in a cohort of 821 patients with HR-positive disease (644 HER2-negative and 157 HER2+) treated in the first-line metastatic setting with either letrozole or letrozole plus lapatinib (24). Patients with HER2-E and Basal-like disease showed worse outcome in terms of progression free survival (PFS) and overall survival (OS) compared to Luminal A disease regardless of the HER2 status and treatment. Compared with the Luminal A subtype, the non-luminal subtypes showed a significantly decreased PFS independently of other clinical-pathological variables. Patients with HER2-E, and Basal-like subtypes had a 2.87, and 2.26 times higher risk of tumor progression, respectively. Median PFS differed across the intrinsic subtypes: Luminal A (16.9 months), Luminal B (11.0 months), HER2-E (4.7 months), and Basal-like (4.1 months). In the second study, PAM50 was performed in 261 tumor samples from the BOLERO-2 phase III trial (41). The subtype distribution was: 46.7% Luminal A, 21.5% HER2-negativeE, 15.7% Luminal B, 14.2% Normal-like and 1.9% Basal-like. Non-luminal disease was independently associated with poor PFS and OS compared to the luminal subtypes. In the third study, PAM50 was performed in 465 tumor samples from the PALOMA-2 phase III trial. Both non-luminal subtypes were associated with worse PFS compared to Luminal A subtype. These results support that non-luminal HR+/HER2-negative tumors are aggressive and require novel therapeutic approaches.

The ability of the Basal-like and HER2-E subtype to predict benefit from anti-estrogen therapy has been evaluated in the neoadjuvant setting. In the Z1031 neoadjuvant trial (16) within ER+/HER2-negative disease, patients with HER2-E or Basal-like disease had persistently high surgical Ki67 levels (20%) after 4–6 months of treatment with an aromatase inhibitor, consistent with high-level estrogen-independent growth. In another retrospective study of 112 postmenopausal women with stages I–IIIB ER+ early breast cancer before and after 2-weeks' anastrozole treatment in a neoadjuvant trial, patients with HER2-E subtype ($n = 9$ [8.0%]) or Basal-like subtype ($n = 3$ [2.7%]) showed a poorer Ki67 response (mean Ki-67 change of−50.7 and +15.3%) compared to Luminal A or B subtypes (mean Ki-67 change of−75%). Interestingly, this study also profiled post-treatment samples. As expected, the vast majority of Luminal A samples (31/32, 97%) continued being Luminal A. However, although the majority of Luminal B tumors became Luminal A (9/17, 53%), 12% (2/17) became HER2-E. Overall, this data, together with the poor PFS of the HER2-E subtype following endocrine therapy in EGF30008, BOLERO-2 and PALOMA 2 trials (22, 24, 41), suggest that both non-luminal subtypes within HR-positive disease might not benefit substantially from anti-estrogen therapy.

The ability of the Basal-like and HER2-E subtype to predict benefit from palbociclib has been recently evaluated in 465 samples of the PALOMA-2 study (22). The increase in median PFS in the HER2-E subtype was modest (2.8 months), compared to the increase in median PFS of 13.4 and 8.6 months in Luminal A and B subtypes, respectively. Regarding Basal-like, only 1 patient was identified and progressed at 6.4 months following letrozole plus palbociclib. This data suggest that non-luminal subtypes do not benefit much from CDK4/6 inhibition. In the neoadjuvant setting, Ma and colleagues conducted the NEOPALANA clinical trial with anastrozole and palbociclib. Two non-luminal tumors were identified by PAM50 (1 HER2-E and 1 Basal-like) and, interestingly, none of the 2 patients responded to the combined treatment (17).

The ability of the Basal-like and HER2-E subtype to predict chemotherapy sensitivity within HR+/HER2- disease has been

evaluated in the neoadjuvant setting. In one study, we evaluated the pathological complete response (pCR) rated in 451 patients with HR+/HER2-negative disease treated with standard multi-agent neoadjuvant chemotherapy (42). The pCR rates in the non-luminal subtype was 23.2% compared to 15% in Luminal B and 5% in Luminal A tumors. In another neoadjuvant study, Prat and colleagues evaluated the residual cancer burden (RCB) 0/1 rates of the intrinsic subtypes in 180 patients with HR+/HER2-negative disease treated with anthracycline/taxane-based chemotherapy (18). Concordant with the first study, the RCB0/1 rates were higher in the non-luminal subtypes (38.1%) compared to Luminal B (20.0%) and Luminal A (9.3%). Overall, this data suggests that within HR+/HER2-negative disease, non-luminal tumors are highly chemo-sensitive.

Our study has several limitations worth noting. For example, determination of ER, PR and Ki67 was not performed centrally in a single lab and, in 2 studies, IHC data was obtained from local pathology reports. In addition, each study used different pathology-based assays. Although this heterogeneity is a limitation, its effects must not be large since the proportion of non-luminal disease across studies was similar and the fact that NOLUS was able to predict non-luminal disease in both the training and testing sets with similar performance. Another limitation is that NOLUS is not a standardized assay; thus, analytical validity is lacking. However, the biomarkers that compose NOLUS (i.e., ER, PR, and Ki67) have not been standardized; thus, NOLUS will suffer from lack of standardization as well. Another aspect is that we did not aim to derive a model that could further distinguish Basal-like from HER2-E subtypes within non-luminal disease. The main reason is that at this point it is unclear what are the clinical implications of each of these entities both from a prognostic and predictive point of view. However, as more data is gathered, NOLUS could be updated in the future to further distinguish these 2 non-luminal subtypes. Finally, we do not provide clinical validation of the NOLUS predictor.

To conclude, NOLUS is a tool that, in the absence of gene expression-based assays, may help identify non-luminal disease within HR+/HER2-negative breast cancer. Overall, the data clearly suggest that both non-luminal subtypes provide additional prognostic and predictive information beyond HR and HER2 status and may support more informed treatment decisions (1). For example, to identify patients who are not good candidates for endocrine therapy alone. Pivotal and large studies evaluating prognosis and treatment benefits can now apply NOLUS and further define the clinical validity and clinical utility of this biomarker.

AUTHOR CONTRIBUTIONS

All authors participated in the design and/or interpretation of the reported results and participated in the acquisition and/or analysis of data. In addition, all authors participated in drafting and/or revising the manuscript and provided administrative, technical, or supervisory support.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2019.00303/full#supplementary-material

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

GENERAL DISCUSSION

Over the past decade, breast cancer mortality has substantially declined. Mainly, advances in molecular biology and genomics have provided helpful information about breast tumors' heterogeneity, helping to establish a more efficient drug development as we pursue the practical deployment of precision medicine. Unfortunately, tumor molecular heterogeneity cannot be fully recapitulated by the three main IHC biomarkers routinely performed in clinical practice (i.e., ER, PR, and HER2). This project provides scientific evidence to identify high-risk mRNA-based molecular subtypes within HR+/HER2- breast tumors when genomic platforms are not available.

1. THE CLINICAL NEED TO IDENTIFY DIFFERENT SUBTYPES WITHIN HR+/HER2- BREAST TUMORS

HR+/HER2- is the most frequent breast cancer type (~70%), and all the intrinsic molecular subtypes can be found in different proportions within this group (18). This heterogeneity has relevant prognostic and predictive implications in early and advanced breast cancer.

First, Luminal A/HR+/HER2- early breast tumors have an excellent prognosis when treated with adjuvant endocrine therapy alone than Luminal B/HR+/HER2- tumors. Thus, the discrimination between Luminal A and Luminal B provides valuable information to guide treatment decisions about the need for (neo)adjuvant chemotherapy.

Second, non-luminal (i.e., HER2-Enriched and Basal-like) HR+/HER2- tumors are associated with estrogen independence and chemosensitivity, especially in the metastatic setting. Consequently, the discrimination between non-luminal and luminal HR+/HER2- disease can help tailor the treatment in these patients.

Third, new correlative studies suggest that the therapeutic benefit of CDK4/6i in non-luminal HR+/HER2- patients, especially Basal-like tumors, is limited compared to the benefit in luminal patients. Therefore, by identifying non-luminal HR+/HER2- breast tumors, we could predict the resistance to these drugs, which are currently the standard first-line treatment in patients with HR+/HER2- metastatic breast cancer.

Finally, non-luminal HR+/HER2- breast tumors are more immune infiltrated than luminal HR+/HER2- ones, being a group of great interest in expanding immunotherapy-based treatment, currently limited to TNBC.

In summary, identifying patients with high-risk HR+/HER2- breast tumors has become a necessity, even when genomic platforms are not available.

2. HOW TO IDENTIFY HIGH-RISK HR+/HER2- BREAST CANCER PATIENTS WHEN GENE EXPRESSION PLATFORMS ARE NOT AVAILABLE

Intrinsic subtyping has demonstrated a high prognostic value beyond clinicopathologic features and HR status. The gold standard to identify the breast tumor intrinsic subtype is the validated PAM50/Prosigna® assay (NanoString Technologies, Seattle, WA). In clinical practice, genomic platforms like Prosigna® used in the appropriate setting have proven to help guide treatment decisions. Moreover, these assays can help reduce overtreatment with chemotherapy, which might improve the cost-effectiveness of this approach (105). However, this is a reality only in high-income countries. As cancer medicine becomes increasingly driven by molecular alterations in high-income populations, low-income populations may become left behind. This is why further efforts on an international scale must be made by researchers, funders, and policymakers to ensure cancer research addresses disease across the world, so models are not limited to expensive technologies (106). In this project, we provide helpful information about how to recognize high-risk molecular tumors (i.e., high-proliferative Luminal B, HER2-Enriched, and Basal-like) by using 4 widely used IHC biomarkers (i.e., ER, PR, HER2, and Ki67) that are routinely performed in most of the pathology laboratories from high and low-income countries. To do this, we have analyzed the concordance between gene expression and protein expression by IHC in the same sample sets from two big cohorts of patients diagnosed with HR+/HER2- breast tumors.

2.1 HOW TO IDENTIFY HIGH-RISK HR+/HER2- LUMINAL B TUMORS BY USING KI67.

Proliferation is the hallmark of cancer that better discriminates Luminal A from Luminal B tumors. Breast cancer pathologists have embraced the IHC staining of Ki67 as the standard method to assess proliferation. In our scientific article entitled “Limitations in predicting PAM50 intrinsic subtype and risk of relapse score with Ki67 in estrogen receptor-positive HER2-negative breast cancer”, we compare the intrinsic subtype and ROR by Prosigna® with the Ki67 IHC levels in the same sample set.

Our results highlight the discrepancy between both biomarkers by describing the presence of 15.3% of Luminal A and 6.5% of Low-ROR in the group of tumors with Ki67 > 30% and, what is more clinically relevant, a 17.7% of Luminal B and 13.5% of High-ROR tumors within Ki67 lower than 10%. These results challenge the notion that gene expression-based assays are not

needed in HR+/HER2- patients with either low (i.e., <10%) or high (i.e.,>20%) Ki67 scores. Moreover, Ki67, as a continuous variable, had a sensitivity of 79.4% to differentiate Luminal A from Luminal B tumors, meaning that using this biomarker, approximately 1 out of 4 patients evaluated would not be classified correctly. Besides these discrepancies between both technics, our study confirms that the optimal Ki67 cutoff for identifying patients with low-risk breast tumors is ~14%, similar to previous studies in which an old qRT-PCR-based PAM50 version was used.

2.2 HOW TO IDENTIFY NON-LUMINAL HR+/HER2- TUMORS BY A COMBINED IHC-BASED PREDICTIVE MODEL.

There is accumulating evidence that non-luminal HR+/HER2-negative disease represents a distinct biological and clinical entity that cannot always be identified in clinical practice due to the absence of gene expression-based assays. In our scientific article entitled “A Pathology-Based Combined Model to Identify PAM50 Non-luminal Intrinsic Disease in Hormone Receptor-Positive HER2-Negative Breast Cancer”, we develop and validate a useful classifier to identify non-luminal HR+/HER2- tumors based on the IHC levels of ER, PR, and Ki67.

Our article showed how ER, PR, and Ki67 distribution were significantly different across tumor intrinsic subtypes in a combined training set of 903 patients with HR+/HER2- tumors (ANOVA p-value < 0.001 all the three IHC biomarkers). Using a multivariate logistic regression approach, we demonstrated that ER, PR, and Ki67, as continuous variables, provided independent and meaningful information to identify non-luminal tumors. Based on the odds ratio (OR) obtained from the multivariate model, we built a combined model: NOLUS score. This model was able to classify a tumor as non-luminal with an accuracy measured by AUC of 0.729 on the train set and 0.902 in the test set. The model was also validated in the entire subpopulation using 10-fold cross-validation, with an average of AUC of 0.97 and a Cohen’s kappa coefficient of agreement of 0.83 between the different cross-validation partitions. Finally, the optimum NOLUS cutoff to define non-luminal vs. luminal HR+/HER2- disease was established at 51.38 by selecting the most significant (Fisher’s exact test) split between luminal and non-luminal disease within the train set. Using this cutoff in the test set, the proportion of non-luminal tumors in NOLUS-positive and NOLUS-negative groups was 76.9% (56.4-91.0) and 2.6% (1.4-4.5), respectively (p-value < 0.01). These results suggest that we can still identify non-luminal HR+/HER2- disease based on three IHC-based biomarkers with pretty good accuracy and high specificity in the absence of genomic platforms.

3. LIMITATIONS.

Our studies have several limitations:

- First, survival data was not collected to test the correlation with the ROR model in our cohorts of patients. Thus, all the analyses are based on a relapse prediction but not on the actual relapse-free survival outcome. Still, we would like to highlight that the analytical and clinical validation of the Prosigna® assay to identify low-risk outcome patients is higher than the validation of Ki67 for the same purpose.
- Second, the IHC determination of ER, PR, and Ki67 was not performed centrally in a single lab, and, in some cases, IHC data was obtained from local pathology reports. Also, each study used different pathology-based assays. However, the effect of this heterogeneity shouldn't be considered statistically significant since the results were similar when adjusted by different cohorts. Moreover, both biomarkers (i.e., Ki67 as a continuous variable and the NOLUS model) preserved their prediction accuracy when performed separately in the different cohorts of patients.
- Third, both study cohorts' sample size was small, especially in patients with breast tumors higher than 2cm. Therefore, findings in this study subpopulation should be interpreted with caution.
- Finally, more analytical and clinical validation is needed for both classifiers.

4. STRENGTHS

- Novelty is probably the biggest strength of our two publications. Our first scientific article uses for the first time the gold standard and validated Prosigna® assay to test the association between intrinsic subtype and the IHC staining of Ki67. Our second scientific article is the first study to integrate the information provided by the IHC staining of ER, PR, and Ki67 into a single pathology-based predictive model to identify non-luminal HR+/HER2- disease.
- A second strength is the big sample size in both studies. These two large cohorts of patients with HR+/HER2- breast cancer have IHC and subtype information obtained by the gold standard Prosigna ® in the same tumor samples. No research versions of the PAM50 have been used in these analyses. Furthermore, the high dynamic range, sensitivity, and reproducibility of the nCounter technology used in the Prosigna® assay is also one strength by itself.

- The third and most relevant strength of this thesis is the easy implementation and clinical applicability of both Ki67 and NOLUS classifiers. In the first case, the Ki67 value at baseline in HR+/HER2- breast tumors could be used to stratify patients in different risk groups, tailoring the (neo)adjuvant treatment. Moreover, Ki67 baseline levels could be helpful in better select the intermediate-risk subpopulation of patients that could benefit the most for a genomic test. Finally, identifying patients with non-luminal HR+/HER2- breast tumors using the NOLUS score will allow us to select the best treatment option in the metastatic setting, offering access to new therapies in the context of clinical trials. One example is the SOLTI study TATEN (NCT04251169), in which patients with non-luminal HR+/HER2- metastatic breast cancer are treated with a combination of paclitaxel and pembrolizumab after CDK4/6i progression

CONCLUSIONS

The prognostic and predictive value of IHC-based biomarkers in breast cancer has been studied for years. Both ER and PR are well-known prognostic factors and robust predictive biomarkers for response to endocrine therapy. HER2 status has traditionally been considered a bad prognostic factor and a predictive factor for several anti-HER2 targeted therapies. Ki67 has repeatedly shown to be prognostic and predictive of chemotherapy response besides its lack of reproducibility. Thus, these biomarkers are routinely reported in all the breast cancer pathology reports to select the best treatment strategy possible for each patient.

Besides, there is increasing evidence of the prognostic and predictive value of breast cancer tumor intrinsic subtypes beyond IHC biomarkers. Thus, in HR+/HER2- breast cancer, the prognostic differences between the molecular tumor intrinsic subtypes have led to the development of multiple gene expression-based tests such as Prosigna®, guiding (neo)adjuvant treatment (19-22). HER2-Enriched and Basal-like HR+/HER2- tumors are associated with a worse prognostic, chemosensitivity, endocrine, and CDK4/6i resistance and a higher immune infiltration (18). However, genomic platforms are not always a reality in middle and low-income countries and, in high-income countries, not always are available due to a lack of reimbursement. Thus, correlation studies between IHC-based biomarkers and molecular subtypes are necessary to pursue precision medicine.

Our studies have proven that:

- Ki67 levels are significantly lower in HR+/HER2- Luminal A tumors than HR+/HER2- Luminal B tumors defined by the gold standard Prosigna® assay.
- Ki67 as a continuous biomarker can discriminate patients with Luminal A and ROR-low defined by Prosigna® with acceptable performance (AUC 0.794 and 0.722, respectively
- In the absence of gene expression platforms, the optimal Ki67 cutoff in identifying low-risk outcome patients (together with tumor size and nodal status) or Luminal A disease remains at 14%. However, it is worth highlighting that ~50% of patients with Luminal A-like disease (e.g., ER+/PR>20%/HER2- and Ki67<14%), node-negative and a tumor size above 2 cm, will not be classified as ROR-low.
- In both our studies, ER and PR are significantly lower in non-luminal (i.e., HER2-Enriched and Basal-like) than luminal (i.e., Luminal A and Luminal B) disease. On the contrary, Ki67 IHC levels are significantly higher in non-luminal vs. luminal tumors.

- The NOLUS score, a model based on the integration of ER, PR, and Ki67 IHC staining, provides accurate identification of non-luminal disease, with an AUC 0.729 in the training set and AUC 0.902 in the test set.

To conclude, advances in molecular biology and genomics allow practical assessments of newly defined and evolving biomarkers, like tumor intrinsic subtype. These biomarkers are necessary for more efficient drug development as we pursue the practical deployment of precision medicine. Unfortunately, gene expression assays are not always available. Thus, the two scientific articles that compose this thesis provide valuable and easy-to-implement classifiers in an attempt to detect patients with high-risk HR+/HER2- breast tumors that could benefit from different treatment strategies.

OTHER PUBLICATIONS RELATED TO THE PROJECT

Reference	Impact Factor*	Rank in the category**
Response and survival of breast cancer intrinsic subtypes following multi-agent neoadjuvant chemotherapy Aleix Prat, Cheng Fan, **Aranzazu Fernández,** Katherine A. Hoadley, Rossella Martinello, Maria Vidal, Margarita Viladot, Estela Pineda, Ana Arance, Montserrat Muñoz, Laia Paré, Maggie C. U. Cheang, Barbara Adamo, Charles M. Perou. BMC Med. 2015 Dec 18; 13: 303 PMID: 26684470 PMCID: PMC4683815 DOI: 10.1186/s12916-015-0540-z	6.782	**Medicine, General & Internal** Q1 (15/165)
Clinical implications of the intrinsic molecular subtypes of breast cancer. Aleix Prat, Estela Pineda, Barbara Adamo, Patricia Galvan, **Aranzazu Fernández**, Lydia Gaba, Marc Díez, Margarita Viladot, Ana Arance, Montserrat Muñoz. Breast. 2015 Nov; 24 Suppl 2: S26-35 PMID: 26253814 DOI: 10.1016/j.breast.2015.07.008	3.754	**Oncology** Q2 (98/244) **Obstetrics & Gynecology** Q1 (10/82)
Clinical implications of the non-luminal intrinsic subtypes in hormone receptor-positive breast cancer. Juan Miguel Cejalvo, Tomás Pascual, **Aranzazu Fernández-Martínez**, Fara Brasó-Maristany, Roger R. Gomis, Charles M. Perou, Montserrat Muñoz, Aleix Prat. Cancer Treat Rev. 2018 Jun; 67: 63-70. PMID: 29763779	8.885	**Oncology** Q1 (22/244)

DOI: 10.1016/j.ctrv.2018.04.015		
Clinical, pathological, and PAM50 gene expression features of HER2-low breast cancer. Francesco Schettini, Nuria Chic, Fara Brasó-Maristany, Laia Paré, Tomás Pascual, Benedetta Conte, Olga Martínez-Sáez, Barbara Adamo, Maria Vidal, Esther Barnadas, **Aranzazu Fernández-Martinez**, Blanca González-Farre, Esther Sanfeliu, Juan Miguel Cejalvo, Giuseppe Perrone, Giovanna Sabarese, Francesca Zalfa, Vicente Peg, Roberta Fasani, Patricia Villagrasa, Joaquín Gavilá, Carlos H. Barrios, Ana Lluch, Miguel Martín, Mariavittoria Locci, Sabino De Placido & Aleix Prat NPJ Breast Cancer. 2021 Jan 4;7(1):1. PMID: 33397968 DOI: 10.1038/s41523-020-00208-2		

Q: quartile. * Journal Citation Reports®, ISI Web of Knowledge; Thompson Reuters. ** At publication date.

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