Clinical Study

Are Breast Cancer Molecular Classes Predictive of Survival in Patients with Long Follow-Up?

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Received 19 June 2013; Accepted 25 September 2013

1. Introduction

Breast carcinoma (BC) encompasses a heterogeneous group of tumors with great variability both at the molecular and at the morphological levels. Clinical outcome is also variable, some tumors are completely cured and others can recur even after more than 10 years from surgical treatment. Gene expression profiling through microarray technology has been designed to improve molecular taxonomy as a tool for supporting clinical management of patients [1–3]. According to molecular classification, BCs were grouped into at least four “intrinsic” subtypes: two ER-positive luminal-like subtypes and two ER-negative subtypes, HER2 overexpressing (HER2 positive nonluminal) and triple negative (TN). These intrinsic subtypes showed different prognosis and outcome [3, 4]. Luminal tumors, divided into A and B subtypes, include more than two-thirds of all BCs [5], while HER2 positive nonluminal and triple negative (TN) make up, respectively, 15–20% and 10–15% of breast tumors [6]. However, due to the limitation of microarray-based molecular classification in clinical practice, immunohistochemical (IHC) classification by the analysis of estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor-2 (HER2), and Ki67 (with a cutoff of 14%) has been proposed and has been shown to correlate with gene expression profiling data [6–11]. Furthermore, the St. Gallen consensus 2011 has strongly supported the clinicopathological determination of ER, PR, HER2, and Ki-67 as useful for defining subtypes, as well as providing treatment recommendations for BC intrinsic subtypes [7].

In normal breast tissue, luminal epithelial cells of ducts and lobules are characterized by the expression of low molecular weight cytokeratin CK8, CK18, CK19, and CK7. The outer layer includes more heterogeneous cells that express...
2. Patients and Methods

2.1. Description of the Patients’ Cohort. All patients were resident in a province in the northern-east area of Italy. Inclusion criteria were (i) diagnosis of BC at least 15 years before the censoring date of the study (December 31 2008), (ii) invasive BC of stage I–III, (iii) age at diagnosis 55 years or younger, and (iv) availability of formalin-fixed and paraffin-embedded (FFPE) tissues. Cases with a second primary breast cancer or other malignancies were excluded from the study. From 380 women initially enrolled, 75 patients with breast cancer or other malignancies were excluded from the study. From 380 women initially enrolled, 75 patients with in situ BC were excluded. Accordingly, 305 patients represented the final cohort for the study, of these 154 (50.4%) presented with lymph node involvement (LN+) at diagnosis. FFPE tissues of the primary tumor obtained by surgical treatment were used. Clinical information was obtained from medical records. Tumors were reviewed and histologically classified according to the World Health Organization (WHO 2003) [16], graded using Elston and Ellis grading system [17] and grouped into stages according to TNM classification [18]. The patients’ cohort was followed for a maximum of 25 years through the local Cancer Registry from diagnosis of BC to death or until censoring date. This study was approved by the Ethical Committee of the University of Trieste.

Fifteen patients (5%) were lost at follow-up during the period of observation because of emigration. The mean age at diagnosis was 47 years (range 26–55). No significant differences in age at diagnosis were observed between the LN− (46.8 y, range 32–55 y) and LN+ (47.0 y, range 26–55 y) groups, but the frequency of patients under 35 years was higher in LN+.

Patients were treated with mastectomy or breast-conserving surgery. All patients submitted to conserving surgery were treated with radiotherapy. All LN+ patients were treated with adjuvant chemotherapy with CMF ( cyclophosphamide, methotrexate, and fluorouracil) or EC/ECF (epirubicin, cyclophosphamide, and fluorouracil) regimens according to standard protocols. ER-positive patients, both LN− and LN+, were submitted to hormone therapy with tamoxifen. No specific treatment with trastuzumab was performed in HER2 positive patients, because this therapy was not available at the time of diagnosis.

The median follow-up time was 16 years (range 0–25). In detail it was 18 years (range 0–25) for the LN− group and 9 years (range 0–24) for the LN+ group (P = 0.000). In the LN− group 49 women (33%) recurred, while in the LN+ group 98 patients (69%) did so. For 4 LN− patients (3%) and 11 LN+ ones (7%) no information about recurrences was given. All clinical and pathological characteristics are reported in Table 1.

2.2. Tissue Microarray. Each patient’s haematoxylin and eosin (H&E) slides were reviewed by an expert pathologist (R. Barbazza), who marked the representative tumor areas to be analyzed. Tissue microarrays (TMAs) were constructed by the use of the FFPE tissues of the entire cohort using the Galileo TMA CK3500 (Integrated Systems Engineering, Milano, Italy), as previously described [19]. Multiple tissue cores were sampled when the tumor presented with histological heterogeneous regions. One section from each TMA block was stained with H&E to confirm the presence of carcinoma.

2.3. Immunohistochemical Staining. IHC staining was performed following the standard procedures [20], according to the manufacturers’ instructions for each MAb used. Immunostaining was performed manually with the Vectastain Universal Elite ABC kit (Vector Laboratories, Burlingame, CA, USA) for ER, PR, CK8, CK5/6, and vimentin. Ready-to-use antibodies for ER (CONFIRM anti-Estrogen Receptor clone SPI; Ventana Medical System, Tucson, AZ, USA), PR (CONFIRM anti-Progesterone Receptor clone 1E2; Ventana), vimentin (CONFIRM anti-Vimentin clone V9; Ventana), and CK5/6 (clone D5&16B4; diluted 1:100 Aczon Biotech, Monte San Pietro, Bologna, Italy) were applied for 60 minutes after 20-minute antigen retrieval in 0.1M pH 8 Tris-Borate 1mM EDTA at high temperature, in water bath. CK8 antibody (clone M20; Abcam, Cambridge, UK) was used at 1:250 dilution with 60-minute incubation, after 20-minute antigen retrieval in 10 mM pH6 Citrate Buffer at high temperature in water bath. For visualization, the DAB Substrate kit for Peroxidase (Vector Laboratories, Burlingame, CA, USA) was used.

Immunostaining for Ki67 (clone MIB-1; 1:200 dilution, DakoDenmark A/S, Glostrup, Denmark) and HER2 (clone CB11; 1:300 dilution Thermo Scientific, Aptomar Runcorn, Cheshire, UK) was performed in Lab Vision Autostainer 480S (Thermo Scientific) with the UltraVision LP Large Volume Detection System HRP Polymer (Lab Vision Corporation, Thermo Scientific) according to manufacturer’s instructions. HER2 gene amplification in case of equivocal staining (2+)
Table 1: Clinical-pathological parameters and immunohistochemistry results of the BC cohort (N = 305) and with respect to lymph node involvement.

| Features                        | Entire cohort (305) n (%) | LN− (151) n (%) | LN+ (154) n (%) | P   |
|---------------------------------|---------------------------|-----------------|-----------------|-----|
| Mean age, years (range)         | 46.9 (26–55)              | 46.8 (32–55)    | 47.0 (26–55)    | 0.2 |
| Age, years ≤35                  | 21 (7)                    | 4 (3)           | 17 (11)         | 0.004* |
| Age, years >35                  | 284 (93)                  | 147 (97)        | 137 (89)        |     |
| Histology                       |                           |                 |                 |     |
| Ductal                          | 250 (83)                  | 117 (77)        | 136 (88)        |     |
| Lobular                         | 28 (9)                    | 13 (9)          | 15 (10)         | 0.001* |
| Medullary                       | 6 (2)                     | 6 (4)           | 0               |     |
| Mucinous                        | 7 (2)                     | 4 (3)           | 3 (2)           |     |
| Tubular                         | 12 (4)                    | 11 (7)          | 0               |     |
| Grade                           |                           |                 |                 |     |
| 1                               | 39 (13)                   | 34 (22)         | 5 (3)           |     |
| 2                               | 145 (47)                  | 86 (57)         | 59 (38)         | 0.000* |
| 3                               | 121 (40)                  | 31 (21)         | 90 (59)         |     |
| Tumor size, cm ≤2               | 181 (60)                  | 109 (72)        | 72 (48)         |     |
| Tumor size, cm 2–5              | 106 (35)                  | 39 (26)         | 67 (44)         | 0.000* |
| Tumor size, cm ≥5               | 15 (5)                    | 3 (2)           | 12 (8)          |     |
| Missing                         | 3                         | 0               | 3               |     |
| Lymph nodes 1–3 lymph nodes     | 97 (63)                   | 0               | 97 (63)         |     |
| Lymph nodes ≥4 lymph nodes      | 56 (37)                   | 0               | 56 (37)         |     |
| Stage                           |                           |                 |                 |     |
| I                               | 107 (35)                  | 107 (71)        | 0               |     |
| II                              | 125 (41)                  | 42 (28)         | 83 (54)         |     |
| III                             | 72 (24)                   | 2 (1)           | 70 (46)         |     |
| Missing                         | 1                         | 0               | 1               |     |
| Type of surgery                 |                           |                 |                 |     |
| Mastectomy                      | 221                       | 88              | 133             | 0.001* |
| Breast conservation             | 84                        | 63              | 21              |     |
| Recurrence                      |                           |                 |                 |     |
| No                              | 143 (49)                  | 98 (67)         | 45 (31)         |     |
| Yes                             | 147 (51)                  | 49 (33)         | 98 (69)         | 0.000* |
| Missing                         | 15                        | 4               | 11              |     |
| BC specific death               | 128 (42)                  | 39 (26)         | 89 (58)         |     |
| Living                          | 143 (47)                  | 99 (65)         | 44 (28)         | 0.000* |
| Other cause death               | 16 (5)                    | 7 (5)           | 9 (6)           |     |
| Lost at FU                      | 18 (6)                    | 6 (4)           | 12 (8)          |     |
| ER                              |                           |                 |                 |     |
| Negative                        | 63 (21)                   | 25 (17)         | 38 (25)         | 0.08 |
| Positive                        | 242 (79)                  | 126 (83)        | 116 (75)        |     |
| PR                              |                           |                 |                 |     |
| Negative                        | 81 (27)                   | 27 (18)         | 54 (35)         | 0.001* |
| Positive                        | 224 (73)                  | 124 (82)        | 100 (65)        |     |
| HER2                            |                           |                 |                 |     |
| Negative                        | 244 (80)                  | 140 (93)        | 104 (67)        | 0.000* |
| Positive                        | 61 (20)                   | 11 (7)          | 50 (33)         |     |
Table 1: Continued.

| Features | Entire cohort (305) n (%) | LN− (151) n (%) | LN+ (154) n (%) | P       |
|----------|--------------------------|-----------------|-----------------|---------|
| Ki67     |                          |                 |                 |         |
| <14%     | 156 (51)                 | 104 (69)        | 52 (34)         | 0.000*  |
| ≥14%     | 149 (49)                 | 47 (31)         | 102 (66)        |         |
| CK8      |                          |                 |                 |         |
| Negative | 29 (10)                  | 11 (7)          | 18 (12)         | 0.3     |
| Positive | 276 (90)                 | 140 (93)        | 136 (88)        |         |
| CK5/6    |                          |                 |                 |         |
| Negative | 235 (77)                 | 109 (72)        | 126 (82)        | 0.04*   |
| Positive | 70 (23)                  | 42 (28)         | 28 (18)         |         |
| Vimentin |                          |                 |                 |         |
| Negative | 196 (64)                 | 106 (70)        | 90 (58)         | 0.03*   |
| Positive | 109 (36)                 | 45 (30)         | 64 (42)         |         |
| AR       |                          |                 |                 |         |
| Negative | 80 (37)                  | 29 (30)         | 51 (43)         | 0.04*   |
| Positive | 136 (63)                 | 69 (70)         | 67 (57)         |         |

ER: estrogen receptor, PR: progesterone receptor, HER2: human epidermal growth factor receptor-2, CK8: cytokeratin 8, CK5/6: cytokeratin 5/6, AR: androgen receptor. * Statistically significant data.

was assessed by SISH assays using the ultra View SISH DNP Detection Kit (Ventana) in Benchmark XT automated slide strainer instrument, as previously described [21].

IHC for androgen receptor (AR) was performed with mouse monoclonal antibody (AR441, DakoDenmark A/S, Glostrup, Denmark) as previously described [22]. Positive and negative control slides were used in each IHC assay.

2.4. Evaluation of Immunohistochemistry and Definition of Intrinsic Subtypes. Immunostaining was quantitatively evaluated by two different observers in a blinded fashion (DP, RB) using light microscopy and counting the positive cells across three high power fields (HPF at 40x magnification), each containing about one hundred cells. Tumors were considered as positive for ER and PR if more than 10% of tumor nuclei were stained independently of staining intensity [10, 23], because at the time of diagnosis this was the cutoff used to submit patients to Tamoxifen treatment. This is a deviation from the St. Gallen consensus and ASCO/CAP guidelines, which defined cases presenting with more than 1% of tumor cells independently of staining intensity as hormonal receptor positive [7, 24]. The cutoff for CK8 and CK5/6 positivity was 10% of cells as well. Tumors were considered as positive for vimentin and AR when more than 1% of tumor cells were stained [22, 25].

HER2 overexpression was scored according to ASCO guideline [26]. Our patients had a diagnosis of BC between 1983 and 1993 and no specific treatment with trastuzumab was performed in those years. Since we had no information about the scoring of HER2 at the time of diagnosis, we adopted the current method. For evaluation of Ki67 immunostaining, the positively stained cancer cells were counted manually across three high power fields (HPF). The mean value was used for Ki67 score. Ki67 threshold of 14% of nuclear staining of cancer cells was used to discriminate low proliferation index (<14%) and high proliferation index (≥14%) [5, 7]. The tumors were then classified into the four main intrinsic subtypes according to the staining profile of the antigen markers: ER, PR, HER2, and Ki67 [5, 7, 11, 27]. CK8, CK5/6, vimentin, and AR were investigated in the entire case study. In detail, cases that were ER positive and/or PR positive, HER2 negative, and low Ki67 were classified as luminal A; cases that were either (i) ER positive and/or PR positive, HER2 negative, and high Ki67 (luminal B HER2 negative) or (ii) ER positive and/or PR positive, HER2 positive (luminal B HER2 positive) were classified as Luminal B; tumors ER negative, PR negative, and HER2 positive were defined as HER2 positive; cases ER negative, PR negative, and HER2 negative, were classified as triple negative (TN), a surrogate for subtype [6]. If the tumors exhibited markers staining that did not meet the abovementioned panel criteria, they were defined as “unclassified.”

2.5. Statistical Analysis. Associations between clinical-pathological data, lymph node involvement, and intrinsic subtypes were tested for significance using the Chi-square test for categorical variables. For continuous variables the parametric Student's t-test, the one-way ANOVA test, or the Kruskal-Wallis test was used. BC specific survival was defined as the time from surgery to breast cancer specific death or end of follow-up, whichever came first. To estimate the joint effects of the analyzed covariates on patients’ survival, the data were analyzed by fitting the Cox proportional hazard regression model. Cox proportional hazard analysis included pathological variables (age at diagnosis, histological type, tumor size, lymph nodes status, and tumor grade) and intrinsic subtypes as covariates. The log-rank test was used to check the dependence of patients’ survival on single variables or on combinations of variables. All P values are two sided with values <0.05 regarded as statistically significant. P values between 0.05 and 0.07 were considered as “borderline.”
3. Results

3.1. Survival according to Intrinsic Subtypes. The BC specific survival curves per intrinsic subtypes for the entire cohort are shown in Figure 1. A significantly better BC specific survival was recorded for luminal A tumors and a worse outcome was observed for HER2 positive nonluminal subtype \( (P = 0.0003) \). In detail, significant differences were found between luminal A and luminal B \((P = 0.009)\), luminal A and HER2 positive (nonluminal) \((P = 0.0003)\), luminal A and TN \((P = 0.01)\), and luminal B and HER2 positive nonluminal \((P = 0.04)\). Therefore no significant differences between HER2 positive nonluminal and TN \((P = 0.3)\), and luminal B and TN \((P = 0.5)\) were detected.

Considering luminal A and B together, PR positive tumors showed a significantly better outcome in comparison with negative ones \((P = 0.02)\), as shown in Figure 2. Vimentin-positive luminal tumors had shorter survival compared to negative ones \((P = 0.03)\) (Figure 3). HER2 \((P = 0.5)\) and CK5/6 \((P = 0.9)\) expression did not seem to influence BC specific survival among luminal tumors. Moreover, luminal A tumors, but not luminal B, with AR-positive expression showed significant longer survival with respect to negative ones \((P = 0.01)\), as shown in Figure 4.

Cox multivariate analysis run for pathological variables and for the molecular subtypes revealed that histological type, tumor grade, lymph node involvement, and luminal tumors (A and B) showed an independent influence on BC specific survival, as reported in Table 2.
Survival by vimentin expression in luminal tumors ($N = 240$, 93 BC specific deaths).

Survival by AR in luminal A tumors ($N = 92$, 32 BC specific deaths).

**Table 2:** Cox multivariate regression analysis for breast cancer survival in the entire cohort ($N = 302$).

| Risk factors            | Hazard ratio | 95% CI*   | $P$  |
|-------------------------|--------------|-----------|------|
| Age at diagnosis        | 1.0          | 0.9-1.0   | 0.4  |
| Histologic type         | 0.8          | 0.6-1.0   | 0.04*|
| Grade                   | 1.4          | 1.0-1.9   | 0.05 |
| Tumor size              | 1.2          | 0.9-1.7   | 0.2  |
| Lymph node              | 2.4          | 1.5-3.7   | 0.000*|
| Luminal A               | 0.2          | 0.1-0.9   | 0.04*|
| Luminal B               | 0.2          | 0.1-0.8   | 0.03*|
| HER2 positive nonluminal| 0.3          | 0.1-1.4   | 0.1  |
| Triple negative         | 0.4          | 1.0-1.6   | 0.2  |

*Statistically significant data.

By comparing luminal patients who had BC specific death within 10 years (68 out of 93) with those who died from BC after 10 years (25 out of 93), tumors with higher Ki67 ($P = 0.02$) and positive to vimentin ($P = 0.02$) tend to cause more frequently "late" BC specific death.

3.2 Clinical Pathological Features of Intrinsic Subtypes. Luminal A was the most frequent molecular subtype with 140 patients (46%), followed by luminal B with 102 women (34%), 38 TN (12%), and 24 HER2 positive (nonluminal) (8%). One case failed to be classified in one of the molecular subtypes, because it exhibited staining for PR, CK8, and high Ki67, but not for ER, HER2, and CK5/6.

Clinical and pathological details stratified per intrinsic subtypes are shown in Table 3.

Significant associations between intrinsic subtypes and age at diagnosis (cutoff 35 years) ($P = 0.03$), histological type ($P = 0.001$), tumor grade ($P = 0.000$), lymph node involvement ($P = 0.000$), tumor stage ($P = 0.000$), and presence of later recurrences ($P = 0.006$) were found. Patients under 35 years at diagnosis more frequently fell into luminal B and HER2 positive (nonluminal) subtypes. Tumor size distribution did not show any difference in the intrinsic subtypes, but it was significantly higher in LN+ group ($P = 0.000$).

An important association between molecular classification and LN involvement was detected ($P = 0.000$). Most luminal A tumors were of stage I at diagnosis, while most luminal B tumors were of stage II and nearly half of HER2 positive (nonluminal) of stage III. TN tumors were equally distributed in the three stages. In the entire case study, luminal A subtype showed the lowest percentage of recurrences, while more than 75% of HER2 positive nonluminal tumors relapsed. Luminal B and TN patients showed a similar intermediate percentage of recurrences.

Vimentin prevailed in triple negative subgroup (75% of positive tumors) as compared to other classes ($P = 0.000$).

AR was evaluated in 216 tumors (tissue cores of 89 patients were not analyzable on TMAs), of which 136 were positive, as reported in Table 1. AR-positive staining was significantly associated with LN− tumors ($P = 0.04$), low-grade tumors ($P = 0.001$), and tumor stage I-II ($P = 0.04$). All medullary tumors were AR negative, whereas 75% of mucinous and 88% of tubular carcinomas were AR positive ($P = 0.02$). No significant association between AR positivity and tumor size was detected; however, the log-rank test revealed a significantly better outcome for patients showing positive AR expression, in comparison to negative ones, on the whole cohort ($P = 0.03$). Moreover, AR positivity was significantly associated with ER-positive ($P = 0.000$) and PR-positive ($P = 0.000$) tumors.

When we analyzed LN− and LN+ groups separately, we observed similar frequency of recurrences for luminal subtypes: 33% and 26% of recurrences in luminal A and luminal B in LN−; 64% and 67% for luminal A and B in LN+, respectively ($P = 0.6$ and $P = 0.8$). The result was also confirmed for cancer specific deaths: frequencies were comparable in luminal A and B subgroups when divided per lymph node involvement ($P = 0.9$ for both). Survival rates of luminal A and B patients were 0.75 at 25 years for both in lymph LN− and in LN+, they were 0.42 for luminal A and 0.34 for luminal B (at 25 years).
| Features                        | Luminal A (140) n (%) | Luminal B (102) n (%) | HER2 (24) n (%) | Triple negative (38) n (%) | P  |
|--------------------------------|-----------------------|-----------------------|-----------------|-----------------------------|----|
| Age at diagnosis, years (range)| 47.4 (30–55)         | 46.6 (30–55)          | 45.9 (28–55)    | 46.5 (26–55)                | 0.7|
| Age, years                     |                       |                       |                 |                             |    |
| ≤35                            | 4 (3)                 | 10 (10)               | 4 (17)          | 3 (8)                       | 0.04*|
| >35                            | 136 (97)              | 92 (90)               | 20 (83)         | 35 (92)                     |    |
| Histology                      |                       |                       |                 |                             |    |
| Ductal                         | 110 (79)              | 89 (87)               | 23 (96)         | 30 (79)                     | 0.002*|
| Lobular                        | 16 (11)               | 10 (10)               | 1 (4)           | 1 (3)                       |    |
| Medullary                      | 0                     | 0                     | 0               | 6 (16)                      |    |
| Mucinous                       | 3 (2)                 | 3 (3)                 | 0               | 1 (3)                       |    |
| Tubular                        | 11 (8)                | 0                     | 0               |                             |    |
| Grade                           |                       |                       |                 |                             |    |
| 1                              | 34 (25)               | 5 (4)                 | 0               | 0                           |    |
| 2                              | 77 (55)               | 49 (48)               | 9 (37)          | 9 (24)                      | 0.000*|
| 3                              | 29 (20)               | 48 (48)               | 15 (63)         | 297 (76)                    |    |
| Tumor size, cm                 |                       |                       |                 |                             |    |
| ≤2                             | 95 (68)               | 50 (49)               | 12 (50)         | 23 (62)                     | 0.1|
| 2–5                            | 39 (28)               | 44 (43)               | 11 (46)         | 12 (32)                     |    |
| ≥5                             | 5 (4)                 | 7 (7)                 | 1 (4)           | 2 (5)                       |    |
| Missing                         | 1                     | 1                     | 0               | 1                           |    |
| Lymph node involvement         |                       |                       |                 |                             |    |
| No                             | 98 (70)               | 28 (27)               | 5 (21)          | 19 (50)                     | 0.000*|
| Yes                            | 42 (30)               | 74 (73)               | 19 (79)         | 19 (50)                     |    |
| Lymph nodes                    |                       |                       |                 |                             |    |
| 1–3                            | 28 (67)               | 51 (69)               | 10 (53)         | 8 (44)                      | 0.1|
| ≥4                             | 14 (33)               | 23 (31)               | 9 (47)          | 10 (56)                     |    |
| Missing                         | 0                     | 0                     | 0               | 1                           |    |
| Stage                           |                       |                       |                 |                             |    |
| I                              | 71 (51)               | 17 (16)               | 4 (16)          | 14 (38)                     | 0.000*|
| II                             | 48 (34)               | 55 (54)               | 9 (38)          | 13 (35)                     |    |
| III                            | 21 (15)               | 30 (30)               | 11 (46)         | 10 (27)                     |    |
| Missing                         | 0                     | 0                     | 0               | 1                           |    |
| Recurrence                      |                       |                       |                 |                             |    |
| No                             | 78 (58)               | 43 (45)               | 5 (23)          | 16 (42)                     | 0.02*|
| Yes                            | 56 (42)               | 53 (55)               | 17 (77)         | 21 (55)                     |    |
| Missing                         | 6                     | 6                     | 2               | 1                           |    |
| Status                          |                       |                       |                 |                             |    |
| BC specific death              | 45 (32)               | 48 (47)               | 14 (58)         | 21 (55)                     |    |
| ER                              |                       |                       |                 |                             |    |
| Negative                       | 0                     | 0                     | 24 (100)        | 38 (100)                    |    |
| Positive                       | 140 (100)             | 102 (100)             | 0               | 0                           |    |
| PR                              |                       |                       |                 |                             |    |
| Negative                       | 6 (4)                 | 13 (13)               | 24 (100)        | 38 (100)                    |    |
| Positive                       | 134 (96)              | 89 (87)               | 0               | 0                           |    |
| HER2                            |                       |                       |                 |                             |    |
| Negative                       | 140 (100)             | 65 (64)               | 0               | 38 (100)                    |    |
| Positive                       | 0                     | 37 (36)               | 24 (100)        | 0                           |    |
### Table 3: Continued.

| Features          | Luminal A (140) n (%) | Luminal B (102) n (%) | HER2 (24) n (%) | Triple negative (38) n (%) | P  |
|-------------------|-----------------------|-----------------------|----------------|-----------------------------|----|
| **Ki67**          |                       |                       |                |                             |    |
| <14%              | 140 (100)             | 6 (6)                 | 4 (17)         | 6 (16)                      |    |
| ≥ 14%             | 0                     | 96 (94)               | 20 (83)        | 32 (84)                     |    |
| **CK8**           |                       |                       |                |                             |    |
| Negative          | 0                     | 0                     | 5 (21)         | 24 (63)                     |    |
| Positive          | 140 (100)             | 102 (100)             | 19 (79)        | 14 (37)                     |    |
| **CK5/6**         |                       |                       |                |                             |    |
| Negative          | 125 (89)              | 89 (87)               | 19 (79)        | 2 (5)                       |    |
| Positive          | 15 (11)               | 13 (13)               | 5 (21)         | 36 (95)                     |    |
| **Vimentin**      |                       |                       |                |                             |    |
| Negative          | 107 (76)              | 65 (64)               | 14 (58)        | 10 (26)                     | 0.000* |
| Positive          | 33 (24)               | 37 (36)               | 10 (42)        | 28 (74)                     |    |
| **AR**            |                       |                       |                |                             |    |
| Negative          | 19 (21)               | 20 (26)               | 14 (70)        | 26 (100)                    | 0.000* |
| Positive          | 73 (79)               | 57 (74)               | 6 (30)         | 0                           |    |

ER: estrogen receptor, PR: progesterone receptor, HER2: human epidermal growth factor receptor, CK8: cytokeratin 8, CK5/6: cytokeratin 5/6, AR: androgen receptor. *Statistically significant data.

### Table 4: Metastasis sites and local recurrence in accordance with BC molecular subtypes.

| Intrinsic subtypes (n) | Local n (%) | Bone n (%) | Liver n (%) | Lung and pleura n (%) | Brain n (%) | Endocrinea n (%) | Distant LB n (%) | Otherc n (%) |
|------------------------|-------------|------------|-------------|------------------------|-------------|-------------------|-----------------|--------------|
| Luminal A (140)        | 22 (16)     | 23 (16)    | 28 (21)     | 25 (18)                | 2 (1)       | 4 (3)             | 4 (3)           | 5 (4)        |
| Luminal B (102)        | 13 (13)     | 29 (28)    | 27 (26)     | 20 (20)                | 8 (8)       | 4 (4)             | 2 (2)           | 6 (6)        |
| HER2 positive (24)     | 7 (29)      | 3 (12)     | 7 (29)      | 6 (25)                 | 4 (17)      | 1 (4)             | 1 (4)           | 1 (4)        |
| TN (38)                | 5 (13)      | 12 (32)    | 13 (34)     | 14 (37)                | 4 (11)      | 1 (3)             | 1 (3)           | 1 (3)        |

a Endocrine sites include thyroid, ovary, and kidney. b Distantlymph nodes are considered the nodes that do not belong to the axilla, including the supraclavicular lymph nodes. c Other sites comprise peritoneum and pericardium.

#### 3.3. Pattern of Local and Distant Metastases.

The pattern of metastatic sites was reported in Table 4. Organ distribution of metastasis among intrinsic subtypes was almost similar, but for bone and brain. HER2 positive (nonluminal) type exhibited a lower rate (12%) of bone metastasis in comparison to other subtypes (P = 0.03). In particular, for bone involvement significant differences were found between HER2 positive (nonluminal) and luminal B (P = 0.01) and TN (P = 0.04). A higher rate of brain metastases was found in both HER2 positive (nonluminal) (17%) and TN (11%) (P = 0.03). In detail, a significant difference for brain metastases was observed between HER2 positive (nonluminal) and luminal A (P = 0.02) and TN and luminal A (P = 0.04).

#### 4. Discussion

Current BC clinical management is based on the assessment of traditional clinical and pathological factors and some molecular markers, such as ER, PR HER2, and Ki67. More recently, the 2011 St Gallen International Breast Cancer Conference [7] suggested using immunohistochemistry for molecular subtyping [5, 7, 8, 14] with the analysis of ER, PR, HER2, and Ki67 to subdivide patients into luminal A and B, HER2 positive nonluminal, and triple negative.

In this study we investigated the prognostic role of BC molecular classification in a cohort of 305 BC patients aged 55 years or younger at diagnosis, to obtain a long follow-up (mean follow-up: 14 years). To this purpose we used the panel of four biomarkers for molecular subgrouping by IHC, ER, PR, HER2, Ki67, [5, 6, 28, 29], plus CK8, CK5/6 (to confirm the luminal and basal-like types), vimentin, and AR as additional markers.

The distribution of intrinsic subtypes obtained by IHC in our patients’ cohort was similar to other previous studies [11, 27, 30]: luminal tumors were the most represented subtype with 80% of cases, followed by TN cancers (12%) and HER2 positive nonluminal (8%). In agreement with other studies, a significant association between clinical-pathological features and molecular subtypes was observed [3, 6, 8, 31]. In particular, most low-grade tumors that displayed features of luminal A, conversely poorly differentiated were mainly luminal B and HER2 positive nonluminal subtypes (P = 0.000). In TN subtype the rate of G3 tumors was significantly higher, as already reported [6, 32]. Regarding BC histology, pure tubular and mucinous types, related to a favorable prognosis [16], were luminal tumors, as well as lobular ones. HER2 positive nonluminal and TN molecular classes tumors were mostly of the ductal histological type, as already reported [29, 33].
Medullary carcinomas, whose histological type is rare, were classified as triple negative tumors [29, 31].

Regarding ER-positive tumors, luminal A type was mainly represented in LN−, while luminal B was more common in the LN+ group, and this is the main clinical difference between the two groups. Luminal B and HER2 positive nonluminal frequently presented at diagnosis with lymph node involvement, in agreement with Wiechmann et al. [30]. Conversely, TN tumors were equally distributed between LN− and LN+ groups, showing that its prognosis is mainly related to their intrinsic biological behavior [34]. Triple negative, as well as HER2 positive nonluminal, subtypes presented with higher rate of brain metastasis in comparison with luminal A tumors, in agreement with other reports [11, 12, 34].

A few luminal A tumors exhibited cancer specific death, while the HER2 positive nonluminal subtype presented with the highest rate of relapses, as already reported [11]. Luminal B and TN showed a similar intermediate frequency of recurrences, also reflected by specific survival (Figure 1(a)). However, if we consider luminal A and B by lymph node involvement, they exhibited in the two groups similar frequencies of recurrences. These data are also confirmed by BC specific survival as shown in Figures 1(b) and 1(c). This finding is related to the fact that the cutoffs to define luminal A and luminal B cancer are arbitrarily set [14], and by IHC they differ for the rate of Ki67 and HER2 overexpression, both characteristic of higher aggressiveness. Our results point out that the recurrence rate and cancer specific deaths are highly dependent on the status of axillary lymph nodes rather than on splitting between luminal A and B subtypes. In agreement with us, recent studies have called into question the very split between luminal A and B subtypes. In agreement with others [25, 35], triple negative, as well as HER2 positive nonluminal, subtypes presented with higher rate of brain metastasis in comparison with luminal A tumors, in agreement with other reports [11, 12, 34].

In our cohort, luminal B tumors were often negative to PR (P = 0.03), but positive to vimentin expression (P = 0.003) with respect to luminal A. Other authors have already reported on vimentin expression in luminal epithelium of the breast and on its positive correlation with ER in BC tumors [25, 35].

Furthermore, vimentin expression and higher Ki67 were significantly associated with worse survival in the subgroup of luminal patients who died from BC after 10 years. This suggests that vimentin and Ki67 could represent long-term unfavorable prognostic markers in ER-positive tumors. We also observed that most ER-positive tumors were positive to AR, which seems to be a marker of good prognosis, as already reported [22], especially in early stages of luminal tumors. Of note in the period of diagnosis of our cohort of patients, LN− patients were not treated with cytotoxic chemotherapy, according to regimens of those years.

To our knowledge this is the first study investigating BC specific survival associated with intrinsic subtypes in a population with up to 25 years of follow-up. As expected, we observed that BC specific survival analysis identified two well separated groups of tumors: ER-positive and ER-negative. ER-positive LN negative cancers have the best outcome, and ER-positive LN positive cases showed an intermediate overall survival, while TN and HER2 positive nonluminal types showed the shortest survival, as already reported [2, 3, 11, 14, 33]. HER2 positive nonluminal and TN subtypes displayed a very aggressive outcome with early BC specific death within 5 and 10 years, respectively. However we recognize that a possible limitation of the study is the low number of patients in the HER2 positive non luminal (24) and TN (38) subtypes.

Cox proportional hazard model confirmed an independent and favorable influence on BC survival for luminal tumors (ER-positive), together with the pathological variables, histological type, tumor grade, and lymph node involvement.

Taken together, our results stress that, in our experience, luminal A and luminal B belong to a unique clinical group of ER-positive tumors, and their clinical differences are mainly related to a different tumor stage at diagnosis. We also observed that the most important predictor of prognosis ER-positive tumors was still the lymph node involvement, which is still reported as one of the most relevant prognostic factors [36].

Although patients with luminal tumors showed significantly longer BC survival, they frequently undergo over 10-year long-term BC deaths [11]. In our case study 10% of luminal BC patients died from BC after 10 years of follow-up. It is possible to hypothesize that ER-positive tumors with high Ki67 and vimentin positivity harbor some specific biological properties that slow down progression for a very long time and may postpone BC specific death even for 20 years.

Therefore we strongly believe that monitoring and follow-up program should be different according to cancer subtypes, and, in case of luminal tumors, prolonged controls are recommended because of possible relapses.

5. Conclusion

Luminal tumors (ER positive) showed longer survival and better prognosis with respect to ER-negative ones, as it is widely accepted. In addition, AR resulted in a significant prognostic marker among luminal A patients. From our long-term survival study ER-positive tumors are confirmed to be a single clinical group, diagnosed in different periods of cancer progression. The prognosis is good for LN− ER-positive patients independently of any further molecular class and very severe for LN+. In this group, general survival after 20 years is very similar to HER2 positive nonluminal tumors after 5 years and to TN after 10 years.

Finally, together with Ki67 the use of PR and vimentin as prognostic markers could help to better stratify patients with ER-positive tumors with respect to clinical outcome.

Conflict of Interests

The authors declare no conflict of interests.
Acknowledgments

The authors would like to thank Dr. Valentina Melita for the language revision of the paper. The authors are also grateful to Dr. Maria Malagoli for providing the oncological therapy regimen. This study was partially supported by the MIUR PRIN 2008, Protocol no. 2008YFRLC8_002.

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