Cancer Chemother Pharmacol (2012) 69:533–546
DOI 10.1007/s00280-011-1730-9

ORIGINAL ARTICLE

Triple-negative phenotype is of adverse prognostic value in patients treated with dose-dense sequential adjuvant chemotherapy: a translational research analysis in the context of a Hellenic Cooperative Oncology Group (HeCOG) randomized phase III trial

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Received: 2 June 2011 / Accepted: 18 August 2011 / Published online: 8 September 2011

Abstract
Purpose It is well recognized that breast cancer is a heterogeneous disease. The purpose of the current study was to classify patients according to the immunohistochemical phenotype of their tumors in an effort to evaluate the outcome of the respective groups of patients and specifically of those with triple-negative breast cancer (TNBC) following dose-dense sequential adjuvant chemotherapy.

Methods A total of 595 patients with high-risk breast cancer were treated with adjuvant anthracycline-based dose-dense sequential chemotherapy with or without paclitaxel in the context of a randomized study. ER, PgR, HER2, Ki67, EGFR, and CK5 protein expression were evaluated in 298 formalin-fixed paraffin-embedded tumor samples by immunohistochemistry (IHC). HER2 was also evaluated by chromogen in situ hybridization (CISH). HER2 status and Ki67 protein expression differentiated luminal IHC subtypes (luminal B tumors being HER2 and/or Ki67-positive).
Results Among the 298 tumors, the immunohistochemical panel classified 37 (12%) as luminal A, 198 (66%) as luminal B, 27 (9%) as HER2 enriched, and 36 (12%) as TNBC. The median follow-up time was 97 months.

The first two authors P. Skarlos and C. Christodoulou have contributed equally to this work.

Electronic supplementary material The online version of this article (doi:10.1007/s00280-011-1730-9) contains supplementary material, which is available to authorized users.

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Patients with luminal A tumors had the best prognosis, with improved disease-free survival (log-rank, $P = 0.033$) and overall survival ($P = 0.006$) compared with the other three tumor subtypes. The three subtypes had an increased risk for relapse and death compared with luminal A in multivariate analysis, as well. No benefit from paclitaxel treatment was detected in any of the four subtypes or the total cohort. Hierarchical clustering based on mRNA expression of ER, PgR, and HER2 by quantitative RT-PCR identified patient groups that were comparable to the subtypes identified by IHC.

Conclusions The results of this study confirm that triple negative, luminal B and HER2-enriched phenotypes identified by IHC are of adverse prognostic value in high-risk breast cancer patients treated with dose-dense sequential adjuvant chemotherapy.

Keywords Immunophenotypic subtypes · mRNA subtypes · mRNA expression · Triple-negative breast cancer · HER2 enriched subtype · Prognostic value

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Introduction

Over the past 20–30 years, adjuvant chemotherapy improved disease-free survival (DFS) and overall survival (OS) in early breast cancer. There has been a 15–25% improvement in outcomes [12]. CMF offered a benefit of 4% [2], anthracyclines an additional 5% [21], while the addition of taxanes further improved DFS and OS [20]. Finally, Intergroup Trials 0148 [17] and C9741 [7] highlighted the importance of the use of dose-dense and sequential chemotherapy in the adjuvant setting of breast cancer.

It is well recognized that breast cancer is a heterogeneous disease that is composed of several molecular subtypes with different characteristics. New technologies have greatly contributed to the understanding of the heterogeneity of breast cancer and provided new classifications (luminal A, luminal B, HER2 enriched, triple negative, basal-like). These classifications represent molecular subtypes with substantial differences in growth rates, invasiveness, and treatment response (reviewed in ref [9]). Estrogen receptors (ER), progesterone receptors (PgR), and HER2 are important determinants of these molecular subtypes because they drive specific targeted treatments, such as antiestrogens or aromatase inhibitors, trastuzumab, and lapatinib. On the other hand, for patients with triple-negative (TN) breast cancer, which is characterized by the lack of expression of both hormone receptors and HER2, chemotherapy is the only therapeutic option.

It is well known that adjuvant hormonal therapy confers survival benefit in hormone receptor positive breast cancer patients. It is also well known that administration of trastuzumab in the adjuvant setting in combination with chemotherapy results in an improvement of DFS and survival in HER2-positive breast cancer patients. But it is not known whether all molecular subtypes derive the same benefit from adjuvant chemotherapy and in particular from dose-dense sequential chemotherapy.

Dose-dense sequential chemotherapeutic regimens are considered to be highly effective and well tolerated for the adjuvant treatment of patients with “intermediate or high-risk” operable breast cancer [15]. Our Group has conducted a randomized trial (HE10/97) in such patients, comparing dose-dense sequential chemotherapy with epirubicin and CMF with or without paclitaxel [14].

In the current study, we classified patients participating in the above-mentioned randomized study with available tumor tissue blocks according to the immunohistochemical phenotype of their tumors in an effort to evaluate the outcome of the respective groups of patients and specifically of those with TN breast cancer. Our focus on the latter group of patients is due to the relative lack of information about their response to dose-dense sequential chemotherapy.

Materials and methods

The clinical study

In 1997, the Hellenic Cooperative Oncology Group (HeCOG) designed and began a randomized phase III trial (HE10/97) in patients with operated, metastasis-free, high-risk node-negative breast cancer, or patients with at least one infiltrated axillary node, comparing dose-dense sequential chemotherapy with epirubicin, followed by CMF, with or without paclitaxel. High-risk disease was defined by at least one of the following: tumor size ≥2 cm, ER/PgR negativity, grade III and lympovascular invasion. The trial was included in the Australian New Zealand Clinical Trials Registry (ANZCTR) and allocated the following Registration Number: ACTRN12611000506998. The primary endpoint of the study was DFS and secondary endpoints were OS, acute toxicity, and quality of life.

Patients were stratified by menopausal status, ER/PgR status, and number of infiltrated axillary nodes and were randomized to receive either three cycles of epirubicin 110 mg/m² every 2 weeks, followed by three cycles of paclitaxel 250 mg/m² every 2 weeks, followed by three cycles of intensified CMF (cyclophosphamide 840 mg/m², methotrexate 57 mg/m², fluorouracil 840 mg/m², experimental arm) or four cycles of epirubicin followed by four cycles of CMF, at the same dose intensity and intervals as in the experimental group (control arm). G-CSF was given prophylactically on days 3–10 of each cycle in both arms. Radiation therapy (RT) was administered to patients with partial mastectomy or patients with ≥4 infiltrated axillary nodes and/or tumor size ≥5 cm, irrespectively of the type of mastectomy. Finally, all patients with hormone receptor positive tumors received oral tamoxifen 20 mg daily for 5 years after the completion of RT. Additionally, LH-RH analogs were given to all premenopausal patients for 2 years. None of the HER2-positive patients received trastuzumab, since it was not approved for treatment in the adjuvant setting at the time of the study. Totally, 595 eligible patients were randomized in the study. Results of the HE10/97 study were previously described [14].

Each patient before randomization signed an informed consent to participate in the study and optionally provide biological material for future research studies. The clinical protocol was approved by appropriate local Institutional Review Boards. The present translational protocol was approved by the Bioethics Committee of the Aristotle University of Thessaloniki School of Medicine.

Molecular and immunohistochemical studies

This was a retrospective translational research study among 595 patients who had been enrolled in a prospective clinical trial. Accordingly, collection of formalin-fixed paraffin-embedded (FFPE) primary tumor tissue samples was possible in 317 patients only, due to logistical/organizational barriers. Gene expression analysis by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was successfully performed in 314 primary tumor samples, while tumor classification by immunohistochemistry (IHC) was available for 298 samples. Data collected for this retrospective experimental study included selected patient and tumor characteristics, treatment given, as well as relapse, survival, and follow-up data.

Tissue microarray (TMA) construction

TMA blocks were constructed (by M.B. and D.T.) as previously described [6, 19, 32]. Each case was represented by 2 tissue cores, 1.5 mm in diameter, obtained from the most representative tumor areas of primary invasive breast carcinoma cases and re-embedded in 17 microarray blocks with the use of a manual arrayer (Model I, Beecher Instruments, San Prairie, WI). Each TMA block contained 38–66 tissue cores from the original tumor tissue blocks, while cores from various neoplastic, non-neoplastic, and reactive tissues were also included, serving as assays controls.

Immunohistochemistry (IHC)

Immunohistochemical staining was performed according to standard protocols with slight modifications, on serial 2.5-μm-thick sections from the TMA blocks using the Bond-Max™ (Leica Microsystems, Wetzlar, Germany and Menarini Diagnostics, Athens, Greece) and i6000 (Biogenex, San Ramon, CA) autostainers. To assure optimal immunoreactivity, the sections of the TMA blocks were stained in one run for each antibody, shortly after mounting of the TMA sections on positively charged glass slides (within 3–10 days).

The deparaffinization was performed by incubation at 60°C for 1 h and subsequent immersion in Ottix Plus (Diapath, Martinengo, Italy) for 2 × 8 min and rehydration in Ottix Shaper (Diapath) for 2 × 5 min. The antigen–antibody complex was visualized using the Bond Polymer Refine and Bond Polymer Refine Red Detection systems (Leica Biosystems, Newcastle, UK); DAB and Fast Red were used as chromogens and Mayer's hematoxylin as a counter-stain. Double IHC was performed for selected antibodies (CK5/p53, CK14/Ki67, CK17/vimentin).

ER, PgR, HER2, Ki67, EGFR, CK5, CK14, CK17, and vimentin protein expressions were evaluated according to established or proposed criteria [4, 10, 16, 30, 35]. The authors responsible for the evaluation of the immunostains were: A.B. for ER and PgR; M.B. for HER2, Ki67, and EGFR; and I.K. for CK5, CK14, CK17, and vimentin. The

Table 1 Proteins, source and dilution of antibodies, staining procedures and patterns, and interpretation analysis

Protein	Antibody clone (source)	Antibody dilution	Pretreatment (t/Epitope retrieval/T/I)	Incubation time	IHC staining detection system/ Chromogen	Scoring system	Cutoff (%)	Staining pattern	Ref.
Intermediate filaments									
CK5	XM26 (1)	1:50	20′/ER2/98°C/HP	20′	Polymer AP + Fast Red	SQ	Any+	C	[11]
CK14	LL002 (1)	1:50	15′/ER2/98°C/HP	20′	Polymer AP + Fast Red	SQ	Any+	C	[11]
CK17	E31 (1)	1:50	15′/ER1/98°C/HP	20′	Polymer AP + Fast Red	SQ	Any+	C	[11]
Vimentin	V9 (2)	1:500	15′/ER1/98°C/HP	20′	Polymer AP + Fast Red	SQ	Any+	C	[11]
Cell proliferation molecule									
Ki67	MIB1 (2)	1:70	15′/ER2/98°C/HP	20′	Polymer HRP + DAB	SQ	14%[a]	N	[4]
Cell receptors									
EGFR	31G7 (3)	1:50	8′/ENZ/37°C/HP	20′	Polymer HRP + DAB	0–3+[b]	1%	M	[30]
HER2	PL (2)	1:200	25′/ER1/98°C/HP	30′	Polymer HRP + DAB	0–3+[c]	30%	M	[35]
Hormonal receptors									
ER	6F11 (1)	1:70	20′/ER1/98°C/HP	20′	Polymer HRP + DAB	H-Score	1%	N	[16]
PgR	1A6 (1)	1:70	20′/ER1/98°C/HP	20′	Polymer HRP + DAB	H-Score	1%	N	[16]

Double IHC was performed for the following antibodies: CK5/p53, CK14/Ki67, CK17/Vimentin. 1: Leica BioSystems, Newcastle, UK; 2: Dako, Glostrup, Denmark; 3: Invitrogen, Carlsbad, CA

AP alkaline phosphatase, *C* cytoplasmic, *DAB* 3,3′-diaminobenzidine, *ENZ* proteolytic enzyme, *ER1* citric acid—pH 6.0, *ER2* ethylenediamine-tetraacetate—pH 8.8, *HP* hot plate at Bond™ autostainer, *HRP* horseradish peroxidase, *I* instrument, *M* membranous, *N* nuclear, *SQ* semi-quantitative, *T* temperature, *t* time

[a] Proliferation Index was evaluated as Low if <14% and High if ≥14%

[b] Scores 1+, 2+ and 3+ were considered to be positive

[c] Score 3+ in >30% of tumor cells was considered to be positive

immunohistochemical target proteins, source and dilution of antibodies used, staining procedures and patterns, and evaluation criteria and cutoffs for each target protein are presented in Table 1. Negative controls were included by omitting the primary antibody. For protein expression of Ki67, 14% was used as cutoff to categorize low (<14%) and high (≥14%) protein status, according to Cheang et al. [4]. Tissue sections stained for ER/PgR were considered as positive when ≥1% of neoplastic cells displayed nuclear immunoreactivity [16]. Histological grade was evaluated according to the Scarff, Bloom and Richardson system.

Chromogen in situ hybridization (CISH)

CISH was performed in all TMAs applying CISH technology protocols from ZYMED™ (Invitrogen, Karlsbad, CA) for the HER2 gene and the centromeric region of chromosome 17. The method was performed as described by the manufacturer with slight modifications, and the sections were evaluated by C.V., O.J., and P.A. Each case was classified according to the number of gene hybridization signals, in more than 50% of cancer cells of the infiltrative tumor component, in one of five CISH ranks; rank 1, monosomy (one signal per nucleus); rank 2, diploid (two gene signals per nucleus); rank 3, low gene gain (3–5 gene copies per nucleus); rank 4, low gene amplification (6–10 signals per nucleus or small clusters); and rank 5, high gene amplification (>10 signals per nucleus or large clusters). CISH was considered to be positive when cases were classified in ranks 4 and 5 [34, 35].

Immunophenotypic classification

For the immunohistochemical classification of tumors, HER2 status determined by IHC and CISH, and protein expression of ER, PgR, and Ki67 were used. More specifically, tumors with positive ER and/or PgR protein expression, negative HER2 status and negative Ki67 protein expression were classified as luminal A, tumors with positive of ER and/or PgR protein expression and either positive HER2 status or positive Ki67 protein expression were classified as luminal B, tumors with negative ER and PgR protein expression and positive HER2 status were classified in the HER2-enriched group, and finally, tumors with negative ER and PgR receptors and negative HER2 status were classified as triple-negative breast cancer (TNBC).

Luminal A and B subtypes were originally identified by gene arrays; however, the terms [luminal A] and [luminal B] will be herein used for the IHC classification of tumors throughout the manuscript, tables, and figures. Tumors with positive ER and/or PgR protein expression and positive HER2 status were classified in the luminal B group, since

they represented a small percentage to stand alone as a group in the analysis and were thought to be similar to the high-proliferative Ki67-positive tumors [4]. Among TNBC tumors, those expressing either CK5 or EGFR were classified in the basal core phenotype (BCP) group [5].

RNA isolation from FFPE tissue samples and qRT-PCR assessment

Hematoxylin–eosin sections from all available FFPE tissue specimens were evaluated histologically by a certified pathologist (M.B.) and recorded for the percentage of tumor cell content. Prior to RNA isolation, macrodissection of tumor areas was performed in most of the sections with <50% tumor cell content.

Sufficient RNA was isolated from 314 FFPE specimens (R.K. and R.M.W.) followed by qRT-PCR, as previously described [27]. From each FFPE section or macrodissected tissue fragments (10 µm thick), RNA was isolated using a fully automated isolation method for total RNA from FFPE tissue, based on silica-coated magnetic beads (VERSANT Tissue Preparation Reagents, Siemens Healthcare Diagnostics, Tarrytown, NY) in combination with a liquid handling robot, as previously described in detail [1]. The method involves DNase I treatment for eliminating DNA contamination in the sample. The tumor cell content of the FFPE sections used for the RNA extraction was >30%, in practically all (97%) of the samples, and >50% in the majority (76%) of the samples.

One-step qRT-PCR was applied (R.K. and R.M.W.) for the assessment of ESR1 (estrogen receptor 1), PgR, and HER2 mRNA expression by using gene-specific TaqMan® based assays. Forty cycles of nucleic acid amplification were applied and the cycle threshold (CT) values of the target genes were identified. CT values were normalized by subtracting the CT value of the housekeeping gene RPL37A from the CT value of the target genes (ΔCT). RNA results were then reported as 40-ΔCT values, which correlate proportionally to the mRNA expression level of the target genes. Samples with average RPL37A CT values <32 were considered eligible for analysis.

Expression of the target genes, as well as the reference gene RPL37A, was assessed in triplicate by qRT-PCR using the SuperScript III PLATINUM One-Step Quantitative RT-PCR System with ROX (Invitrogen, Karlsruhe, Germany) in an ABI PRISM 7900HT (Applied Biosystems, Darmstadt, Germany) [24]. The quality and quantity of RNA was checked by measuring RPL37A expression as a surrogate for amplifiable mRNA by qRT-PCR. The lengths of the amplicons detected by the estrogen receptor 1 (ESR1), PgR, HER2, and RPL37A assays were 73 bp, 95 bp, 87 bp, and 65 bp, respectively, with PCR efficiencies [$E = 1^{(10\text{-slope})}$] of 95.9, 87.4, 95.7, and 86.0%, respectively.

The Primer/Probe (FAM/TAMRA-labeled) sets used for amplification of the target and reference genes were the following (5′ → 3′):

ESR1 Probe ATGCCCTTTTGCCGATGCA

Forward Primer GCCAAATTGTGTTTGATGGATTAA
Reverse Primer GACAAAACCGAGTCACATCAGT AATAG

PgR Probe TTGATAGAAACGCTGTGAGCTCGA

Forward Primer AGCTCATCAAGGCAATTGGTTT
Reverse Primer ACAAGATCATGCAAGTTATCAA GAAGTT

HER2 Probe ACCAGGACCCACCAGAGCGGG

Forward Primer CCAGCCTTCGACAACCTCTATT
Reverse Primer TGCCGTAGGTGTCCCTTTG

RPL37A Probe TGGCTGGCGGTGCCTGGA

Forward Primer TGTGGTTCCTGCATGAAGACA
Reverse Primer GTGACAGCGGAAGTGGTATTGT AC

Human reference total RNA pooled from ten human cell lines (Stratagene, La Jolla, California, USA) was used as a positive control. No-template controls were assessed in parallel to exclude contamination.

Statistical methodology

For immunohistochemical classification of tumors, HER2 status determined by IHC and CISH and protein expression of ER, PgR, and Ki67 were used. Unsupervised hierarchical clustering analysis using ER, PgR, and HER2 mRNA expression was conducted in order to identify the subgroup of "triple-negative" tumors. Differences in the clinicopathological features between patients assigned to the four breast cancer subtypes were examined using the χ^2 test for categorical data and the Kruskall–Wallis test for continuous data. Statistical measures for the performance of mRNA classification relative to immunophenotypic classification were also presented.

DFS was measured from the date of randomization until recurrence of the tumor, or secondary neoplasm, or death from any cause [18]. OS was measured from the date of randomization until death from any cause. Surviving patients were censored at the date of last contact. Time-to-event distributions were estimated using Kaplan–Meier curves and compared using log-rank tests. Multivariate Cox regression analysis was performed using a backward selection procedure with $P > 0.10$ as a removal criterion based on the likelihood ratio test, in order to identify significant clinicopathological variables among the following: age (≤34 vs. 35–50 vs. >50), treatment group (E-CMF vs. E-T-

CMF), menopausal status (post vs. pre), tumor grade (III-undifferentiated vs. I–II), tumor size (≤2 cm vs. 2–5 cm vs. >5 cm), number of positive axillary nodes (≥4 vs. 0–3), histological type (other vs. mixed vs. invasive lobular vs. invasive ductal), and immunohistochemical or mRNA tumor classification.

Results of this study are presented according to reporting recommendations for tumor marker prognostic studies [23]. This design of the study is prospective–retrospective as described in Simon et al. [31]. All statistical tests are two sided, and $P < 0.05$ was considered statistically significant. The statistical analysis was conducted using SPSS (SPSS for Windows, version 15.0, SPSS Inc.), JMP version 8 (SAS, Institute Inc., Cary, NC), and the freeware statistical package R version 2.10.0.

Results

Patients and immunohistochemically defined subtypes

Selected patient and tumor characteristics for the 298 patients according to chemotherapeutic regimen delivered are depicted in Table 1S (*supplementary material, online only*). The two arms of treatment were well balanced in basic clinicopathological parameters except for tumor grade ($P = 0.002$) and HER2 immunostaining ($P = 0.035$). The imbalance in grade was also reported in the 595 patients presented in the clinical paper [14]. Furthermore, there were no significant differences in patient and tumor characteristics and DFS and OS between the group of patients with available tumor tissue blocks ($N = 298$) and those with no block available ($N = 297$), except for the number of examined nodes (mean 19 vs. 17, respectively, $P = 0.003$) and the number of positive nodes (mean 7 vs. 5, respectively, $P = 0.048$), indicating that the cohort evaluated in the present study is representative of the total cohort.

Half of the women were postmenopausal (49%), most with more than four positive nodes (65%). The majority of tumors were ductal (71%) of stage II or III (95%). Positive ER status was observed in 74% of the patients, while 23% of the tumors were HER2-positive (IHC 3+ and/or CISH amplified). There were 9 cases with discordant HER2 results (two IHC 3+ cases that were not found to be CISH amplified and 7 IHC 0 or 1+ cases that were CISH amplified). These nine patients were considered to be HER2-positive, according to our definition (IHC 3+ and/or CISH amplified). Among the 298 tumors, the immunohistochemical panel classified 37 (12%) as luminal A, 198 (66%) as luminal B, 27 (9%) as HER2 enriched, and 36 (12%) as TNBC. In the latter group, there were 25 tumors (69%) with BCP, expressing either CK5 or EGFR, while 26 of 36 cases (72%) expressed at least one basal marker among EGFR, CK5, CK14, CK17, and vimentin. Patient and tumor characteristics according to breast cancer subtypes are shown in Table 2. There were no significant differences in selected clinicopathological characteristics such as age, number of positive or removed nodes, menopausal status, tumor size, and histological classification between the four subtypes ($P > 0.05$ in all cases), except for tumor grade (Fisher's exact test, $P = 0.048$). Table 2 also includes the distribution, across the four subtypes, of protein expression of all markers evaluated by IHC, as well as HER2 gene amplification evaluated by CISH. Representative IHC stains are shown in Fig. 1.

Prognosis of immunohistochemically defined subtypes

After a median follow-up time of 97 months (range 94.8–99.8), 104 patients (35%) relapsed and 78 (26%) died. The vast majority of disease relapses were distant (90%), while only a 10% of the patients developed locoregional relapse. The 5-year DFS rate was 70% (95% confidence interval [CI]: 64.9–75.5) and the 5-year survival rate was 84% (95% CI: 80.0–88.2). No benefit of paclitaxel treatment was seen in the total cohort of the 298 evaluated patients in terms of DFS or OS (Wald's $P > 0.05$ for both).

The 5-year DFS and OS rates according to tumor subtype are given in detail in Table 3. As expected, patients with luminal A tumors had the best prognosis (Fig. 2) in terms of DFS (log-rank, $P = 0.033$) and OS (log-rank, $P = 0.006$). No significant differences in DFS or OS were observed between patients in the other three tumor subtypes. There was no interaction of immunohistochemical subtypes with paclitaxel treatment for either DFS or OS (Wald's $P > 0.05$ for both).

Hierarchical clustering analysis using mRNA expression

Unsupervised hierarchical clustering analysis was performed in 314 tumors based on mRNA expression of ESR1, PgR, and HER2 assessed by qRT-PCR. As shown in the dendogramm (Fig. 3), five groups were identified that had similarities but also significant differences compared with the subtypes identified by IHC. The first three groups were "luminal" tumors, since at least one of the hormonal receptors (ESR1 or PgR) was highly expressed. The red group was characterized as ESR1-high and PgR/HER2-intermediate (n = 81, "luminal" tumors), the petrol group was ESR1-high, PgR-intermediate and HER2-high (n = 31, "luminal HER2-positive" tumors), and the blue group was ESR1/PgR-high and HER2-intermediate (n = 129, "luminal" tumors). For the immunohistochemical classification of tumors, Ki67 protein expression was used for the distinction between luminal A and B tumors. However, MKI67 mRNA

Table 2 Selected patient and tumor characteristics ($N = 298$), according to breast cancer subtypes identified by IHC

	Luminal A ($N = 37$)	Luminal B ($N = 198$)	HER2 enriched ($N = 27$)	TNBC ($N = 36$)	BCP[a] ($N = 25$)
Age (years)					
Median (range)	53 (34–76)	50 (22–78)	54 (24–72)	52 (31–70)	53 (31–66)
N of nodes examined					
Median (range)	18 (4–42)	20 (5–57)	23 (10–56)	17 (4–59)	19 (5–59)
N of positive nodes					
Median (range)	7 (1–17)	6 (0–43)	10 (1–35)	5 (0–54)	6 (0–54)
	N (%)	*N* (%)	*N* (%)	*N* (%)	*N* (%)
Age					
≤34	1 (2.7)	12 (6.1)	1 (3.7)	1 (2.8)	1 (4.0)
35–50	16 (43.2)	83 (41.9)	10 (37.0)	15 (41.7)	9 (36.0)
>50	20 (54.1)	99 (50.0)	16 (59.3)	19 (52.8)	14 (56.0)
Missing data	–	4 (2.0)	–	1 (2.8)	1 (4.0)
Menopausal status					
Premenopausal	18 (48.6)	107 (54.0)	12 (44.4)	16 (44.4)	10 (40.0)
Postmenopausal	19 (51.4)	91 (46.0)	15 (55.6)	20 (55.6)	15 (60.0)
Tumor size (cm)					
≤2	13 (35.1)	70 (35.4)	7 (25.9)	10 (27.8)	7 (28.0)
2–5	16 (43.2)	97 (49.0)	14 (51.9)	19 (52.8)	14 (56.0)
>5	8 (21.6)	31 (15.7)	6 (22.2)	7 (19.4)	4 (16.0)
N of positive nodes					
0	–	3 (1.5)	–	1 (2.8)	1 (4.0)
1–3	7 (18.9)	41 (20.7)	5 (18.5)	11 (30.6)	6 (24.0)
4–9	15 (40.5)	96 (48.5)	8 (29.6)	17 (47.2)	13 (52.0)
>9	15 (40.5)	58 (29.3)	14 (51.9)	7 (19.4)	5 (20.0)
Histology					
Invasive ductal	22 (59.5)	140 (70.7)	21 (77.8)	27 (75.0)	19 (76.0)
Invasive lobular	8 (21.6)	22 (11.1)	2 (7.4)	5 (13.9)	3 (12.0)
Mixed	6 (16.2)	23 (11.6)	2 (7.4)	–	–
Other	–	10 (5.1)	2 (7.4)	3 (8.3)	2 (8.0)
Missing data	1 (2.7)	3 (1.5)	–	1 (2.8)	1 (4.0)
Grade[b]					
I	3 (8.1)	8 (4.0)	1 (3.7)	1 (2.8)	1 (4.0)
II	22 (59.5)	97 (49.0)	7 (25.9)	11 (30.6)	5 (20.0)
III	12 (32.4)	91 (46.0)	19 (70.4)	23 (63.9)	18 (72.0)
Undifferentiated	–	2 (1.0)	–	–	–
Missing data	–	–	–	1 (2.8)	1 (4.0)
Ki67					
<14	37 (100)	3 (1.5)	2 (7.4)	8 (22.2)	3 (12.0)
≥14	–	195 (98.5)	24 (88.9)	27 (75.0)	21 (84.0)
Missing data	–	–	1 (3.7)	1 (2.8)	1 (4.0)
ER					
Negative	2 (5.4)	13 (6.6)	27 (100)	36 (100)	25 (100)
Positive	35 (94.6)	184 (92.9)	–	–	–
Missing data	–	1 (0.5)	–	–	–

Table 2 continued

	N (%)	*N* (%)	*N* (%)	*N* (%)	*N* (%)
PgR					
Negative	6 (16.2)	33 (16.7)	27 (100)	36 (100)	25 (100)
Positive	31 (83.8)	164 (82.8)	–	–	–
Missing data	–	1 (0.5)	–	–	–
HER2 score (IHC)					
0–1+	33 (89.2)	138 (69.7)	3 (11.1)	32 (88.9)	22 (88.0)
2+	3 (8.1)	23 (11.6)	3 (11.1)	4 (11.1)	3 (12.0)
3+	–	31 (15.7)	21 (77.8)	–	–
Missing data	1 (2.7)	6 (3.0)	–	–	–
HER2 (CISH)					
Non-amplified	35 (94.6)	148 (74.7)	2 (7.4)	33 (91.7)	23 (92.0)
Amplified	–	39 (19.7)	25 (92.6)	–	–
Missing data	2 (5.4)	11 (5.6)	–	3 (8.3)	2 (8.0)
HER2 status					
Negative	37 (100)	156 (78.8)	–	36 (100)	25 (100)
Positive	–	40 (20.2)	27 (100)	–	–
Missing data	–	2 (1.0)	–	–	–
CK5					
Negative	34 (91.9)	176 (88.9)	23 (85.2)	18 (50.0)	7 (28.0)
Positive	2 (5.4)	18 (9.1)	4 (14.8)	18 (50.0)	18 (72.0)
Missing data	1 (2.7)	4 (2.0)	–	–	–
CK14					
Negative	36 (97.3)	190 (96.0)	25 (92.6)	33 (91.7)	22 (88.0)
Positive	1 (2.7)	7 (3.5)	2 (7.4)	3 (8.3)	3 (12.0)
Missing data	–	1 (0.5)	–	–	–
CK17					
Negative	37 (100)	192 (97.0)	27 (100)	30 (83.3)	19 (76.0)
Positive	–	4 (2.0)	–	5 (13.9)	5 (20.0)
Missing data	–	2 (1.0)	–	1 (2.8)	1 (4.0)
EGFR					
Negative	34 (91.9)	175 (88.4)	12 (44.4)	17 (47.2)	6 (24.0)
Positive	3 (8.1)	22 (11.1)	15 (55.6)	19 (52.8)	19 (76.0)
Missing data	–	1 (0.5)	–	–	–
Vimentin					
Negative	35 (94.6)	168 (84.8)	22 (81.5)	22 (61.1)	13 (52.0)
Positive	2 (5.4)	27 (13.6)	5 (18.5)	14 (38.9)	12 (48.0)
Missing data	–	3 (1.5)	–	–	–

BCP basal core phenotype, *N* number, *TNBC* triple-negative breast cancer

[a] Defined by triple negativity, CK5 and/or EGFR positivity

[b] $P = 0.048$ (Fisher's exact test)

expression data were not available for the hierarchical clustering analysis; therefore, the two "luminal" groups identified by RT-PCR were not in anyway comparable to the luminal A and B tumors identified by the IHC data. Furthermore, the "luminal HER2-positive" tumors, identified as a separate group here, were placed in the luminal B group in the immunohistochemical classification of tumors, since such tumors represented a small percentage to stand alone as a group in the analysis and were thought to be similar to the high-proliferative Ki67-positive tumors [4]. The last two groups shown on the right of the hierarchical clustering dendogram (Fig. 3) were very comparable to their respective subtypes identified by IHC. The green group was ESR1/PgR/HER2-low (n = 40, "triple-negative" tumors), and the orange group was ESR1/PgR-low and HER2-high (n = 33, "HER2 enriched" tumors).

In the examined population with complete IHC data ($N = 298$), there were 37 cases with missing mRNA data, and therefore, concordance of the mRNA classification with the immunohistochemical classification was evaluated in 261 tumors (Table 2S, *supplementary material, online only*). Of the 28 true triple-negative tumors defined by IHC,

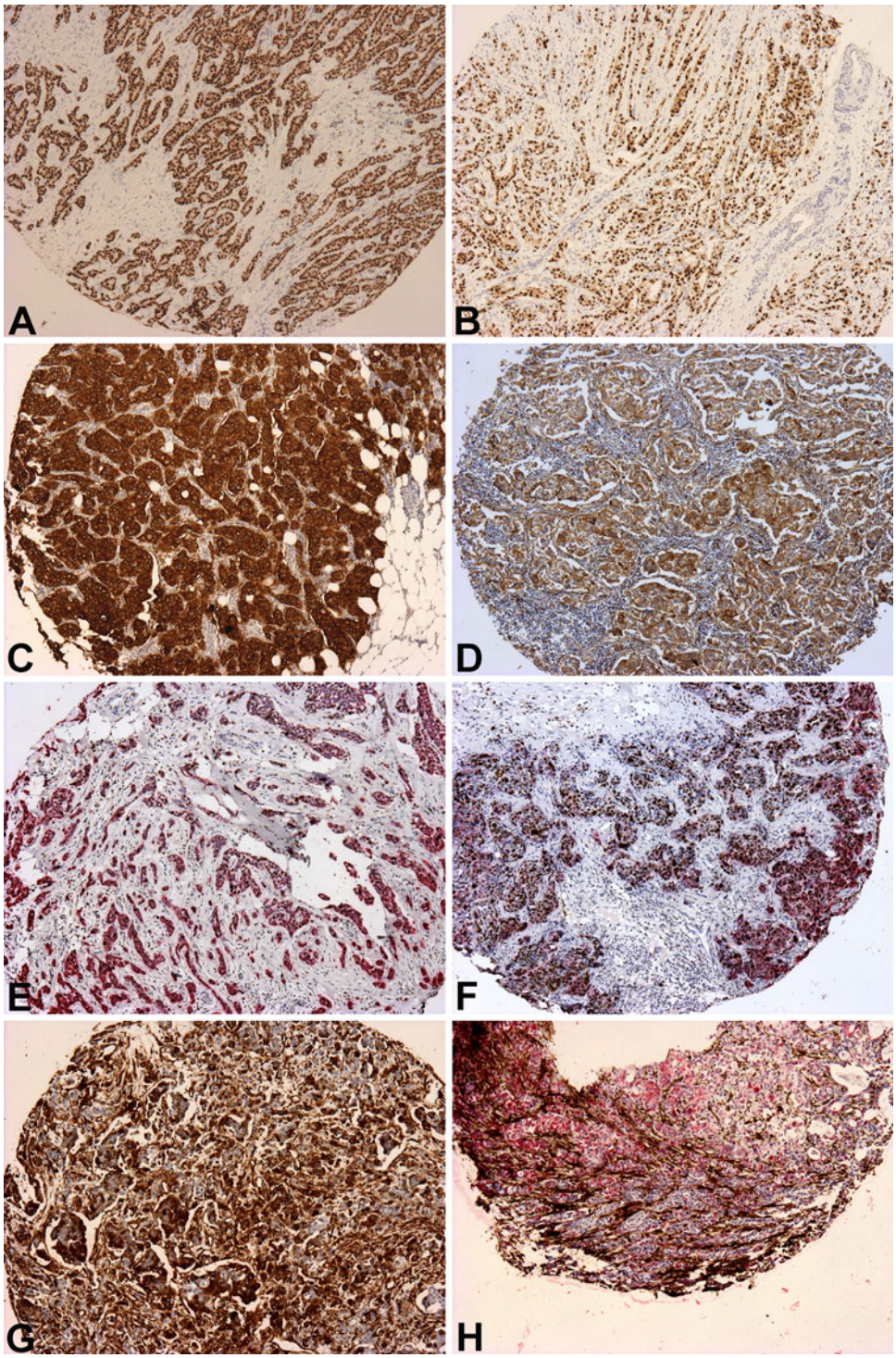

Fig. 1 Representative TMA immunostains. **a** ER-positive staining (DAB was used as a chromogen); **b** PgR positive staining (DAB), **c** HER2 positive 3+ staining (DAB); **d** EGFR positive staining (DAB); **e** positive double immunostaining for CK5 (*Fast Red* was used as a chromogen) and p53 (DAB); **f** positive double immunostaining for CK14 (*Fast Red*) and Ki67 (DAB); **g** double immunostaining negative for CK17 (*Fast Red*) and positive for vimentin (DAB); and **h** double immunostaining positive for CK17 (Fast Red) and negative for vimentin (DAB). Magnification ×40

21 cases were also identified to be "triple negative" by the mRNA classification (75%). Concerning the rest of the clusters, 20 of the 26 cases (77%) classified in the orange group as "HER2 enriched" were truly HER2 enriched by IHC, while the majority (96%) of the 178 cases classified in the red and blue "luminal" groups were truly luminal by IHC. Totally, only 21 cases (8%) were misclassified by the mRNA hierarchical clustering method using mRNA expression. The sensitivity of the qRT-PCR method was 75% with a specificity of 94%.

Multivariate analysis

In the multivariate Cox regression analysis (Table 4) immunohistochemical classification of tumor subtypes was prognostic for both DFS (Wald's $P = 0.016$) and OS (Wald's

Table 3 Survival status according to breast cancer subtypes identified by IHC

	Luminal A (*N* = 37)	Luminal B (*N* = 198)	HER2 enriched (*N* = 27)	TNBC (*N* = 36)	BCP[a] (*N* = 25)
Disease-free survival					
Relapses *N* (%)	6 (16.2)	70 (35.4)	11 (40.7)	17 (47.2)	12 (48.0)
Events *N* (%)	7 (16.2)	74 (37.4)	11 (40.7)	17 (47.2)	12 (48.0)
5-year rate (%)	94.4	70.0	58.8	55.6	56.0
Range (months)	36.6–121.5	4.0–109.7	6.9–45.7	10.3–84.0	13.6–84.0
Median (95% CI)	NE	NE	NE	NE	NE
Overall survival					
Deaths *N* (%)	2 (5.4)	53 (26.8)	9 (33.3)	14 (38.9)	10 (40.0)
5-year rate (%)	97.2	86.3	69.6	69.4	68.0
Range (months)	49.6–108.5	12.1–110.8	13.0–98.3	13.6–88.3	13.6–88.3
Median (95% CI)	121.5	NE	NE	NE	NE
Site of relapse					
Locoregional *N* (%)	–	7 (3.5)	1 (3.7)	2 (5.6)	–
Distant	5 (13.5)	62 (31.3)	11 (40.7)	16 (44.4)	12 (48.0)
Locoregional and distant	–	7 (3.5)	1 (3.7)	2 (5.6)	–
Median time to death following relapse (in months)	32.8	28.0	29.8	21.3	15.8

BCP basal core phenotype, *N* number, *NE* not estimated yet, *TNBC* triple-negative breast cancer

[a] Defined by triple negativity, CK5 and/or EGFR positivity

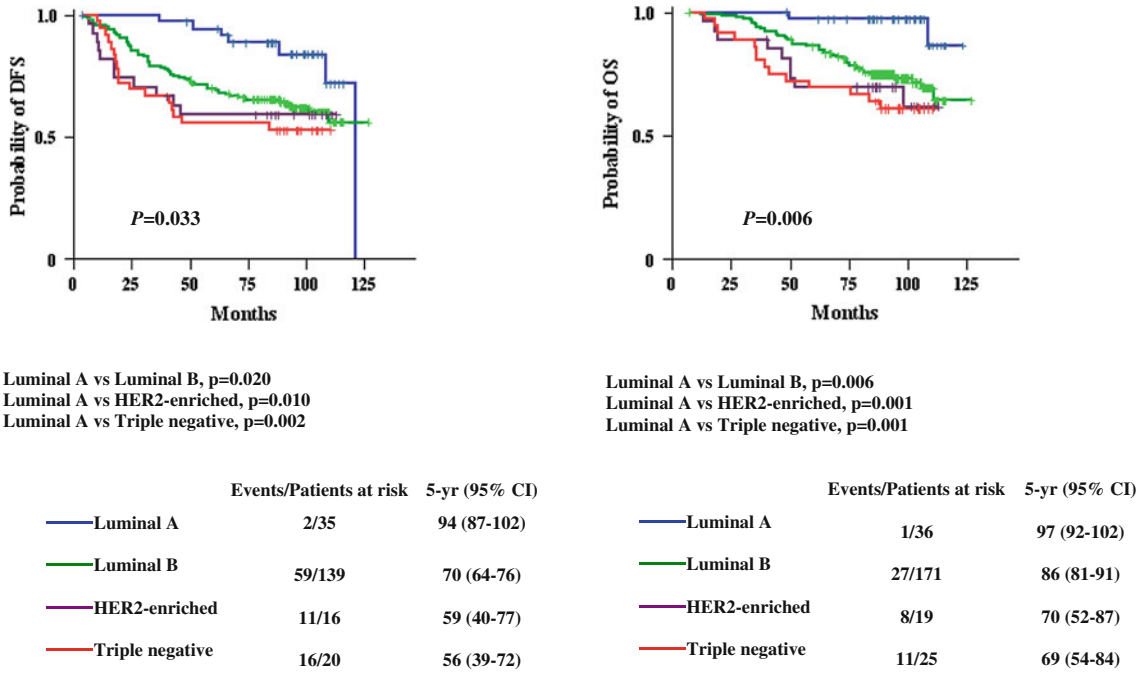

Fig. 2 Disease-free survival and overall survival of patients according to breast cancer subtype; Luminal A (*blue line*), Luminal B (*green line*), HER2 enriched (*violet line*), Triple negative (*red line*)

$P = 0.032$). More specifically, luminal B, HER2 enriched and TNBC tumors were associated with significantly increased risk of relapse and death compared with luminal A tumors (for hazard ratios and *P* values see Table 4). Moreover, tumor size was of adverse prognostic significance for survival (Wald's $P = 0.042$), while four or more positive nodes were associated with increased risk of relapse (Wald's $P < 0.001$) and death (Wald's $P = 0.022$). When we examined the prognostic significance of mRNA classification of tumor subtypes in the concept of the multi-

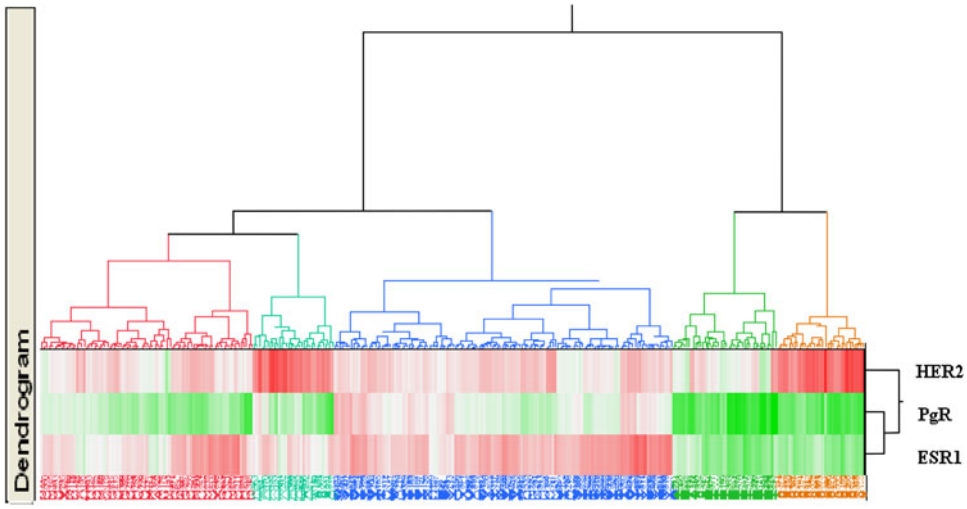

Fig. 3 Hierarchical clustering analysis based on ESR1, PgR and HER2 mRNA expression levels, revealing five main clusters. In the colored map, gene expression levels ranged from low (*green*), to moderate (*white*), to high (*red*)

variate analysis, no association was observed with either DFS or OS.

Discussion

We conducted a phase III study in high-risk patients with operated breast cancer to evaluate the role of paclitaxel when integrated in dose-dense sequential chemotherapy [14]. In the context of this study, we examined the outcome of patients according to their immunophenotypic classification. In order to determine the immunophenotypic classification of the tumors, we used antibodies against ER, PgR, HER2, and Ki67 proteins. HER2 status was determined both by IHC and CISH, whereas the BCP group was identified using basal cell cytokeratins, vimentin, and EGFR.

Patients with luminal A tumors had the best prognosis with a 5-year OS rate of 97.2% and a 5-year DFS rate of 94.4%. No significant differences in DFS or OS were observed between patients in other tumor subtypes. Patients with luminal B tumors had a better 5-year OS and DFS rate (86.3 and 70%, respectively), as compared to HER2 enriched (69.6 and 58.8%, respectively), TNBC (69.4 and 55.6%, respectively) and BCP (68 and 56%, respectively). However, this difference was not statistically significant due to small number of patients. Moreover, the luminal B subtype included 19.7% of the patients with HER2-amplified tumors as assessed with CISH. Patients with luminal B/HER2-positive tumors, as well as those with HER2-enriched tumors did not receive adjuvant trastuzumab, which was not available when the study was conducted. Finally, it is of note that the addition of paclitaxel to the dose-dense adjuvant E-CMF regimen did not improve outcome in any immunohistochemical subtype.

Limited information is available regarding the outcome of various molecular subtypes among patients with breast cancer receiving adjuvant chemotherapy and particularly dose-dense sequential chemotherapy. On the contrary, there is information regarding outcome following adjuvant hormonal therapy and neoadjuvant chemotherapy.

There are a few studies evaluating the response of the molecular subtypes to neoadjuvant chemotherapy, as determined by gene expression [26, 29], as well as using IHC [3]. These studies highlight the higher chemo-sensitivity of basal-like and HER2-enriched subtypes and the chemo-resistance of the luminal subtypes. However, despite the lower rates of pathological complete response (pCR) to neoadjuvant chemotherapy, disease-free survival was better for patients with ER-positive tumors due to higher rates of relapse in triple-negative and HER2-positive/ER-negative patients with residual disease. This hypothesis was further tested and confirmed by Liedtke et al. [22].

There is a different outcome of patients with luminal A breast cancer as compared to luminal B, when treated with adjuvant tamoxifen alone. Luminal B tumors are characterized by higher proliferation and poorer prognosis than luminal A tumors. HER2 status and Ki67 index, determined immunohistochemically, appear to distinguish luminal A from luminal B breast cancer subtypes. In a cohort of 4,046 patients with breast cancer, 2847 had hormone receptor positive tumors. Using immunohistochemistry to determine HER2 status and Ki67 index, with a cutoff of 13.25% for Ki67, 59% of hormone receptor positive tumors were classified as luminal A, 33% as luminal B/Ki67-positive and 9% as luminal B/HER2-positive; the 10-year breast cancer-specific survival was 79, 64 and 57%, respectively, with tamoxifen as their sole adjuvant systemic treatment [4]. It is unlikely that adjuvant treatment with aromatase inhibitors might change the relative hormone-resistance of luminal B

Table 4 Multivariate Cox regression analysis

	HR	95% CI	Wald's *P*
Disease-free survival			
Treatment group			
E-T-CMF	1		
E-CMF	1.11	0.76–1.63	0.58
Number of positive nodes			
0–3	1		
≥4	3.09	1.68–5.67	<0.001
Tumor subtypes			
Luminal A	1		0.016
Luminal B	2.54	1.15–5.59	0.021
HER2 enriched	3.13	1.20–8.18	0.020
TNBC	4.20	1.71–10.31	0.002
Overall survival			
Treatment group			
E-T-CMF	1		
E-CMF	1.57	0.99–2.50	0.058
Grade			
I–II	1		
III–Undif	1.55	0.96–2.48	0.070
Tumor size (cm)			
≤2	1		0.042
2–5	1.27	0.73–2.22	0.39
>5	2.24	1.17–4.28	0.015
Number of positive nodes			
0–3	1		
≥4	2.21	1.22–4.37	0.022
Tumor subtypes			
Luminal A	1		0.032
Luminal B	5.51	1.34–22.68	0.018
HER2 enriched	6.64	1.43–30.91	0.016
TNBC	8.99	2.01–40.13	0.004

CI confidence interval, *HR* hazard ratio, *TNBC* triple-negative breast cancer

tumors, as shown in the TransATAC study [11]. In our study, the vast majority of patients (66%) had luminal B tumors, which is in contrast to the published literature. This is attributed to the fact that the published literature is based upon registries, including non-selected populations, whilst our study included a high-risk population. In addition, the luminal B subtype, in our study, included both luminal B/Ki67-positive and luminal B/HER2-positive tumors.

Tan et al. [33] showed that metastasis-free and breast cancer-specific survival remained poor in patients with TNBC receiving adjuvant chemotherapy. This finding is in contrast to the excellent response rates reported with anthracyclines in neoadjuvant studies [3, 29]. It has been suggested that the negative prognosis of the TNBC phenotype may be alleviated by adjuvant chemotherapy comprised of CMF [28]. This regimen has been found to be particularly active in TNBC patients, both in the neoadjuvant [13] and adjuvant settings [8].

Tumors classified to the TNBC phenotype are further subdivided to BCP and unclassified tumors. The poor prognosis of TNBC phenotype is conferred almost entirely by tumors positive for basal markers (EGFR, basal cytokeratins). Even in TNBC patients treated with adjuvant anthracycline-based chemotherapy, those with BCP had a significantly worse outcome [5].

Basal core phenotype is associated not only with worse outcome but with worse clinical and pathological characteristics (high-grade tumors, younger age, etc.) as well. In our study, there were no significant differences in age, number of positive lymph nodes, menopausal status, and tumor size/grade between various subtypes of breast cancer. This could be attributed, not only to the small number of patients in our study, but to the selected population, which was high-risk by definition. The outcome of patients with BCP was not statistically worse as compared to luminal B, HER2 enriched, and TNBC. Again, the small number of patients could be an explanation; another speculation is the fact that all our patients were treated with dose-dense adjuvant chemotherapy including CMF, which is a particularly active regimen for BCP patients, as mentioned above.

Apart from the immunophenotypic classification, we performed hierarchical clustering analysis in formalin-fixed paraffin-embedded (FFPE) primary tumors, based on mRNA expression of three molecular markers: ESR1, PgR, and HER2. We examined the concordance of the mRNA classification with the immunohistochemical classification. We obtained a high specificity (93%) and sensitivity (75%) for triple-negative tumors.

Nielsen et al. had performed hierarchical clustering analysis based on mRNA expression of six molecular markers, ER, PgR, HER2, HER1, C-Kit, and cytokeratins 5/6 and compared the results with the protein expression of the above markers, as assessed with immunohistochemistry. They obtained a slightly higher specificity (100%) and an almost identical sensitivity (77%) for basal-like tumors, as compared to our results [25].

In conclusion this was one the first studies to confirm that triple-negative, luminal B and HER2 enriched phenotypes, identified by IHC, are of adverse prognostic value in high-risk breast cancer patients treated with dose-dense sequential adjuvant chemotherapy, with patients in the luminal A subtype having a better outcome compared with the other immunohistochemical subtypes. The addition of paclitaxel to the dose-dense adjuvant E-CMF regimen did not improve outcome in any immunohistochemical phenotype. In addition, we have shown that there is a very good concordance between immunohistochemical classification and mRNA expression classification based on three molecular

markers (ESR1, PgR and HER2) assessed by qRT-PCR in FFPE primary breast cancer tumors.

Acknowledgments The authors are deeply indebted to all patients who participated in the study and provided biological material for translational research. The authors also wish to thank Evita Fragou and Dimitra Katsala for monitoring the study, Maria Moschoni for coordinating the data management, and Thalia Spinari for tissue sample collection and Ioanna Panou for secretarial assistance. On behalf of the Hellenic Foundation for Cancer Research, Athens, Greece, the senior investigator author (GF) has pending patent applications with Siemens Healthcare Diagnostics, Tarrytown, NY, USA. Translational research was supported by a HeCOG research grant: HE TRANS_BR. The senior investigator (GF) has received Commercial Research Funding by Roche Hellas SA and Genesis Pharma SA, Athens, Greece.

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