Defining Breast Cancer Intrinsic Subtypes by Quantitative Receptor Expression

Maggie C.U. Cheang,[a,c] Miguel Martin,[d] Torsten O. Nielsen,[e] Aleix Prat,[f] David Voduc,[g] Alvaro Rodriguez-Lescure,[h] Amparo Ruiz,[i] Stephen Chia,[g] Lois Shepherd,[j] Manuel Ruiz-Borrego,[k] Lourdes Calvo,[l] Emilio Alba,[m] Eva Carrasco,[n] Rosalia Caballero,[n] Dongsheng Tu,[j] Kathleen I. Pritchard,[o] Mark N. Levine,[p] Vivien H. Bramwell,[q] Joel Parker,[a,b] Philip S. Bernard,[r] Matthew J. Ellis,[s] Charles M. Perou,[a,b] Angelo Di Leo,[t] Lisa A. Carey[a]

[a]Lineberger Comprehensive Cancer Center and [b]Department of Genetics, University of North Carolina, Chapel Hill, North Carolina, USA; [c]Clinical Trials and Statistics Unit at The Institute of Cancer Research, London, United Kingdom; [d]Servicio de Oncología Médica, Instituto de Investigacion Sanitaria Hospital Universitario Gregorio Marañón, Facultad de Medicina, Universidad Complutense, Madrid, Spain; [e]Department of Pathology, University of British Columbia, Vancouver, British Columbia, Canada; [f]Translation Genomics Unit, Vall d´Hebron Institute of Oncology, Barcelona, Spain; [g]British Columbia Cancer Agency, Vancouver, British Columbia, Canada; [h]Department of Medical Oncology, Hospital General Universitario de Elche, Alicante, Spain; [i]Department of Medical Oncology, Instituto Valenciano de Oncologia, Valencia, Spain; [j]National Cancer Institute of Canada Clinical Trials Group, Kingston, Ontario, Canada; [k]Department of Medical Oncology, Hospital Universitario Virgen del Rocio, Seville, Spain; [l]Department of Medical Oncology, Complejo Hospitalario Universitario A Coruña, Coruña, Spain; [m]Department of Medical Oncology, Hospital Universitario Virgen de la Victoria, Málaga, Spain; [n]GEICAM, Madrid, Spain; [o]Sunnybrook Odette Cancer Centre, University of Toronto, Toronto, Ontario, Canada; [p]McMaster University, Hamilton, Ontario, Canada; [q]Department of Oncology, Tom Baker Cancer Centre, University of Calgary, Calgary, Alberta, Canada; [r]Department of Pathology, University of Utah Health Sciences Center, Salt Lake City, Utah, USA; [s]Department of Medicine, Washington University, St. Louis, Missouri, USA; [t]Department of Oncology, Hospital of Prato, Istituto Toscani Tumori, Florence, Italy
Disclosures of potential conflicts of interest may be found at the end of this article.

Abstract

Purpose. To determine intrinsic breast cancer subtypes represented within categories defined by quantitative hormone receptor (HR) and HER2 expression.

Methods. We merged 1,557 cases from three randomized phase III trials into a single data set. These breast tumors were centrally reviewed in each trial for quantitative ER, PR, and HER2 expression by immunohistochemistry (IHC) stain and by reverse transcription-quantitative polymerase chain reaction (RT-qPCR), with intrinsic subtyping by research-based PAM50 RT-qPCR assay.

Results. Among 283 HER2-negative tumors with <1% HR expression by IHC, 207 (73%) were basal-like; other subtypes, particularly HER2-enriched (48, 17%), were present. Among the 1,298 HER2-negative tumors, borderline HR (1%–9% staining) was uncommon ($n = 39$), and these tumors were heterogeneous: 17 (44%) luminal A/B, 12 (31%) HER2-enriched, and only 7 (18%) basal-like. Including them in the definition of triple-negative breast cancer significantly diminished enrichment for basal-like cancer ($p < .05$). Among 106 HER2-positive tumors with <1% HR expression by IHC, the HER2-enriched subtype was the most frequent (87, 82%), whereas among 127 HER2-positive tumors with strong HR (>10%) expression, only 69 (54%) were HER2-enriched and 55 (43%) were luminal (39 luminal B, 16 luminal A). Quantitative HR expression by RT-qPCR gave similar results. Regardless of methodology, basal-like cases seldom expressed ER/*ESR1* or PR/*PGR* and were associated with the lowest expression level of HER2/*ERBB2* relative to other subtypes.

Conclusion. Significant discordance remains between clinical assay-defined subsets and intrinsic subtype. For identifying basal-like breast cancer, the optimal HR IHC cut point was <1%, matching the American Society of Clinical Oncology and College of American Pathologists guidelines. Tumors with borderline HR staining are molecularly diverse and may require additional assays to clarify underlying biology. ***The Oncologist*** 2015;20:474–482

Implications for Practice: This study pooled centrally reviewed hormone receptor (HR) and HER2 data and individual gene expression and intrinsic subtyping from three cooperative group trials. The results indicated that the optimal cut point for defining triple-negative breast cancer, if the goal is to enrich for basal-like biology, is to adopt the guideline of <1% staining. Tumors with borderline HR expression are highly biologically heterogeneous, which raises the question of whether these tumors should be considered indeterminate. A proportion of clinically defined HER2-negative tumors were defined as molecular HER2-enriched subtype; however, whether they are suitable for anti-HER2 therapy needs to be determined.

INTRODUCTION

Breast cancer clinical decision making is based on assays for ER and PR by immunohistochemistry (IHC) and HER2 by IHC and/or fluorescence in situ hybridization (FISH). The American Society of Clinical Oncology (ASCO) and the College of American Pathologists (CAP) issued a joint guideline in 2010 regarding IHC determination of hormone receptor (HR; ER and PR, collectively) status [1] that recommended the most restrictive cut points of ≥1% staining for calling a tumor ER-positive or PR-positive. Similarly, ASCO and CAP issued clinical practice guidelines for HER2 in 2007 [2], 2012 [3], and 2013 [4]. Together, these guidelines define the use of endocrine therapy in HR-positive and HER2-targeted therapy in HER2-positive disease and the populations of interest for novel approaches in clinical trials.

The molecular entities within these clinical subsets and how they might vary across quantitative HR or HER2 categories have not been established, although considerable evidence exists regarding molecular heterogeneity within identifiable clinical subsets [5–9]. Triple-negative breast cancer (TNBC), for example, is characterized by the absence of HR and HER2 expression clinically but represents a diverse population of biologic entities [10–12]. The majority of TNBCs are of the basal-like intrinsic subtype [13, 14], although other molecular subtypes such as luminal or HER2-enriched are also represented within TNBC [10, 12]. Several definitions of ER and PR negativity (from <1% to <10%) have been used as entry criteria for clinical trials in TNBC; borderline (1%–9%) hormone receptor staining is sometimes included in TNBC and sometimes not.

In this study, we compared centrally performed clinical assays of quantitative ER, PR, and HER2 expression with molecular intrinsic subtypes identified by PAM50, an open-source, centroid-based, 50-gene subtype predictor [15], in tumors collected across three phase III randomized clinical trials and determined the molecular populations within strongly hormone receptor-positive tumors, borderline tumors, and triple-negative tumors.

MATERIALS AND METHODS

Study Populations

Molecular (PAM50 intrinsic subtyping) and clinical-pathological data (including ER, PR, HER2, and outcome data) were collected from three phase III randomized adjuvant treatment trials and merged into a single data set for analysis. In GEICAM/9906, 1,246 women with node-positive breast cancer were randomized to fluorouracil, epirubicin, and cyclophosphamide (FEC) alone for six cycles versus FEC for four cycles followed by weekly paclitaxel for eight cycles, with tamoxifen given to HR-positive patients [16]. In National Cancer Institute of Canada Clinical Trials Group (NCIC CTG) trial MA.5, 710 premenopausal women with node-positive breast cancers were randomized to oral cyclophosphamide, methotrexate, and fluorouracil versus oral cyclophosphamide, epirubicin, and fluorouracil [17]. In NCIC CTG MA.12, 672 premenopausal women received adjuvant anthracycline-based or CMF chemotherapy and then were randomized to tamoxifen versus placebo for 5 years [18]. The CONSORT diagram for these three trials is provided in

Figure 1. Together, tumors collected across the three trials formed a combined cohort of 1,690 cases with formalin-fixed paraffin embedded tumor samples with intrinsic subtyping, and 1,557 of these cases had quantitative ER, PR, and HER2 centrally reviewed in each trial. Previous analyses found no statistically significant differences in clinicopathological characteristics or outcomes between subtyped trial subsets and the overall trial cohorts [19–21]. All biomarker studies were approved by the institutional research ethics board of the University of North Carolina at Chapel Hill.

Intrinsic Subtyping

The previously published reverse transcription-quantitative polymerase chain reaction (RT-qPCR)-based intrinsic subtypes were used [19–21] and included the original log-expression ratios of the 50 PAM50 genes normalized to the 5 housekeeper genes (open-source version of the PAM50 intrinsic subtype classifier). The tumor specimens used for RT-qPCR were obtained from the same tissue blocks used for building tissue microarrays. These data also included quantitative individual gene expression levels for standard clinical markers (*ESR1* for ER, *PGR* for PR, and *ERBB2* for HER2). The RNA preparation from paraffin cores, the RT-qPCR assay for the PAM50 panel and reference genes, and the classification of tumor samples into the intrinsic subtypes (luminal A, luminal B, HER2-enriched, basal-like, and normal-like subtypes) have been described previously [15].

Central ER, PR, and HER2 Determination

Data on ER, PR, and HER2 expression, assessed centrally in each trial, were included in the combined data set. Semiquantitative ER and PR expression (percentage of positive tumor nuclei) were determined by visual assessment of IHC staining by corresponding study pathologists; tumor samples from NCIC CTG MA.5 and MA.12 were assessed by T.O.N. and GEICAM/9906 by I. Aranda. The same scoring criteria were used by the two pathologists; however, no interobserver variation study was performed. HER2 expression was determined by IHC, and the amplification ratio was determined by FISH for NCIC CTG MA.5 and MA.12 and by chromogenic in situ hybridization (CISH) for GEICAM 9906, according to ASCO/CAP guidelines [2]. Technical details of the IHC methodology are provided in supplemental online Table 1.

For our analysis, HER2 negativity by clinical assay was defined as IHC 0/1+ or 2+ confirmed by a FISH/CISH amplification ratio <2.0. ER and/or PR IHC expression of 1%–9% was considered "borderline," and ≥10% expression was considered positive. If ER and PR fell into discrepant categories or one was borderline, the higher score defined the hormone receptor status for that case.

Statistical Analysis

In the combined data set, the PAM50 gene expression data were standardized across the three trials using z scores. The population means and standard deviations of parameters (gene expression levels in this scenario) were estimated from a stratified randomized subpopulation of the three trials based on the percentage of clinically ER-positive tumors ($n = 1,185$). This
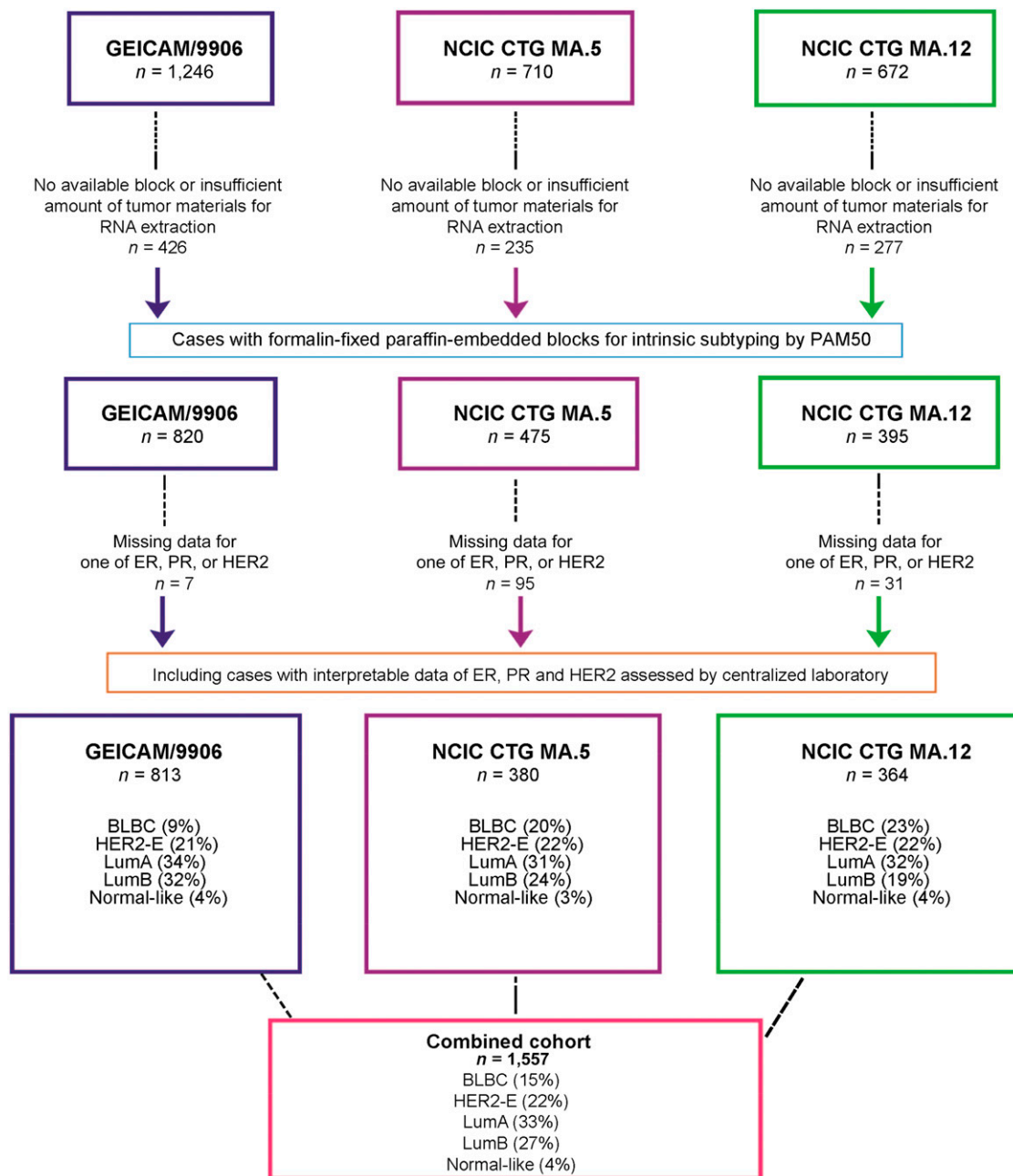
Figure 1. REMARK diagram, including intrinsic subtype frequencies for each trial.
Abbreviations: BLBC, basal-like subtype; HER2-E, HER2-enriched subtype; LumA, luminal A subtype; LumB, luminal B subtype.

subpopulation included all cases from NCIC CTG MA.5 ($n = 380$) and MA.12 ($n = 364$) and 441 cases from GEICAM/9906 (including all the ER-negative cases [$n = 171$]). For balance, 270 ER-positive tumors were randomly selected from GEICAM/9906. The mean and standard deviations were then estimated from this subpopulation ($n = 1,185$) and used to transform to z scores for the entire combined cohort ($n = 1,557$). The expression profiles were used to calculate the luminal signatures and proliferation index.

The standardized values of *ESR1*, *PGR*, and *ERBB2* gene expression levels were also rescaled to a relative scale of 1–10. In brief, each value was recalculated as (V − minV) / (maxV − minV) × 10, in which V represents the value of the gene expression, *min* indicates minimal, and *max* indicates

maximal. This allows *ESR1, PGR,* and *ERBB2* to have different means and standard deviations but equal ranges.

The frequencies of the PAM50 intrinsic subtype among cases clinically ER/PR <1% and HER2-negative versus ER/PR 1%–9% and HER2-negative were compared and tested using chi-square statistics and Fisher's exact test, as appropriate. The variation of quantitative ER and PR expression measured by IHC and gene expression of *ESR1, PGR,* and *ERBB2* measured by RT-qPCR across the intrinsic subtypes were examined using box-and-whisker plots and analysis of variance. Pairwise Student *t* tests were used to compare the differences in expression levels between groups, and the reported *p* values were adjusted for multiple comparisons by the Benjamini and Hochberg method [22]. All statistical tests were two-sided.

RESULTS

ER, PR, and HER2 Categories and Intrinsic Subtype

Table 1 summarizes the clinical-pathological characteristics and adjuvant treatments administered to the study cohorts overall and within each trial.

Among the 1,557 tumors (Fig. 1), 1,298 (83.4%) were centrally HER2 negative. Of these 1,298 HER2-negative tumors, 283 (21.8%) were classified as ER or PR negative (<1% staining), 39 (3%) were ER or PR borderline (1%–9% staining), and 976 (75.2%) were strongly ER or PR positive (≥10%).

Of 259 centrally confirmed HER2-positive tumors in the cohort, 106 (40.9%) were HR negative, 26 (10.0%) were HR borderline, and 127 (49.0%) were HR positive.

In the overall cohort (1,557 tumors), 228 (15%) tumors were basal-like, 338 (22%) were HER2-enriched, 510 (33%) were luminal A, 421 (27%) were luminal B, and 60 (4%) were normal-like. Table 2 summarizes the distribution of intrinsic subtypes by ER/PR and HER2 categories.

Within HER2-negative tumors, regardless of whether the ER and PR cut point was <1% or <10%, most HR-negative, HER2-negative tumors were identified as basal-like; however, all other intrinsic tumor subtypes (luminal A, luminal B, and HER2-enriched) were also identified. The basal-like subtype composed 73% (207) of the 283 HER2-negative tumors with <1% ER/PR staining; <10% of these ER/PR <1%, HER2-negative tumors were luminal. The 39 borderline (1%–9%) ER-, PR-, or HER2-negative tumors were heterogeneous: only 18% (7) were basal-like, 44% (17) were either luminal A or B, and 31% (12) were HER2-enriched. These borderline tumors included significantly fewer basal-like tumors than tumors identified by <1% HR cut point ($p < .0001$). If these borderline tumors were included in defining TNBC, only 66% (214) of the total 322 HER2-negative tumors with either <1% or 1%–9% HR would be basal-like. Interestingly, depending on the ER or PR cut point used, 10%–30% of HER2-negative tumors were classified by gene profiling as the HER2-enriched subtype (Table 2).

Among the 106 HER2-positive tumors that were HR negative using the <1% cut point, most (82%, 87) were HER2-enriched. Conversely, among the 127 HER2-positive tumors that were strongly HR positive (>10% ER or PR staining), only 54% (69) were HER2-enriched and 43% (55) were either luminal A or B, with luminal B appearing more frequently than luminal A (39 luminal B, 16 luminal A). Among the 26 HER2-positive tumors that were ER or PR borderline, most (77%, 20) were HER2-enriched (Table 2).

Quantitative ER, PR, and HER2 Expression by IHC and RT-qPCR

Among the 228 basal-like tumors, 93.4% (213 of 228) had <1% ER or PR staining by IHC (Table 2; Fig. 2, 3). This subtype had low expression of *ESR1* and *PGR* by RT-qPCR (supplemental online Fig. 1A, 2B). Ten (4%) basal-like tumors had ER staining; 2 of these had borderline (1%–9%) expression and eight had high ER staining (50%–100% of cells positive). Eleven (5%) basal-like tumors had PR staining; 6 of these had borderline (1%–9%) expression. Overall, basal-like breast tumors were significantly associated with low

expression of *ESR1 and PGR* (gene expression of ER and PR measured by qPCR).

ESR1 gene expression correlated moderately well with ER protein expression by IHC (supplemental online Fig. 1B) (Pearson's correlation: 0.68; 95% confidence interval [CI]: 0.66–0.71; $p < .0001$). The majority of tumors with high *ESR1* expression by RT-qPCR fell into the luminal A or B subtype (supplemental online Fig. 1). Among the 415 low (or no) ER expressors, that is, tumors with *ESR1* expression level below the median and no ER expression (0% positive tumor cells) detected by IHC, the majority of cases were basal-like (216, 52.0%) or HER2-enriched (153, 36.9%), with only a small proportion luminal A (16, 3.9%), luminal B (20, 4.8%), or normal-like (10, 2.4%) (supplemental online Fig. 1B). In contrast, among the 45 tumors with *ESR1* expression levels above the median but without ER staining by IHC, the most frequent subtypes were luminal A (20, 44.4%) and luminal B (14, 31.1%), followed by HER2-enriched (7, 15.6%), basal-like (2, 4.4%), and normal-like (2, 4.4%). As demonstrated in Figure 3A, the percentage of ER-positive cells did not discriminate luminal A from luminal B tumors, as illustrated by similar mean ER IHC expression values (66% vs. 67%) (Fig. 2A) and nearly identical distributions (Fig. 3A).

The majority of PR-positive tumors by IHC also had higher *PGR* gene expression than those that were PR negative (supplemental online Fig. 2B) (Pearson's correlation: 0.59; 95% CI: 0.56–0.62; $p < .0001$). Similar to *ESR1*/ER, among the 464 low (or no) PR expressors, that is, tumors with *PGR* gene expression level below the median value (4.1) and no PR expression (0% positive tumor cells) detected by IHC, the majority of cases were basal-like (196, 42.2%) or HER2-enriched (163, 35.1%), with a small proportion categorized as other subtypes: luminal A (18, 3.9%), luminal B (78, 16.8%), and normal-like (9, 1.9%). Compared with *ER/ESR1*, a larger subset of tumors ($n = 102$) was PR negative by IHC but expressed high *PGR* at the mRNA level. Among this subset, the frequencies of other intrinsic subtypes were comparable: basal-like (21, 20.6%), HER2-enriched (25, 24.5%), luminal A (23, 22.5%), luminal B (24, 23.5%), and normal-like (9, 8.8%) (supplemental online Fig. 2B). In contrast to ER, PR discriminated between the two luminal subtypes by genomic assay. Luminal A tumors had significantly more PR-positive cells than luminal B tumors (mean 67% vs. 38%, $p < .0001$) (Fig. 2B), and the distribution of PR values between the two luminal subtypes was clearly different (Fig. 3B).

Clinically HER2-positive tumors had similar variations in hormone receptor expression by subtype (test of equal densities, $p = .03$) (Fig. 3C, 3D); however, the differences were smaller than in HER2-negative tumors. Within HER2-positive tumors, ER expression by IHC was slightly lower in luminal A compared with luminal B (mean 32% vs. 50%, $p = .06$), and PR expression was slightly higher (mean 49% vs. 31% positive, $p = .08$) (Fig. 3C). *ERBB2* gene expression was significantly higher in HR-negative cases compared with HR-positive tumors ($p = .004$) (supplemental online Fig. 3). The lowest *ERBB2* gene expression among all groups was seen in HR <1% and HER2 negative. As anticipated, the HER2-enriched genomic subtype had the highest HER2/ERBB2 mRNA expression level, which was significantly higher ($p < .0001$ in all cases) than the other subtypes.

Table 1. Characteristics and treatments of the combined cohort of 1,557 patients subjected to intrinsic subtyping by PAM50 and central quantitative hormone receptor and HER2 testing

Characteristic	GEICAM/9906 (n = 813)	NCIC.CTG MA.5 (n = 380)	NCIC.CTG MA.12 (n = 364)	Total (n = 1,557)
Nodal status				
Negative	0	0	92 (25.3)	92 (5.9)
1–3	502 (61.7)	222 (58.4)	201 (55.2)	925 (59.4)
≥4	311 (38.3)	158 (41.6)	71 (19.5)	540 (34.7)
Tumor status				
T1 (≤2 cm)	337 (41.5)	137 (36.1)	149 (40.9)	623 (40)
T2 (2.1–5 cm)	430 (52.9)	194 (51.1)	188 (51.6)	812 (52.2)
T3 (>5 cm)	46 (5.7)	21 (5.5)	26 (7.1)	93 (6.0)
T4 (locally advanced)	0	0	1 (0.3)	1 (0.1)
NA	0	28 (7.4)	0	28 (1.8)
Adjuvant chemotherapy				
CMF	0	194 (51.1)	151 (41.5)	345 (22.2)
CEF/AC	0	186 (48.9)	213 (58.5)	399 (25.6)
FEC	413 (50.8)	0	0	413 (26.5)
FEC-P	400 (49.2)	0	0	400 (25.7)
Adjuvant endocrine therapy				
No	169 (20.8)	380 (100)	183 (50.3)	732 (47)
Yes	644 (79.2)	0	181 (49.7)	825 (53)
ER				
Negative	171 (21)	148 (38.9)	141 (38.7)	460 (29.5)
Positive (>1%)	642 (79)	232 (61.1)	223 (61.3)	1097 (70.5)
PR				
Negative	247 (30.4)	156 (41.1)	163 (44.8)	566 (36.4)
Positive (>1%)	566 (69.6)	224 (58.9)	201 (55.2)	991 (63.6)
HER2				
Negative or normal	697 (85.7)	304 (80)	297 (81.6)	1,298 (83.4)
Overexpression or amplified	116 (14.3)	76 (20)	67 (18.4)	259 (16.6)

Data are shown as number (percentage).
Abbreviations: AC: doxorubicin (Adriamycin) and cyclophosphamide; CEF: cyclophosphamide, epirubicin, and fluorouracil; CMF, cyclophosphamide, methotrexate, and fluorouracil; FEC: fluorouracil, epirubicin, and cyclophosphamide; NA, not available; P, paclitaxel.

Table 2. Frequencies of intrinsic subtypes within centrally confirmed ER/PR and HER2 categories

	ER/PR− (<1%)		ER/PR borderline (1%–9%)		ER/PR+ (≥10%)	
Subtype	HER2− (n = 283)	HER2+ (n = 106)	HER2− (n = 39)	HER2+ (n = 26)	HER2− (n = 976)	HER2+ (n = 127)
Basal-like	207 (73.1)	6 (5.7)	7 (17.9)	0	8 (0.8)	0
HER2-E	48 (17)	87 (82.1)	12 (30.8)	20 (76.9)	102 (10.5)	69 (54.3)
Luminal A	6 (2.1)	3 (2.8)	7 (17.9)	1 (3.8)	477 (48.9)	16 (12.6)
Luminal B	15 (5.3)	8 (7.5)	10 (25.6)	3 (11.5)	346 (35.5)	39 (30.7)
Normal-like	7 (2.5)	2 (1.9)	3 (7.7)	2 (7.7)	43 (4.4)	3 (2.4)

Data are shown as number (percentage).
Abbreviations: −, negative; +, positive; E, enriched.

ER, PR, and HER2 Categories and Proliferation Index and Luminal Signatures

Intrinsic subtype gene expression is an integrated evaluation; the subtypes vary by not only hormone receptor and HER2-related gene expression but also by sets of genes related to other functions such as proliferation, which is itself prognostic of outcome in cohorts treated only with endocrine therapy, independent of subtype [7]. In evaluating the proliferation index and luminal gene signatures by PAM50 across the clinical IHC subgroups (Fig. 4A, 4B), ER/PR-negative (<1%), HER2-negative tumors had the highest proliferation index; ER/PR-borderline (1%–9%), HER2-negative tumors had mean proliferation index levels that were significantly lower ($p = .02$) than true triple negatives and significantly higher than HR-positive, HER2-negative tumors ($p = .0001$), although this was highly variable. Similar to the intrinsic subtype findings, borderline tumors had intermediate luminal signatures that were significantly higher than true triple negative ($p < .0001$) and
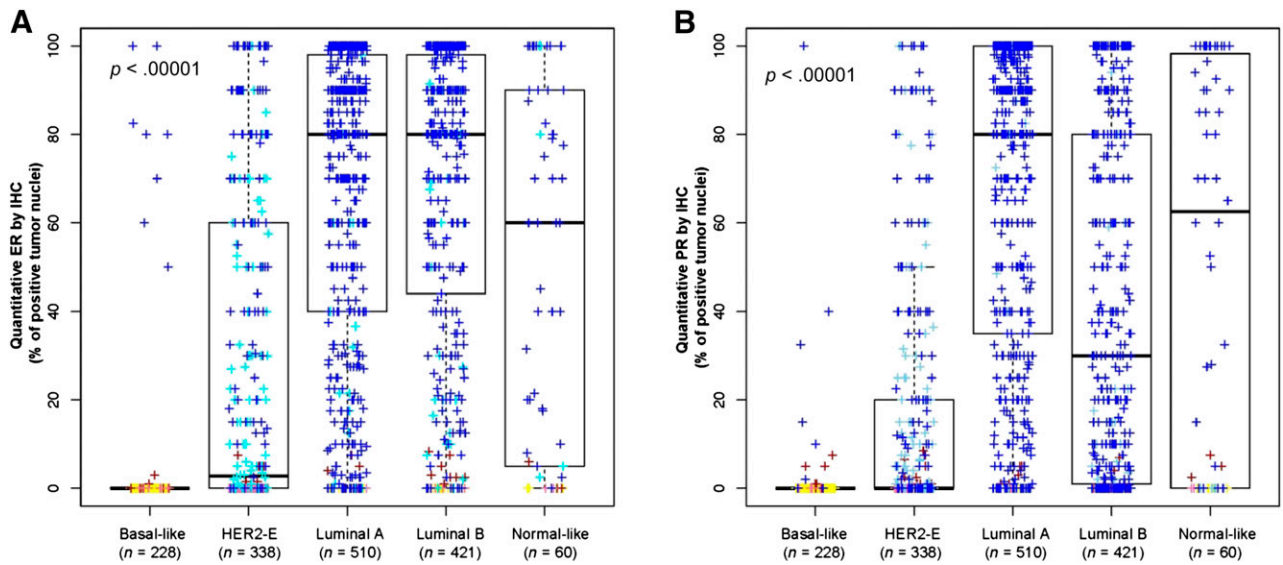
Figure 2. Quantitative IHC showing ER **(A)** and PR **(B)** expression by intrinsic subtype in 1,557 breast tumors demonstrating rare ER or PR expression in basal-like breast cancer and substantial heterogeneity of expression among other subtypes. Individual tumors are colored according to their clinical hormone receptor and HER2 status: ER/PR <1%, HER2 negative (yellow); ER/PR borderline (1%–9%), HER2 negative (red); ER/PR negative (<1%), HER2 positive (pink); ER/PR positive (≥1%), HER2 positive (light blue); ER/PR positive (≥10%), HER2 negative (dark blue).

Abbreviations: HER2-E, HER2-enriched subtype; IHC, immunohistochemistry.
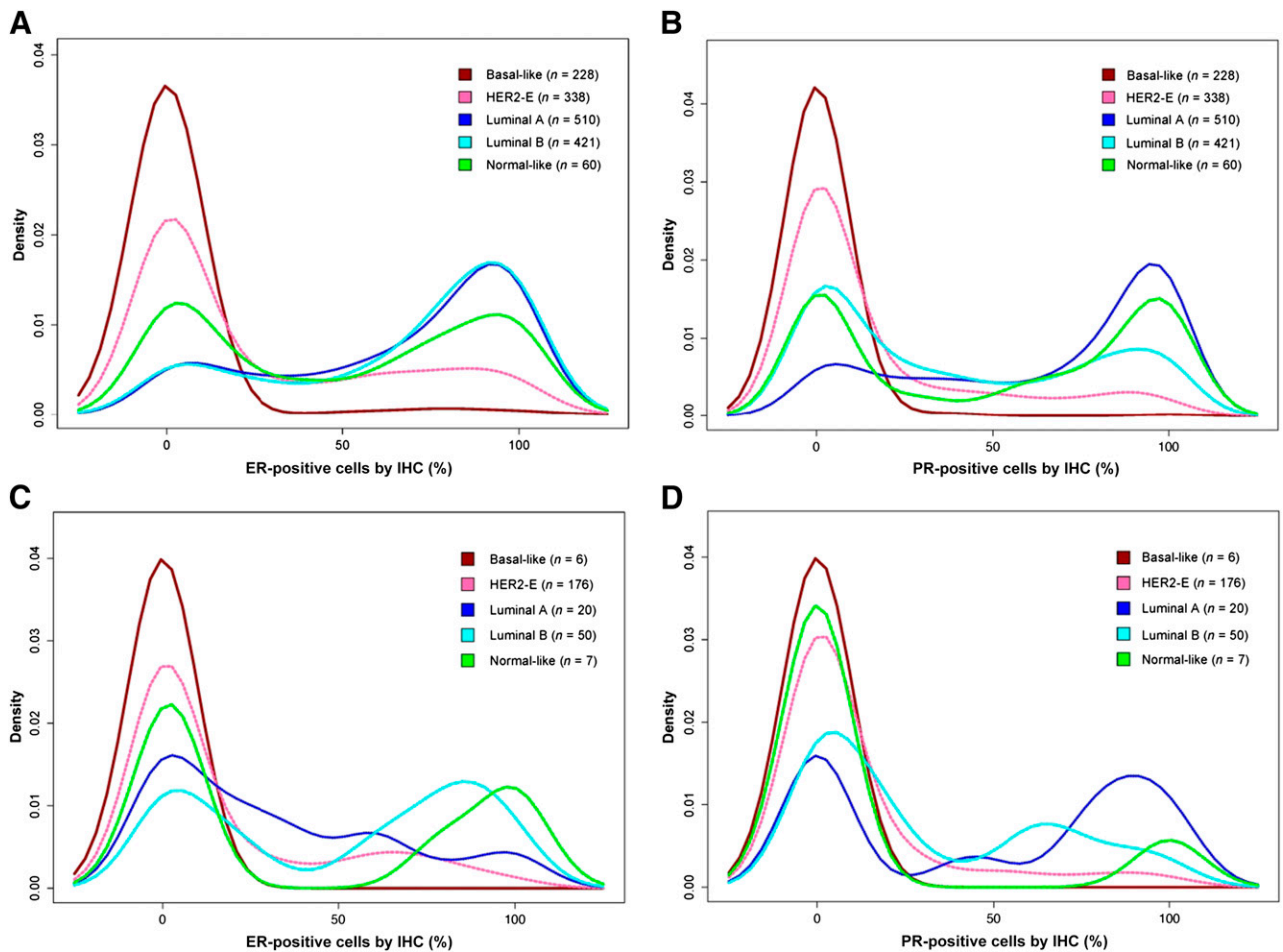
Figure 3. Density plot illustrating quantitative hormone receptor staining across the intrinsic subtypes of all 1,557 breast tumors—ER **(A)** and PR **(B)**—and for 259 clinically HER2-positive tumors—ER **(C)** and PR **(D)**. Although basal-like and HER2-E are typified by low or no staining, luminal A and B have similar staining distributions for ER; however, PR is significantly lower in luminal B than luminal A.

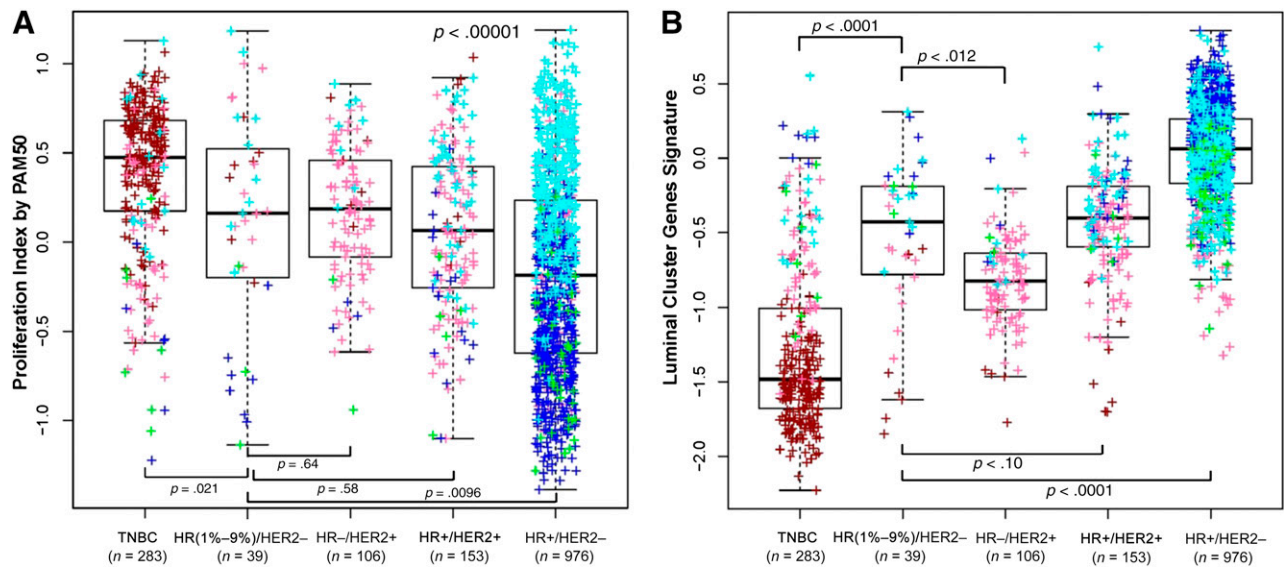Abbreviations: HER2-E, HER2-enriched subtype; IHC, immunohistochemistry.

Figure 4. Proliferation index **(A)** and luminal signature **(B)** gene expression levels across immunohistochemistry-based groups: TNBC (defined as ER/PR negative [<1%], HER2 negative); ER/PR borderline (1%–9%), HER2 negative; HR negative, HER2 positive; HR positive (≥1%), HER2 positive; and HR positive (≥10%), HER2 negative. Individual tumors were colored according to their intrinsic subtypes: basal-like (red), HER2-E (pink), luminal A (dark blue), luminal B (light blue), and normal-like (green). The *p* values were determined from Student *t* tests.
     Abbreviations: HR, hormone receptor; TNBC, triple-negative breast cancer.

significantly lower than HR-positive, HER2-negative tumors (*p* < .0001). This result provides additional evidence that borderline tumors differ in prognostically relevant gene expression from true triple negatives and strongly hormone receptor-positive tumors.

Discussion

This study examined the heterogeneity of breast cancer intrinsic subtypes within quantitative hormone receptor categories by either IHC or RT-qPCR. The definition of triple-negative breast cancer for clinical trials has been variable, including up to 9% ER or PR staining. We found that including borderline ER or PR in the definition of triple-negative breast cancer significantly diluted the proportion that were basal-like; a substantial proportion of those that would be included by this looser definition of TNBC were luminal breast cancers. Basal-like breast cancer composed 73% of TNBC defined by ER/PR <1% staining but only 66% if up to 10% ER or PR staining was included. This supports using the ASCO/CAP-recommended cut point of ER/PR <1% to define HR-negative breast cancer and TNBC. Basal-like tumors rarely expressed hormone receptors, using either IHC or RT-qPCR. Our findings are in keeping with other studies that have found that the percentage of basal-like breast cancers within clinical TNBC can vary from 50% to 70% depending on the population studied [12].

     In our centrally reviewed data, borderline ER/PR staining (1%–9%) represented a rare group, with only 65 borderline cases identified among all 1,557 tumors, but they were a mixed group that included all intrinsic subtypes. Less than 20% of the 39 HER2-negative, HR-borderline tumors were basal-like; almost one-third were HER2-enriched, and nearly half were luminal. The same was true of HER2-positive, HR-borderline tumors: most were HER2-enriched, but >15% were luminal. These findings contrast to a degree with those of a study by Iwamoto et al. [23], who used the Affymetrix U133A chip (Affymetrix, Santa Clara, CA, http://www.affymetrix.com; which includes only 45 of the 50 genes needed for the PAM50

algorithm [24]) and found that, among 16 ER-borderline (1%–9% staining) HER2-negative tumors, only 2 were luminal and 12 were basal-like.

     Our study cannot address therapeutic sensitivity, for example, whether HR-borderline luminal tumors are endocrine sensitive or whether lengthy endocrine therapy can be omitted for HR-borderline basal-like tumors. However, this study raises the question of whether borderline HR staining should be considered indeterminate, requiring additional assays to clarify underlying biology.

     Several limitations of this study should be noted. We used a research version of the open-source PAM50 intrinsic subtype classifier; this is one method of classifying tumors that can reliably identify intrinsic subtypes such as the luminal, HER2-enriched, and basal-like subtypes but cannot identify other subtypes such as basal-like subsets [10], Claudin-Low tumors [25], molecular apocrine tumors [26], or immunomodulatory subsets [10]. Another limitation of this study was that the central immunostaining for GEICAM/9906 was done in Spain, whereas that for NCIC CTG MA.5 and MA.12 was performed in Canada. No cross-comparison studies were done between the two central laboratories for ER and PR determination.

     Regardless of ER/PR cut point, we found that 10%–30% of HER-negative tumors by clinical assay were HER2-enriched by gene expression assay. This finding is consistent with previous studies demonstrating that not all HER2-enriched tumors are clinically HER2 positive [12]. In our study, clinically HER2-positive tumors had different intrinsic subtype distributions. Among HER2-positive, HR-negative tumors, >80% were HER2-enriched. Among HER2-positive, strongly HR-positive tumors, barely more than half were HER2-enriched, with the remainder largely luminal subtypes. With recent studies suggesting that the HER2-enriched subtype is particularly sensitive to HER2-targeting agents, this finding may be clinically meaningful [27–29]. Recent studies suggest that subtype markedly affects response to HER2 targeting [30] and tumor behavior more

strongly than clinical HER2 status [29]. An older classification system used in several seminal studies [31–33] that categorized luminal A as HR positive and HER2 negative and luminal B as HR positive and HER2 positive enriches the populations of interest; however, our data suggest that this approach misclassifies luminal A 47% of the time and luminal B 40% of the time and should not be used.

We also found that luminal A and B tumors have nearly identical distributions of quantitative ER staining overall; however, they differed significantly in PR staining by both IHC and RT-qPCR. In NCIC CTG MA.12, a data set included in this study, PR by IHC but not ER was significantly associated with disease-free survival [34], which may in part reflect the segregation of luminal A and B by PR in that data set. Prat and colleagues also suggested that quantitative PR staining could be used to differentiate luminal A and B tumors [35]. In that analysis of an endocrine therapy-treated cohort, PR staining not only differentiated subtype but also found that, within luminal A, those with higher PR had worse outcome. The study reported in this paper included those 536 luminal A/B tumors with the addition of 395 luminal A/B tumors from the NCIC studies, finding similar relationships of subtype and quantitative PR staining.

This study found that quantitative receptor categories can enrich for intrinsic subtypes of interest but that intrinsic subtype heterogeneity still persists within these categories. This study pooled centrally reviewed HR and HER2 data and RT-qPCR-based individual gene expression and intrinsic subtyping from three cooperative group trials and found that the optimal cut point for enriching for basal-like breast cancer is <1% staining for either hormone receptor, although even with those cut points, <75% were basal-like in our study. This aligns with the ASCO/CAP guideline of <1% HR staining. Tumors with borderline quantitative hormone receptor staining were heterogeneous from all standpoints; they were approximately equally divided between luminal and nonluminal subtypes. This raises the question of whether borderline HR staining should be considered indeterminate and require additional assays to clarify underlying biology. We also found, as others have, that >10% of clinically HER2-negative tumors were molecularly classified as HER2-enriched, but whether they are suitable for anti-HER2 therapy needs to be determined.

Conclusion

Quantitative hormone receptor expression can help identify molecular populations of interest. Borderline-expressing tumors are heterogeneous and do not fit well into molecular categories. While endocrine sensitivity in these tumors is not known, nearly half were luminal subtypes, supporting both current recommendations for endocrine therapy use as well as efforts to better identify sensitive tumors.

Author Contributions

Conception/Design: Maggie C.U. Cheang, Torsten O. Nielsen, Mark N. Levine, Angelo Di Leo, Lisa A. Carey
Provision of study material or patients: Miguel Martin, Torsten O. Nielsen, David Voduc, Alvaro Rodriguez-Lescure, Amparo Ruiz, Stephen Chia, Emilio Alba, Eva Carrasco, Rosalia Caballero, Dongsheng Tu, Mark N. Levine, Vivien H. Bramwell
Collection and/or assembly of data: Maggie C.U. Cheang, Miguel Martin, Torsten O. Nielsen, Aleix Prat, David Voduc, Alvaro Rodriguez-Lescure, Amparo Ruiz, Stephen Chia, Lois Shepherd, Manuel Ruiz-Borrego, Lourdes Calvo, Emilio Alba, Eva Carrasco, Rosalia Caballero, Dongsheng Tu, Kathleen I. Pritchard, Mark N. Levine, Vivien H. Bramwell, Joel Parker, Philip S. Bernard, Matthew J. Ellis, Charles M. Perou, Angelo Di Leo, Lisa A. Carey
Data analysis and interpretation: Maggie C.U. Cheang, Miguel Martin, Torsten O. Nielsen, Aleix Prat, Alvaro Rodriguez-Lescure, Amparo Ruiz, Stephen Chia, Lois Shepherd, Emilio Alba, Rosalia Caballero, Kathleen I. Pritchard, Mark N. Levine, Vivien H. Bramwell, Joel Parker, Philip S. Bernard, Matthew J. Ellis, Charles M. Perou, Angelo Di Leo, Lisa A. Carey
Manuscript writing: Maggie C.U. Cheang, Mark N. Levine, Charles M. Perou, Angelo Di Leo, Lisa A. Carey
Final approval of manuscript: Maggie C.U. Cheang, Miguel Martin, Torsten O. Nielsen, Aleix Prat, David Voduc, Alvaro Rodriguez-Lescure, Amparo Ruiz, Stephen Chia, Lois Shepherd, Manuel Ruiz-Borrego, Lourdes Calvo, Emilio Alba, Eva Carrasco, Rosalia Caballero, Dongsheng Tu, Kathleen I. Pritchard, Mark N. Levine, Vivien H. Bramwell, Joel Parker, Philip S. Bernard, Matthew J. Ellis, Charles M. Perou, Angelo Di Leo, Lisa A. Carey

References

1. Hammond ME, Hayes DF, Dowsett M et al. American Society of Clinical Oncology/College of American Pathologists guideline recommendations for immunohistochemical testing of estrogen and progesterone receptors in breast cancer (unabridged version). Arch Pathol Lab Med 2010; 134:e48–e72.

2. Wolff AC, Hammond ME, Schwartz JN et al. American Society of Clinical Oncology/College of American Pathologists guideline recommendations for human epidermal growth factor receptor 2 testing in breast cancer. J Clin Oncol 2007;25:118–145.

3. Wolff AC, Hammond ME, Hayes DF. Re: Predictability of adjuvant trastuzumab benefit in

N9831 patients using the ASCO/CAP HER2-positivity criteria. J Natl Cancer Inst 2012;104: 957–958.

4. Wolff AC, Hammond ME, Hicks DG et al. Recommendations for human epidermal growth factor receptor 2 testing in breast cancer: American

Society of Clinical Oncology/College of American Pathologists clinical practice guideline update. J Clin Oncol 2013;31:3997–4013.

5. Loi S, Haibe-Kains B, Desmedt C et al. Definition of clinically distinct molecular subtypes in estrogen receptor-positive breast carcinomas through genomic grade. J Clin Oncol 2007;25:1239–1246.

6. Paik S, Shak S, Tang G et al. A multigene assay to predict recurrence of tamoxifen-treated, node-negative breast cancer. N Engl J Med 2004;351:2817–2826.

7. Nielsen TO, Parker JS, Leung S et al. A comparison of PAM50 intrinsic subtyping with immunohistochemistry and clinical prognostic factors in tamoxifen-treated estrogen receptor-positive breast cancer. Clin Cancer Res 2010;16:5222–5232.

8. Dowsett M, Sestak I, Lopez-Knowles E et al. Comparison of PAM50 risk of recurrence score with Oncotype DX and IHC4 for predicting risk of distant recurrence after endocrine therapy. J Clin Oncol 2013;31:2783–2790.

9. Dubsky P, Filipits M, Jakesz R et al. EndoPredict improves the prognostic classification derived from common clinical guidelines in ER-positive, HER2-negative early breast cancer. Ann Oncol 2013;24:640–647.

10. Lehmann BD, Bauer JA, Chen X et al. Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies. J Clin Invest 2011;121:2750–2767.

11. Shah SP, Roth A, Goya R et al. The clonal and mutational evolution spectrum of primary triple-negative breast cancers. Nature 2012;486:395–399.

12. Prat A, Adamo B, Cheang MC et al. Molecular characterization of basal-like and non-basal-like triple-negative breast cancer. *The Oncologist* 2013;18:123–133.

13. Perou CM, Sørlie T, Eisen MB et al. Molecular portraits of human breast tumours. Nature 2000;406:747–752.

14. Sørlie T, Perou CM, Tibshirani R et al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci USA 2001;98:10869–10874.

15. Parker JS, Mullins M, Cheang MC et al. Supervised risk predictor of breast cancer based on intrinsic subtypes. J Clin Oncol 2009;27:1160–1167.

16. Martín M, Rodríguez-Lescure A, Ruiz A et al. Randomized phase 3 trial of fluorouracil, epirubicin, and cyclophosphamide alone or followed by Paclitaxel for early breast cancer. J Natl Cancer Inst 2008;100:805–814.

17. Levine MN, Pritchard KI, Bramwell VH et al. Randomized trial comparing cyclophosphamide, epirubicin, and fluorouracil with cyclophosphamide, methotrexate, and fluorouracil in premenopausal women with node-positive breast cancer: Update of National Cancer Institute of Canada Clinical Trials Group Trial MA5. J Clin Oncol 2005;23:5166–5170.

18. Bramwell VH, Pritchard KI, Tu D et al. A randomized placebo-controlled study of tamoxifen after adjuvant chemotherapy in premenopausal women with early breast cancer (National Cancer Institute of Canada—Clinical Trials Group Trial, MA.12). Ann Oncol 2010;21:283–290.

19. Cheang MC, Voduc KD, Tu D et al. Responsiveness of intrinsic subtypes to adjuvant anthracycline substitution in the NCIC.CTG MA.5 randomized trial. Clin Cancer Res 2012;18:2402–2412.

20. Chia SK, Bramwell VH, Tu D et al. A 50-gene intrinsic subtype classifier for prognosis and prediction of benefit from adjuvant tamoxifen. Clin Cancer Res 2012;18:4465–4472.

21. Martín M, Prat A, Rodríguez-Lescure A et al. PAM50 proliferation score as a predictor of weekly paclitaxel benefit in breast cancer. Breast Cancer Res Treat 2013;138:457–466.

22. Benjamini Y, Hochberg H. Controlling the false discovery rate: A practical and powerful approach to multiple testing. J R Stat Soc Series B Stat Methodol 1995;57:289–300.

23. Iwamoto T, Booser D, Valero V et al. Estrogen receptor (ER) mRNA and ER-related gene expression in breast cancers that are 1% to 10% ER-positive by immunohistochemistry. J Clin Oncol 2012;30:729–734.

24. Prat A, Parker JS, Fan C et al. PAM50 assay and the three-gene model for identifying the major and clinically relevant molecular subtypes of breast cancer. Breast Cancer Res Treat 2012;135:301–306.

25. Prat A, Parker JS, Karginova O et al. Phenotypic and molecular characterization of the claudin-low intrinsic subtype of breast cancer. Breast Cancer Res 2010;12:R68.

26. Lehmann-Che J, Hamy AS, Porcher R et al. Molecular apocrine breast cancers are aggressive estrogen receptor negative tumors overexpressing either HER2 or GCDFP15. Breast Cancer Res 2013;15:R37.

27. Carey LA, Berry DA, Ollila DW et al. Clinical and translational results of CALGB 40601: A neoadjuvant phase III trial of weekly paclitaxel and trastuzumab with or without lapatinib for HER2-positive breast cancer. J Clin Oncol 2013;31 (suppl):500a.

28. Prat A, Bianchini G, Thomas M et al. Research-based PAM50 subtype predictor identifies higher responses and improved survival outcomes in HER2-positive breast cancer in the NOAH study. Clin Cancer Res 2014;20:511–521.

29. Prat A, Carey LA, Adamo B et al. Molecular features and survival outcomes of the intrinsic subtypes within HER2-positive breast cancer. J Natl Cancer Inst 2014;106:dju152.

30. Carey LA, Barrry WT, Pitcher BN et al. Gene expression signatures in pre- and post-therapy (Rx) specimens from CALGB 40601 (Alliance), a neo-adjuvant phase III trial of weekly paclitaxel and trastuzumab with or without lapatinib for HER2-positive breast cancer (BrCa). J Clin Oncol 2014;32 (suppl):506a.

31. Carey LA, Perou CM, Livasy CA et al. Race, breast cancer subtypes, and survival in the Carolina Breast Cancer Study. JAMA 2006;295:2492–2502.

32. von Minckwitz G, Untch M, Blohmer JU et al. Definition and impact of pathologic complete response on prognosis after neoadjuvant chemotherapy in various intrinsic breast cancer subtypes. J Clin Oncol 2012;30:1796–1804.

33. Ali AM, Provenzano E, Bartlett JM et al. Prognosis of early breast cancer by immunohistochemistry defined intrinsic sub-types in patients treated with adjuvant chemotherapy in the NEAT/BR9601 trial. Int J Cancer 2013;133:1470–1478.

34. Chapman JA, Nielsen TO, Ellis MJ et al. Effect of continuous statistically standardized measures of estrogen and progesterone receptors on disease-free survival in NCIC CTG MA.12 Trial and BC Cohort. Breast Cancer Res 2013;15:R71.

35. Prat A, Cheang MC, Martín M et al. Prognostic significance of progesterone receptor-positive tumor cells within immunohistochemically defined luminal A breast cancer. J Clin Oncol 2013;31:203–209.

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