Cellular Oncology 31 (2009) 261–271
DOI 10.3233/CLO-2009-0464
IOS Press

The prognostic value of the proliferation marker Phosphohistone H3 (PPH3) in luminal, basal-like and triple negative phenotype invasive lymph node-negative breast cancer

Ivar Skaland [a,b], Emiel A.M. Janssen [a], Einar Gudlaugsson [a,b], Lydia Hui Ru Guo [c] and Jan P.A. Baak [a,b,d,*]

[a] *Department of Pathology, Stavanger University Hospital, Stavanger, Norway*
[b] *The Gade Institute, University of Bergen, Bergen, Norway*
[c] *Department of Oncology, Longhua Hospital, Shanghai, P.R. China*
[d] *Free University, Amsterdam, The Netherlands*

Abstract. *Purpose*: Prognostic comparison of phosphohistone-H3 (PPH3) with Cytokeratin 5/6 and/or 14 positive (= basal-CK), triple (ER, PR, HER2)-negative (= TNP) and basal-like (= TNP and basal-CK positive) phenotype in invasive breast cancers.

Patients and methods: Classical variables, PPH3, ER, PR, basal-CK and HER2 in 240 $T_{1-2}N_0M_0$ patients under 71 years.

Results: TNP and basal-like cancers had higher PPH3 expression than the other cancers (mean 48 versus 11, $P < 0.001$). Fifteen percent of the patients in the whole group, but 32–38% of TNP and basal-like cancers recurred. With multivariate analysis, PPH3 < 13 ($n = 156$) versus $\geqslant 13$ ($n = 84 = 35\%$ of all cases) was the strongest and only prognosticator (10-year survival 96% and 64%, $P \leqslant 0.001$, Hazard ratio $= 9.0$).

Conclusion: PPH3 is the strongest prognosticators in luminal, Triple negative and basal-like $T_{1-2}N_0M_0$ invasive breast cancers.

Keywords: Breast cancer, prognosis, proliferation, Phosphohistone H3, basal-like

1. Introduction

Breast cancer is a heterogeneous disease, encompassing a number of distinct biological entities. Clinically, invasive lymph node negative breast carcinomas are treated according to tumor size, grade, lymph and blood vessel invasion, age, HER2 and/or hormone receptor status. Recent gene expression studies have shown that breast cancers can be classified into five main groups with prognostic and predictive value; Luminal A and B, Normal breast-like, HER2 positive, and basal-like [20,29–31]. Since gene expression profiling is not widely available as a routine diagnostic method, many investigators have used surrogate immunohistochemical markers to obtain a similar classification. Cancers are classified as Luminal when ER and or PR is expressed with or without expression of HER2, as triple negative (triple negative profile = TNP) when ER, PR, and HER2 are all negative, as basal-like (= BLC) when TNP and basal cytokeratin (= basal-CK) and/or EGFR is expressed, and as HER2 positive when ER and PR are negative and HER2 is overexpressed. The normal-like breast cancers do not express basal cytokeratins, EGFR or HER2 but a TNP without expression of these markers is not necessarily a normal-like tumor as classified by gene expression arrays [1,8,19,23].

Most series contain 10–17% TNPs and up to 15% of BLCs [24,32]. Clinically, both TNPs and BLCs have a poor prognosis and lack predictive markers for

*Address for correspondence: Prof. Dr. J.P.A. Baak, Department of Pathology, Stavanger University Hospital, Box 8100, 4068 Stavanger, Norway. Tel.: +47 51 519378; Fax: +47 51 519920; E-mail: jpabaak@yahoo.com.

known target therapies. It has been claimed that TNPs and BLCs are synonymous [16], but the expression patterns differ. The classification of cancers by gene expression profiling and immunohistochemical methods shows reasonably good agreement, although differences do occur. For example, ER expression has been seen in 5–45% [7,21,25] and HER2 expression in 14% of cancers classified as BLCs by gene expression profiling [25]. On the other hand, 19% of immunohistochemically TNPs were negative for basal Cytokeratin and EGFR, while 7% of non-TNPs were positive for basal Cytokeratin markers [32].

TNPs and BLCs are in general, associated with high proliferation, many apoptotic cells, lack of tubule formation, cellular pleomorphism, prominent nucleoli, high grade, young age, aggressive clinical behavior, early relapse at 3–5 years, with an unusually high frequency of visceral, brain and lung, though less frequent bone, liver and axillary lymph nodes metastases [21,24]. Interestingly, BLCs are over-represented amongst the interval breast cancers [9].

In lymph node negative breast cancers the mortality is relatively low (10–30%). Taking into account the much higher rates of relapse and mortality found in TNP and BLCs [21,24], these subtypes which constitute up to about 17% of all breast cancers, could be responsible for a significant proportion of the deaths within this group. On the other hand, proliferation activity measured by Mitotic activity index (MAI) or phosphorylated histone H3 (PPH3) is currently the strongest prognosticators in lymph-node negative breast cancer patients under 71 years of age [3–5,14, 15,26–28,34]. Whether TNP, basal-CK or BLC and proliferation have additional prognostic value together, is unknown. Therefore, we compare the prognostic value of MAI and PPH3 expression with luminal, TNP, basal-CK positive and basal-like cancers in $T_{1-2}N_0M_0$ invasive breast cancer patients without adjuvant therapy, less than 71 years of age and with long-term follow-up. This special subgroup of patients was chosen because of the obvious potential therapeutic consequences.

2. Materials and methods

2.1. Patients

The study was approved by the Regional Ethics Committee, the Norwegian Social Science Data Service, and the Norwegian Data Inspectorate. Paraffin-embedded material from 684 consecutive invasive breast cancer patients less than 71 years of age with operable breast cancer treated between 1990 and 1997 was provided by the Department of Pathology at the Stavanger University Hospital (Stavanger, Norway). Of these patients, 384 were node negative breast cancers of which the following patients were excluded: 90 patients with adjuvant treatment, carcinoma *in situ* only or extensive carcinoma *in situ* with a small microinvasive component $<$ 1 mm that was ineligible for MAI or PPH3 evaluation ($n = 18$), patients with recurrence within 6 months of follow-up, since it is likely that these patients at the time of diagnosis had undetected metastases and we wanted to analyze a pure group of lymph node negative patients ($n = 3$), Paget's disease ($n = 1$), bilateral breast cancer ($n = 4$), or previous other malignancies ($n = 2$). For 5 patients no follow-up data were available. Material was not available for 21 patients, leaving 240 patients for analysis. There was no difference in age or tumor size in the 240 patients when compared to the original 384 patients. All patients were treated with modified radical mastectomy ($n = 131$) or breast-conserving therapy ($n = 109$) always with adequate lymph node dissection (at least 10, median 13 nodes were examined). Locoregional radiotherapy was administered to patients who underwent breast-conserving therapy or had medially localized tumors.

2.2. Pathology

The post-surgical size of the tumor was measured in fresh specimens. Tumors were cut into 0.5 cm slices, fixed in 4% buffered formaldehyde, and embedded in paraffin. Paraffin sections were carefully cut by the same experienced technician to achieve even section thickness at 4 μm for hematoxylin–eosin (H&E) and immunostaining.

Histological type was assessed according to World Health Organization criteria [33]. Grade (Grade 1 = 3, 4 or 5; Grade 2 = 6 or 7; Grade 3 = 8 or 9) was assessed according to the Nottingham modification [11], calculated as the sum of tubule formation ($>$75% = 1, 10–75% = 2 and $<$10% = 3), nuclear atypia (mild = 1, moderate = 2 and marked = 3), and mitotic activity (class 0–5 = 1, 6–10 = 2, and $>$10 = 3). The Mitotic Activity Index (MAI) was assessed as described elsewhere [3]. Briefly, all unambiguous mitoses were counted in 10 consecutive neighboring fields of vision (FOV) in the most cell-dense area (1.59 mm^2 at specimen level), usually in the peripheral growing zone. The MAI is reproducible and insensitive to variations in tissue processing [3–5,34].

2.3. Immunohistochemistry

Antibody dilution and IHC protocols were optimized prior to the study onset. To ensure uniform handling of samples, each antibody was stained in two batches on following days using the same antibody dilution and detection reagents. Paraffin sections adjacent to the H&E sections used for assessment of MAI and histology were mounted onto Superfrost Plus slides (Menzel, Braunschweig, Germany) and dried overnight at 37°C followed by 1 h at 60°C. Sections were deparaffinized in xylene and rehydrated in decreasing concentrations of alcohol. Antigen was retrieved with a highly stabilized retrieval system (ImmunoPrep, Instrumec, Oslo, Norway) using 10 mM TRIS/1 mM EDTA (pH 9.0) as the retrieval buffer. Sections were heated for 3 min at 110°C followed by 10 min at 95°C and cooled to 20°C. Rabbit polyclonal anti-phosphohistone H3 (ser 10) (Upstate #06-570; Lake Placid, NY) was used at a dilution of 1:1500. Cytokeratin 5/6 (Clone D5/16 B4, Dako, Glostrup, Denmark) was used at a dilution of $1/100$, and Cytokeratin 14 (Clone LL002, Novocastra, Newcastle Upon Tyne, UK) at a dilution of $1/40$. ER (clone SP1, Neomarkers/LabVision, Fremont, CA) was used at a dilution $1/400$. PR (Clone SP2, Neomarkers/LabVision, Fremont, CA) was used at a dilution of $1/1000$. Anti-phosphohistone H3 was incubated for 60 min at 22°C. All other antibodies were incubated for 30 min at 22°C. Dako antibody diluent (S0809) was used. Endogenous peroxidase activity was blocked with a peroxidase blocking reagent (S2001; Dako) for 10 min. The immune complex was visualized with the Dako REAL EnVision Detection System, Peroxidase/DAB, Rabbit/Mouse (K5007; Dako). Sections were incubated with EnVision/HRP, Rabbit/Mouse for 30 min and diaminobenzidine (DAB+) chromogen for 10 min. The sections were counterstained with hematoxylin, dehydrated, and mounted. All steps were performed using Dako Autostainer and TBS (S1968; Dako) with 0.05% Tween 20 as wash buffer. For HER2 assessments Dako HercepTest™ and The PathVysion HER-2 DNA Probe Kit was used according to the manufacturers procedures.

2.4. Quantification of PPH3, basal Cytokeratin, ER, PR and HER2

The PPH3 index was assessed using the same counting protocol as for the MAI. Two independent pathologists counted the number of PPH3-positive objects (nuclei and mitoses) in 10 adjacent FOVs, with a $\times 40$ objective, as described above for mitoses. Nuclei with fine granular PPH3 staining were not counted, as these cells are not in the G2 phase [6]. PPH3-rich areas are usually localized in the periphery (i.e., growing zone) of the cancers. If the counts of two observers differed by more than 3 figures, the count was repeated with a multi-head microscope and a consensus score was obtained. In addition to performing subjective counts, PPH3 expression was evaluated using the fully automated VIS analysis system (Visiopharm, Hørsholm, Denmark), using the same image processing principles described before [27]. Reproducibility of the PPH3 measurements between subjective counts by two observers, and between subjective and digital image analysis results was high ($R = 0.94$–0.98). Not surprisingly, the reproducibility of the PPH3 counts by the automated digital image analysis on different days by different observers was close to perfect ($R = 0.99$). For this reason, in the statistical analysis the image analysis counts were used.

The percentage of CK5/6 and CK14 positive tumor cells in each cancer was scored using a continuous scale of 0–100%. ER and PR were scored as positive when nuclear staining was present in $>10\%$ of the tumor cells. HER2 was scored according to the Dako HercepTest™ scoring protocol. All 2+ and 3+ cases were tested by the PathVysion HER-2 DNA Probe Kit, and only HER2 amplified cases were regarded as positive. All stainings were scored independently by two pathologists; in any discrepancies consensus was achieved using a multi-head microscope.

2.5. Data analysis

Correlations were calculated using the Spearman and kappa tests. For survival analysis, the main endpoints were distant metastases recurrence and overall distance metastases-related survival. To determine the probability that patients would remain free of distant metastases, we defined recurrence as any first recurrence at a distant site. Patients were censored from the date of the last follow-up visit for death from causes other than breast cancer, local or regional recurrences, or the development of a second, primary cancer, including contralateral breast cancer. If a patient's status during follow-up indicated a confirmed metastasis without a recurrence date, the follow-up visit date was used. Age, time to first recurrence, and survival time were calculated relative to the primary diagnosis date. For the MAI, three sets of previously established prog-

nostic thresholds (<6, 6–10, ⩾11, <10 versus ⩾10; and <3, 3–9 and ⩾10) were examined [3–5]. For PPH3 the recently validated cut-off <13 versus ⩾13 positive late G2/mitotic cell/1.59 mm^2 was used [26,27].

For the basal cytokeratins (basal-CK) CK5/6 and CK 14 receiver operating curves were applied to identify the objectively optimal prognostic threshold. TNP was defined as negative for ER, PR, and HER2. Tumours were classified as BLC when TNP (ER/PR/HER2 negative) and one or both of the basal-CK (CK 5/6, CK 14) were positive in >0% of the tumor cells. Kaplan–Meier survival curves were constructed, and between-group differences were tested using the log-rank test. The relative importance of potential prognostic variables was tested using Cox-proportional hazard analysis and expressed as a hazard ratio (HR) with a 95% confidence interval (CI).

3. Results

Of all 240 patients, 36 (15%) developed distant metastases and 28 (12%) died. Phosphohistone H3 expression occurred preferentially in the peripheral growing front. PPH3 correlated with age, tumor diameter, grade, tubular formation, nuclear atypia, MAI, ER, PR, TNP, basal-CK, and BLC (Table 1). Triple-negative cell type (ER, PR, HER2 negative) cancers occurred in 28/240 (12%) patients of which nine(32%) developed distant metastases and seven (25%) died. Of the basal cytokeratin positive cases (CK5/6 and/or CK14 positive, 27/240=11%), ten (37%) showed distant recurrence and nine (33%) died. Of the 21 basal-like cancers (triple negative and basal cytokeratin positive), eight (38%) recurred and seven (33%) died. Most (213) cancers were completely negative for CK5/6 and/or CK14 and therefore classified as TNP, HER2+ or luminal according to ER, PR and HER2 expression (Figs 1 and 2).

ROC curve analysis was used to assess the optimal prognostic threshold for basal cytokeratin expression. In agreement with others we found that negative versus positive (which is easy to define and well reproducible) was the best cut-off value [8,19]. We also investigated the commonly used cut off <10% versus ⩾10% positive tumor cells [22]. Only 4 cases had between 0 and 10% positive tumor cells, and the prognostic results were comparable with the threshold of 0, indicating that basal cytokeratin expression as negative versus positive is a robust and reliable prognostic feature.

TNP, basal-CK positive and BLC cancers had a significantly ($P<0.001$) higher PPH3 expression (means: 48, 43, 48) compared to the luminal (ER and/or PR positive) cancers (mean 11).

Classical features, PPH3 $<$ 13 versus ⩾13 ($P \leqslant 0.001$, HR $= 9.0$, CI $= 3.7$–21.8)), MAI $<$ 10 versus ⩾10 ($P \leqslant 0.001$, HR $= 5.5$, CI $= 2.7$–11.0), basal-CK positive ($P \leqslant 0.001$, HR $= 4.0$, CI $= 1.9$–8.3), TNP- ($P = 0.01$, HR $= 2.5$, CI $= 1.2$–5.3) and BLC-phenotype ($P \leqslant 0.001$, HR $= 3.7$, CI $= 1.7$–9.3) were all strong independent prognostic factors (Table 2, Fig. 3). In the total group, the PPH3 prognostic threshold H3 $<$ 13 ($n = 156$; 65% of all cases) versus ⩾13 ($n = 84$; 35% of all cases) was associated with 96% and 64% 10-year distant metastases recurrence-free survival. Generally, if MAI, PPH3, ER, TNP, basal-CK and BLC had unfavorable values, each of these features identifies a subgroup with a 10 year recurrence free survival of between 60–70%. In agreement with this, in a multivariate analysis, including all the abovementioned prognostic factors studied, PPH3 $<$ 13 versus ⩾13 was the strongest prognosticator ($P \leqslant 0.001$, HR $= 9.0$, CI $= 3.7$–21.8).

Grade 1 versus Grade 2 and 3 had weak additional prognostic value to PPH3 but only in PPH3 $\geqslant$ 13 ($P = 0.03$) and not in the patients with PPH3 $<$ 13 ($P = 0.25$). The other features (TNP, basal-CK, BLC, tumor size, ER, PR and HER2) were not significant (Tables 3 and 4).

4. Discussion

The results confirm earlier studies and show strong prognostic and predictive value of the TNP, basal-CK and BLC phenotype and proliferation markers, especially PPH3. It is well known that TNP, basal-CK and BLC cancers have a poor prognosis, which was also found in our material. The patient group studied therefore is representative for $T_1N_0M_0$ operable invasive breast cancer. Interestingly, a much higher PPH3 proliferation index occurred in the TNP, basal-CK and basal-like cancers, than in luminal type cancers. On the other hand, not all TNP/basal-CK and BLC cancers had high proliferation. Although the numbers were small, the difference between low and high PPH3 in TNP, basal-CK and BLC cancers showed a clear trend similar to that found in luminal cancers. In agreement with this, multivariate analysis selected PPH3 as the factor with overriding strong prognostic value. Taken together, these results suggest that the poor prognostic

Table 1
Correlation of the PPH3 index and clinico-pathologic features

		PPH3 $<$ 13 (n = 156)		PPH3 $\geqslant$ 13 (n = 84)		P
		n	%	n	%	
Age	<55	58	55	48	45	0.003
	⩾55	98	73	36	27	
Tumor diameter	⩽2 cm	141	69	63	31	0.001
	>2 cm	15	42	21	58	
Surgery	BCT	67	62	42	38	0.3
	MRM	89	68	42	32	
Grade	1	73	90	8	10	<0.001
	2	76	70	32	30	
	3	7	14	44	86	
Tubular formation	>75%	20	83	4	17	<0.001
	10–75%	54	84	10	16	
	<10%	82	54	70	46	
Nuclear atypia	Mild	31	94	2	6	<0.001
	Moderate	94	72	37	28	
	Marked	31	41	45	59	
MAI	0–5	136	93	11	7	<0.001
	6–10	15	52	14	48	
	⩾10	5	8	59	92	
ER	Positive	146	73	53	27	<0.001
	Negative	10	24	31	76	
PR	Positive	116	70	49	30	0.01
	Negative	40	53	35	47	
TNP	Not TNP	151	71	61	29	<0.001
	TNP	5	18	23	82	
CK5/6	Negative	153	71	62	29	<0.001
	Positive	3	12	22	88	
CK14	Negative	153	69	68	31	<0.001
	Positive	3	16	16	84	
Basal	Negative	152	71	61	29	<0.001
Cytokeratin	Positive	4	15	23	85	
Basal-like	Not BLC	154	70	65	30	<0.001
	BLC	2	10	19	90	

P, probability of no difference; PPH3, phosphohistone H3; BCT, breast conserving therapy; MRM, modified radical mastectomy; MAI, mitotic activity index; ER, estrogen receptor; PR, progesterone receptor; CK5/6, Cytokeratin 5/6; CK14, Cytokeratin 14; TNP, triple negative; BLC, basal-like carcinoma.

value of basal cell type cancers is due to high proliferation, whereas the overriding prognostic criterion of luminal type cancers is low proliferation and is therefore associated with a much better prognosis (of course, exceptions occur of luminal type cancers with high PPH3 which have poor prognosis). Once the difference in PPH3 proliferation of the TNP, basal and luminal cell types is taken into account, the other characteristics (ER, PR, CK5/6, CK14) do not have additional prognostic value.

Some therapeutic decision making programs for node negative breast cancer use ER, tumour diameter

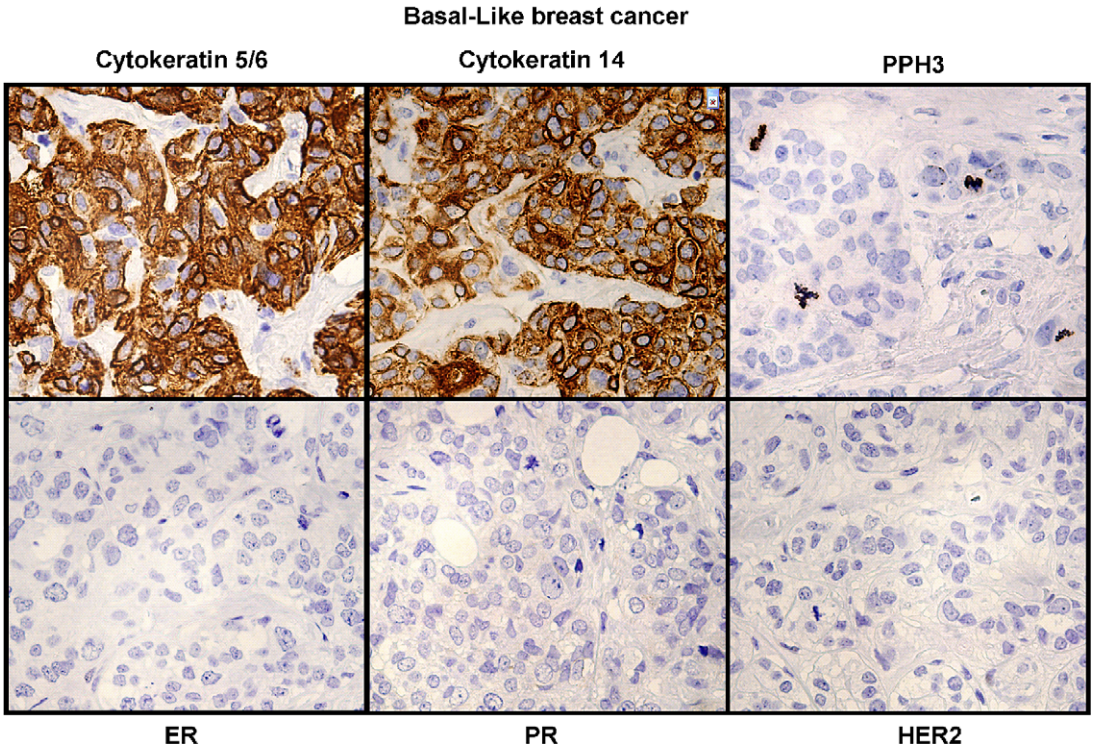

Fig. 1. Example of a basal-like breast cancer stained with Cytokeratin 5/6, Cytokerratin 14, Phosphohistone H3 (PPH3), Estrogen receptor (ER), Progesterone receptor (PR) and HER2.

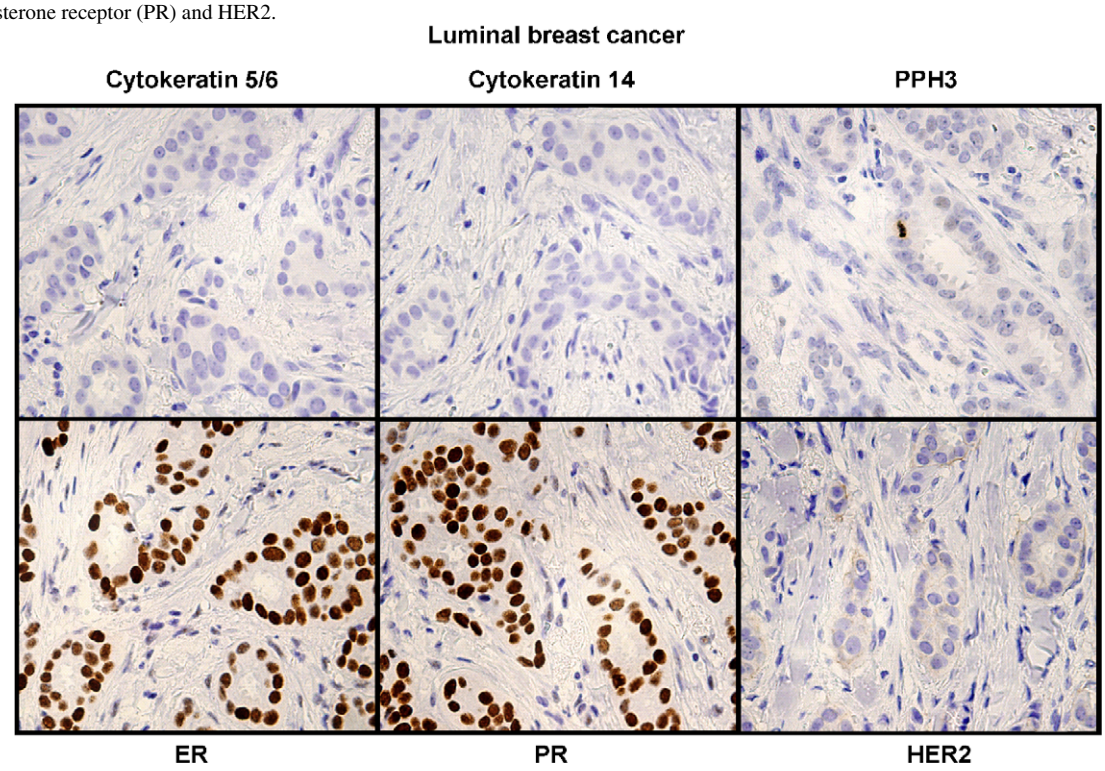

Fig. 2. Example of a luminal breast cancer stained with Cytokeratin 5/6, Cytokerratin 14, Phosphohistone H3 (PPH3), Estrogen receptor (ER), Progesterone receptor (PR) and HER2.

Table 2

Prognostic value (long-term, recurrence-free survival and Disease Related Mortality) of clinicopathologic features, MAI, steroid receptors, and PPH3

Characteristic		Recurrence				Disease Related Mortality			
		Events at risk	% AW	Logrank *P*-value	HR (95% CI)	Events/ at risk	% AW	Logrank *P*-value	HR (95% CI)
Age	<55	20/106	81			15/106	86		
	⩾55	16/134	88	0.41	0.8 (0.4–1.5)	13/134	90	0.45	0.8 (0.4–1.6)
Surgery	BCT	17/109	84			13/109	88		
	MRM	19/131	86	0.96	1.0 (0.5–1.9)	15/131	89	0.8	1.1 (0.5–2.2)
Tumor diameter	⩽2 cm	26/204	87			19/204	91		
	>2 cm	10/36	72	0.02	2.3 (1.1–4.8)	9/36	75	0.01	2.7 (1.2–6.0)
ER status	Positive	23/199	88			17/199	92		
	Neg	13/41	68	0.001	3.0 (1.5–6.0)	11/41	73	0.001	3.5 (1.6–7.4)
PR status	Positive	18/165	89			13/165	92		
	Neg	18/75	76	0.008	2.4 (1.2–4.5)	15/75	80	0.006	2.7 (1.3–5.7)
HER2	Neg	27/214	87			22/214	90		
	Positive	9/26	65	0.002	3.1 (1.5–6.7)	6/26	77	0.05	2.4 (1.0–5.8)
Grade	I	1/81	99			1/81	99		
	II	17/108	84		11.4 (1.5–86.0)	13/108	88		8.7 (1.1–66.9)
	III	18/51	65	<0.001	24.7 (3.3–185.3)	14/51	73	<0.001	20.7 (2.7–157.3)
MAI	<10	12/176	93			9/176	95		
	⩾10	24/64	63	<0.001	5.5 (2.7–11.0)	19/64	70	<0.001	5.9 (2.7–13.0)
MAI	0–5	5/147	97			4/147	97		
	6–10	7/29	76		6.6 (2.1–21.1)	5/29	83		5.9 (1.6–22.0)
	>10	24/64	63	<0.001	10.8 (4.1–28.4)	19/64	70	<0.001	10.9 (3.7–32.2)
MAI	<3	2/115	98			2/115	98		
	3–9	10/61	84		9.9 (2.1–45.5)	7/61	89		6.4 (1.3–30.9)
	⩾10	24/64	63	<0.001	21.8 (5.1–92.2)	19/64	70	<0.001	17.2 (4.0–73.7)
Nuclear atypia	Mild	1/33	97			1/33	97		
	Moderate	13/131	90		2.7 (0.4–21.0)	9/131	93		2.0 (0.3–15.8)
	Marked	22/76	71	0.003	7.3 (1.0–54.0)	18/76	76	0.002	6.6 (0.9–49.7)
Tubular formation	>75%	1/24	96			1/24	96		
	10–75%	3/64	95		0.8 (0.09–8.1)	1/64	98		0.3 (0.02–4.9)
	<10%	32/152	79	0.006	4.1 (0.6–30.0)	26/152	83	0.004	3.6 (0.5–26.6)
PPH3	<13	6/156	96			5/156	97		
	⩾13	30/84	64	<0.001	9.0 (3.7–21.8)	23/84	73	<0.001	8.9 (3.4–23.5)
Triple Negative	Not TNP	27/212	87			21/212	90		
	TNP	9/28	68	0.01	2.5 (1.2–5.3)	7/28	75	0.02	2.6 (1.1–6.2)
Basal-CK	Negative	26/213	88			19/213	91		
	Positive	10/27	63	<0.001	4.0 (1.9–8.3)	9/27	67	<0.001	4.3 (1.9–10.0)
Basal-like	Not BLC	28/219	87			21/219	90		
	BLC	8/21	62	<0.001	3.7 (1.7–8.3)	7/21	68	0.001	3.9 (1.7–9,2)

AW, alive and well; *P*, probability of no difference; HR, Hazard ratio; CI, confidence interval; BCT, breast conserving therapy; MRM, modified radical mastectomy; ER, estrogen receptor; PR, progesterone receptor; MAI, mitotic activity index; PPH3, phosphohistone H3; TNP, triple negative for ER, PGR and HER2; Basal-CK, cytokeratin 5/6 and/or cytokeratin 14; BLC, basal-like.

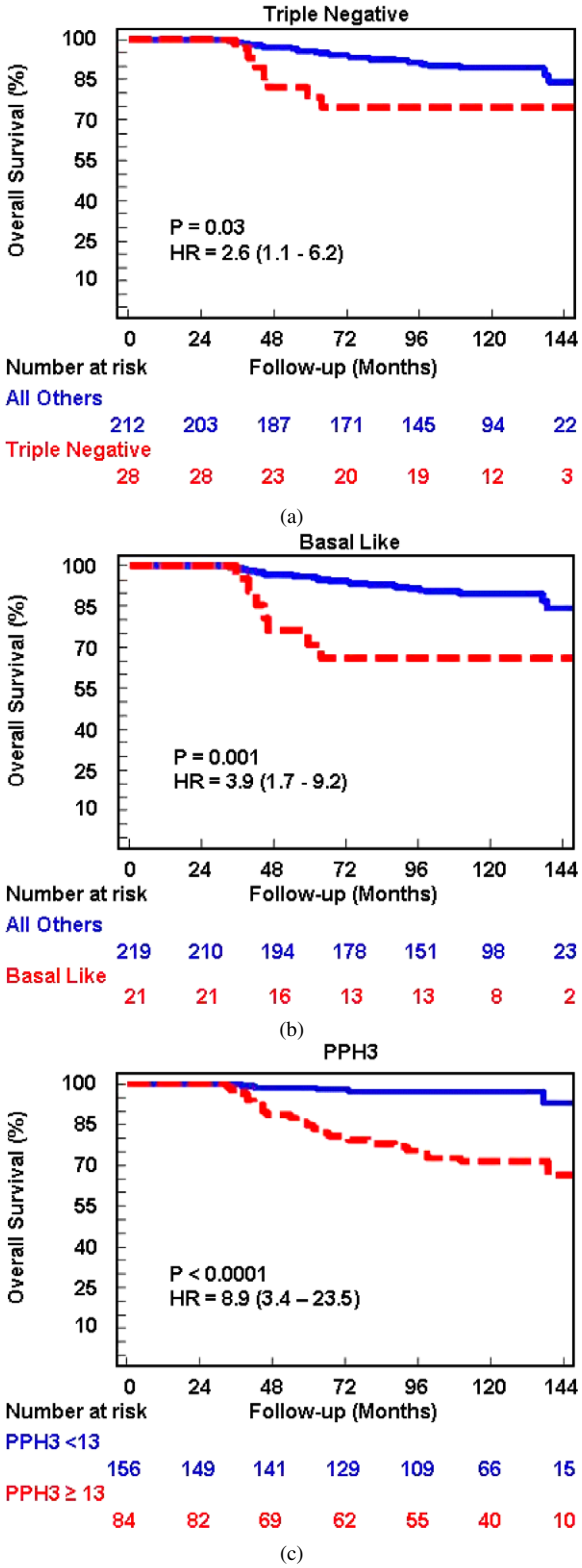

Fig. 3. Survival curves of patients stratified by (a) Triple negative phenotype, (b) Basal-like cancer phenotype and (c) PPH3.

and age as the most important prognostic factors, while the use of basal versus luminal type cancers is also widespread. The previous and current results raise the question of whether proliferation markers, especially PPH3 should not be used instead for this purpose when the results are confirmed in other studies. The excellent identification by the PPH3 stain of mitoses, the robustness of the staining and the wide range of strong prognostic value of other thresholds around 13, reduces the risk of prognostically classifying patients erroneously [26,27]. Steroid receptors are often used as primary prognostic and therapeutic classifiers in node-negative breast cancer patients (see www.adjuvantonline.com). Indeed, in older patients (>70 years) presence of estrogen receptors is a stronger predictor than the proliferation marker MAI and therefore ER can be used as a primary prognostic variable in the elderly patient. However, this is not the case for patients under 71 where proliferation (either measured as the MAI or PPH3 index) is stronger [4,27]. Nearly two-thirds of the LN-negative patients less than 71 years have a low, and only one-third a high, (prognostically unfavorable) PPH3 labeling. This means that if selection of $T_{1-2}N_0M_0$ invasive breast cancer patients for adjuvant therapy would be done according to PPH3 (i.e., treat patients with PPH3 $\geqslant$ 13), overtreatment would be much less than with other widely used prognosticators with a lower sensitivity and specificity. The result is very low under-treatment and at the same time as low over-treatment as possible.

In spite of the strong prognostic value of PPH3 and the excellent prognosis of patients with a low PPH3 index (65% of the patients), 60% of the remaining 35% of patients with PPH3 $\geqslant$ 13 and unfavorable prognostic probability nonetheless survived without signs of distant metastases at long follow-up. This suggests that other factors than proliferation also play a major role. Candidate markers may include hypoxia-related factors [2], vascular tumor factors [10], PAI-1/UpA [13], immune response [17], bone marrow micrometastases [12], activation or deactivation of transcription factors involved in proliferation, differentiation and apoptosis (Stat5 and E2F1) [18,35], or dysregulation of tumor suppressors and signaling pathways. It is important to investigate the prognostic value of these factors in comparison to PPH3.

5. Conclusion

The poor prognostic value of triple negative and basal-like phenotype was confirmed in the current

Table 3

Results of the multivariate survival analysis. Grade 1 versus Grade 2 and 3 have additional prognostic value to PPH3 ($P = 0.04$) but none of the other features analyzed do ($P > 0.05$)

	P	HR	95% CI
PPH3 $<$ 13 vs $\geqslant$13	$<$0.001	9.0	3.8–21.8
Grade 1 vs 2 and 3	0.04	8.7	1.2–65.4
MAI 10	0.76	–	–
ER	0.70	–	–
PR	0.12	–	–
HER2	0.32	–	–
Basal-like	0.36	–	–
TNP	0.89	–	–
Basal-CK	0.27	–	–
Tumor size $\leqslant$ 2 vs $>$2 cm	0.33	–	–

P, probability of no difference; HR, Hazard ratio; CI, confidence interval; PPH3, phosphohistone H3; PR, progesterone receptor; TNP, triple negative for ER, PGR and HER2; HER2, Epidermal growth factor receptor 2; ER, estrogen receptor; Basal-CK, cytokeratin 5/6 and/or cytokeratin 14; BLC, basal-like.

Table 4

Grade 1 versus Grade 2 and 3 stratified for PPH3 $<$ 13 and $\geqslant$ 13. Grade 1 versus Grade 2 and 3 have additional prognostic value to PPH3 but only in PPH3 $\geqslant$ 13 ($P = 0.03$) and not in the patients with PPH3 $<$ 13 ($P = 0.25$)

		Total n	n of events	Censored		P
				n	Percent	
PPH3 $<$ 13	Grade 1	73	1	72	98.6	
	Grade 2 and 3	83	5	78	94.0	0.25
PPH3 $\geqslant$ 13	Grade 1	8	0	8	100	
	Grade 2 and 3	76	30	46	60.5	0.03

PPH3, phosphohistone H3; P, probability of no difference.

group of invasive breast cancer patients less than 71 years. However, the prognostic value of nuclear PPH3 expression overshadows and explains these features.

Conflicts of interest

None of the authors have any financial or other relationships with entities that have investment, licensing, or other commercial interests in the subject matter under consideration in this article.

Acknowledgements

This work was supported by grant 911108 from Helse Vest and grant 08-578 from the Stichting Bevordering Diagnostische Morfometrie.

We thank Marit Nordhus, Bianca van Diermen and Anne-Elin Varhaugvik for excellent technical assistance.

References

[1] J.B.Arnes, K. Collett and L.A. Akselen, Independent prognostic value of the basal-like phenotype of breast cancer and associations with EGFR and candidate stem cell marker BMI-1, *Histopathology* **52** (2008), 370–380.

[2] J.P. Baak, C.G. Colpaert, P.J. van Diest, E. Janssen, B. van Diermen, E. Albernaz, P.B. Vermeulen and E.A. Van Marck, Multivariate prognostic evaluation of the mitotic activity index and fibrotic focus in node-negative invasive breast cancers, *Eur. J. Cancer* **41** (2005), 2093–2101.

[3] J.P. Baak, P.J. van Diest, F.J. Voorhorst, E. van der Wall, L.V. Beex, J.B. Vermorken and E.A. Janssen, Prospective multicenter validation of the independent prognostic value of the mitotic activity index in lymph node-negative breast cancer patients younger than 55 years, *J. Clin. Oncol.* **23** (2005), 5993–6001.

[4] J.P. Baak, P.J. van Diest, F.J. Voorhorst, E. van der Wall, L.V. Beex, J.B. Vermorken, E.A. Janssen, E. Gudlaugsson and other collaborators of the Multicenter Morphometric Mammary Carcinoma Project (MMMCP), The prognostic value of proliferation in lymph-node-negative breast cancer patients is age dependent, *Eur. J. Cancer* **43** (2007), 527–535.

[5] J.P. Baak, P.J. van Diest, E.A. Janssen, E. Gudlaugsson, F.J. Voorhorst, E. van der Wall, J.B. Vermorken and Collaborators of Multicenter Morphometric Mammary Carcinoma

Project (MMMCP), Proliferation accurately identifies the high-risk patients among small, low-grade, lymph node-negative invasive breast cancers, *Ann. Oncol.* **19** (2008), 649–654.

[6] C. Bossard, A. Jarry, C. Colombeix, K. Bach-Ngohou, A. Moreau, D. Loussouarn, J.F. Mosnier and C.L. Laboisse, Phosphohistone H3 labelling for histoprognostic grading of breast adenocarcinomas and computer-assisted determination of mitotic index, *J. Clin. Pathol.* **59** (2006), 706–710.

[7] S. Calza, P. Hall, G. Auer, J. Björhle, S. Klaar, U. Kronenwett, E.T. Liu, L. Miller, A. Ploner, J. Smeds, J. Bergh and Y. Pawitan, Intrinsic molecular signature of breast cancer in a population-based cohort of 412 patients, *Breast Cancer Res.* **8** (2006), R34.

[8] M.C. Cheang, D.Voduc, C. Bajdik, S. Leung, S. McKinney, S.K. Chia, C.M. Perou and T.O. Nielsen, Basal-like breast cancer defined by five biomarkers has superior prognostic value than triple-negative phenotype, *Clin. Cancer Res.* **14** (2008), 1368–1376.

[9] K. Collett, I.M. Stefansson, J. Eide, A. Braaten, H. Wang, G.E. Eide, S.Ø. Thoresen, W.D. Foulkes and L.A. Akslen, A basal epithelial phenotype is more frequent in interval breast cancers compared with screen detected tumors, *Cancer Epidemiol. Biomarkers Prev.* **14** (2005), 1108–1112.

[10] J.S. de Jong, P.J. van Diest and J.P. Baak, Hot spot microvessel density and the mitotic activity index are strong additional prognostic indicators in invasive breast cancer, *Histopathology* **36** (2000), 306–312.

[11] C.W. Elston and I.O. Ellis, Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up, *Histopathology* **19** (1991), 403–410.

[12] R.K. Farmen, O. Nordgård, B. Gilje, F.V. Shammas, J.T. Kvaløy, S. Oltedal and R. Heikkilä, Bone marrow cytokeratin 19 mRNA level is an independent predictor of relapse-free survival in operable breast cancer patients, *Breast Cancer Res. Treat.* **108** (2008), 251–258.

[13] F. Jänicke, A. Prechtl, C. Thomssen, N. Harbeck, C. Meisner, M. Untch, C.G. Sweep, H.K. Selbmann, H. Graeff and M. Schmitt, Randomized adjuvant chemotherapy trial in high-risk, lymph node-negative breast cancer patients identified by urokinase-type plasminogen activator and plasminogen activator inhibitor type 1, *J. Natl. Cancer Inst.* **93** (2001), 913–920.

[14] E.A. Janssen, H. Søiland, I. Skaland, E. Gudlaugson, K.H. Kjellevold, A. Nysted, J.A. Søreide and J.P. Baak, Comparing the prognostic value of PTEN and Akt expression with the Mitotic Activity Index in adjuvant chemotherapy-treated node-negative breast cancer patients aged $<$ 55 years, *Cell Oncol.* **29** (2007), 25–35.

[15] E.A. Janssen, P.J. van Diest, H. Søiland, E. Gudlaugson, A. Nysted, F.J. Voorhorst, J.B. Vermorken, J.A. Søreide and J.P. Baak, Success predictors of adjuvant chemotherapy in node-negative breast cancer patients under 55 years, *Cell Oncol.* **28** (2006), 295–303.

[16] B. Kreike, M. van Kouwenhove, H. Horlings, B. Weigelt, H. Peterse, H. Bartelink and M.J van de Vijver, Gene expression profiling and histopathological characterization oftriple-negative/basal-like breast carcinomas, *Breast Cancer Res.* **9** (2007), R65.

[17] G. La Rocca, R. Anzalone, S. Corrao, F. Magno, F. Rappa, S. Marasà, A.M. Czarnecka, L. Marasà, C. Sergi, G. Zummo and F. Cappello, CD1a down-regulation in primary invasive ductal breast carcinoma may predict regional lymph node invasion and patient outcome, *Histopathology* **52** (2008), 203–212.

[18] M.T. Nevalainen, J. Xie, J. Torhorst, L. Bubendorf, P. Haas, J. Kononen, G. Sauter and H. Rui, Signal transducer and activator of transcription-5 activation and breast cancer prognosis, *J. Clin. Oncol*. **22** (2004), 2053–2060.

[19] T.O. Nielsen, F.D. Hsu, K. Jensen, M. Cheang, G. Karaca, Z. Hu, T. Hernandez-Boussard, C. Livasy, D. Cowan, L. Dressler, L.A. Akslen, J. Ragaz, A.M. Gown, C.B. Gilks, M. van de Rijn and C.M. Perou, Immunohistochemical and clinical characterization of the basal-like subtype of invasive breast carcinoma, *Clin. Cancer Res.* **10** (2004), 5367–5374.

[20] C.M. Perou, T. Sørlie, M.B. Eisen, M. van de Rijn, S.S. Jeffrey, C.A. Rees, J.R. Pollack, D.T. Ross, H. Johnsen, L.A. Akslen, O. Fluge, A. Pergamenschikov, C. Williams, S.X. Zhu, P.E. Lønning, A.L. Børresen-Dale, P.O. Brown and D. Botstein, Molecular portraits of human breast tumours, *Nature* **406** (2000), 747–752.

[21] E.A. Rakha, M.E. El-Sayed, J. Reis-Filho and I.O. Ellis, Pathobiological aspects of basal-like breast cancer, *Breast Cancer Res. Treat.* (2008) Mar. 9. [Epub ahead of print.]

[22] E.A. Rakha, M.E. El-Sayed, A.R. Green, E.C. Paish, A.H. Lee and I.O. Ellis, Breast carcinoma with basal differentiation: a proposal for pathology definition based on basal cytokeratin expression, *Histopathology* **50** (2007), 434–438.

[23] E.A. Rakha, M.E. El-Sayed, A.R. Green, A.H. Lee, J.F. Robertson and I.O. Ellis, Prognostic markers in triple-negative breast cancer, *Cancer* **109** (2007), 25–32.

[24] J.S. Reis-Filho and A.N. Tutt, Triple negative tumours: a critical review, *Histopathology* **52** (2008), 108–118.

[25] R. Rouzier, C.M. Perou, W.F. Symmans, N. Ibrahim, M. Cristofanilli, K. Anderson, K.R. Hess, J.Stec, M. Ayers, P. Wagner, P. Morandi, C. Fan, I. Rabiul, J.S. Ross, G.N. Hortobagyi and L. Pusztai, Breast cancer molecular subtypes respond differently to preoperative chemotherapy, *Clin. Cancer Res.* **11** (2005), 5678–5685.

[26] I. Skaland, E.A. Janssen, E. Gudlaugsson, J. Klos, K.H. Kjellevold, H. Søiland and J.P. Baak, Phosphohistone H3 expression has much stronger prognostic value than classical prognosticators in invasive lymph node-negative breast cancer patients less than 55 years of age, *Mod. Pathol.* **20** (2007), 1307–1315.

[27] I. Skaland, E.A. Janssen, E. Gudlaugsson, J. Klos, K.H. Kjellevold, H. Søiland and J.P. Baak, Validating the prognostic value of proliferation measured by Phosphohistone H3 (PPH3) in invasive lymph node-negative breast cancer patients less than 71 years of age, *Breast Cancer Res. Treat.* (2008) Mar. 29. [Epub ahead of print.]

[28] I. Skaland, P.J. van Diest, E.A. Janssen, E. Gudlaugsson and J.P. Baak, Prognostic differences of World Health Organization – assessed mitotic activity index and mitotic impression by quick scanning in invasive ductal breast cancer patients younger than 55 years, *Hum. Pathol.* **39** (2008), 584–590.

[29] T. Sorlie, R. Tibshirani, J. Parker, T. Hastie, J.S. Marron, A. Nobel, S. Deng, H. Johnsen, R. Pesich, S. Geisler, J. Demeter, C.M. Perou, P.E. Lønning, P.O. Brown, A.L. Børresen-Dale

and D. Botstein, Repeated observation of breast tumor subtypes in independent gene expression data sets, *Proc. Natl. Acad. Sci. USA* **100** (2003), 8418–8423.

[30] T. Sørlie, C.M. Perou, R. Tibshirani, T. Aas, S. Geisler, H. Johnsen, T. Hastie, M.B. Eisen, M. van de Rijn, S.S. Jeffrey, T. Thorsen, H. Quist, J.C. Matese, P.O. Brown, D. Botstein, P. Eystein Lønning and A.L. Børresen-Dale, Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications, *Proc. Natl. Acad. Sci. USA* **98** (2001), 10869–10874.

[31] C. Sotiriou and M.J. Piccart, Taking gene-expression profiling to the clinic: when will molecular signatures become relevant to patient care, *Nat. Rev. Cancer* **7** (2007), 545–553.

[32] D.S. Tan, C. Marchió, R.L. Jones, K. Savage, I.E. Smith, M. Dowsett and J.S. Reis-Filho, Triple negative breast cancer: molecular profiling and prognostic impact in adjuvant anthracycline-treated patients, *Breast Cancer Res. Treat.* (2007) Oct. 6 [Epub].

[33] F.A. Tavassoli and P. Devilee, Pathology and genetics: Tumours of the breast and female genital organs, WHO classification of tumours series, IARC Press, Lyon, 2003, pp. 9–112.

[34] P.J. van Diest, E. van der Wall and J.P. Baak, Prognostic value of proliferation in invasive breast cancer: a review, *J. Clin. Pathol.* **57** (2004), 675–681.

[35] V. Vuaroqueaux, P. Urban, M. Labuhn, M. Delorenzi, P. Wirapati, C.C. Benz, R. Flury, H. Dieterich, F. Spyratos, U. Eppenberger and S. Eppenberger-Castori, Low E2F1 transcript levels are a strong determinant of favorable breast cancer outcome, *Breast Cancer Res.* **9** (2007), R33.