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Chapter 2

The prognostic value of apoptotic and proliferative markers in breast cancer

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ABSTRACT

Introduction

Increasing ability of early breast cancer diagnosis leading to more early stage detection, better survival and low relapse marks one of the milestones achieved over the decades. Foregoing poses a challenge for clinicians regarding optimal treatment, in which over- and under-treatment should be avoided. Classical prognostic and predictive factors fall short for individualized adjuvant therapy selection in this patient group. The key to better characterization may be found in the biology underlying individual tumors. We hypothesized that markers related to cellular proliferation and apoptosis and the balance between these two processes in tumor development will be predictive for clinical outcome.

Material and Method

Our study population (n=822) consisted of all early stage breast cancer patients primarily treated with surgery in our center between 1985-1996. Sections of available tumor tissue (87%, 714/822) were immunohistochemically stained for expression of p53, active-caspase-3 and Ki67. In 43% (304/714) and 18% (126/714) of this cohort respectively a biochemical C2P® risk prediction and caspase-3 assay were performed.

Results

Expression data of the mentioned markers, single or combined, were analyzed. Results showed that both single and combined markers, whether of apoptotic or proliferative origin had associations with clinical outcome. An additive effect was seen for the hazard ratios when data on p53, active caspase-3 and Ki67 status were combined. The assembled prognostic apoptotic-proliferative subtype showed significant association for both the OS (p=0.024) and RFP (p=0.001) in the multivariate analyses of grade I breast tumors.

Conclusion

Combined markers of tumor cell apoptosis and proliferation represents tumor aggressiveness. The apoptotic-proliferative subtypes that we present in this study represent a clinical prognostic profile with solid underlying biological rationale and poses a promising method for accurate identification of grade I breast cancer patients in need of an aggressive therapeutic approach, thus contributing to precision medicine in breast cancer disease.

INTRODUCTION

The introduction of population-based screening for breast cancer (BC) with the aid of mammography led to a shift towards early-stage (<2cm) node-negative BC detection with better prognosis [1]. This development contributed to a continuous decline in BC-related deaths despite the increasing incidence of BC in developed countries over the past decades. Nevertheless BC still remains one of the leading causes of cancer death in women in the western world [1].

Early diagnosis poses a challenge for clinicians regarding optimal treatment. With a relatively low relapse rate in patients detected with early BC, individual estimation of the therapeutic benefit for these patients is of crucial importance, in which over- and under-treatment has to be avoided. Defining individual tumor-specific characteristics could lend a helping hand in this consideration.

Classical prognostic and predictive factors like tumor size, histology, tumor grade, lymph node and hormone receptor status are routinely assessed for every BC patient. Nonetheless, characterizing the tumor by identification of new or additional (bio)markers may lead to a better insight into the tumor biology and thus to its clinical behavior.

It is widely accepted that the presence of certain local factors determine tumor development, such as angiogenesis and the level of tumor cell proliferation and apoptosis. The inability to undergo apoptosis is thought to contribute to tumorigenesis and tumor progression [2]. Recent work showed that identification of the proliferation marker Ki67 proved to be of fixed prognostic value, even in an independent fashion [3;4]. Bearing in mind that healthy tissue signifies a fine proliferative-apoptotic balance, we propose that tumor growth may be more accurately determined by the outcome of the balance between tumor cell proliferation on one side and apoptosis on the other. It is for this reason that we in this study aimed to identify clinically relevant biomarkers quantifying apoptosis and proliferation in breast tumors, which could be of major prognostic and predictive value. To achieve this we assessed the presence of p53, active caspase-3 and the proliferative markers Ki67 and C2P® (Sysmex, Kobe,Japan) in post-operative tumor material of early stage BC patients. Lastly, we constructed an apoptotic-proliferative subtype risk model based on the combination and rate of expressed markers. Reporting was done according to the REMARK criteria [5].

PATIENTS AND METHODS

Patients and tumors

Our retrospectively analyzed patient population comprised of all non-metastasized BC patients primarily treated with surgery, with or without adjuvant systemic therapy in the Leiden University Medical Center between 1985 and 1996 (n=822). Exclusion criteria were bilateral tumors or a prior history of cancer (other than basal cell carcinoma or cervical carcinoma *in situ*). The following data were known: age at diagnosis, tumor grade, histological tumor type, TNM stage, time of locoregional/distant tumor recurrence, survival time and expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) [6]. Perioperative formalin fixed paraffin embedded (FFPE) tumor material was used for immunohistochemistry (IHC) and fresh frozen tumor material for biochemical assays. An experienced BC pathologist (VS) graded all tumors according to current pathological standards. All samples were handled in a coded fashion, according to national ethical guidelines ("Code for Proper Secondary Use of Human Tissue", Dutch Federation of Medical Scientific Societies).

Immunohistochemistry

Stainings were performed according to previously described standard protocols [7]. For each staining, all sections were stained simultaneously to avoid inter-assay variation. Mouse monoclonal antibodies against p53 protein (M700101 clone D-07: Dako, NL, 0.01M EDTA buffer (pH 8.0)) and Ki67 (M7240 Clone MIB-1: Dako, NL, 0.01M EDTA buffer (pH 8.0)) were used. For active caspase-3 detection an immunohistochemical staining was performed with antibodies directed against cleaved caspase-3 (Anti-Asp175 #9661: Cell Signaling, USA, citrate buffer 0.1M (pH 6.0)). Tonsil and colorectal carcinoma sections served as positive control for p53, Ki67 and active caspase-3 staining respectively. Negative controls underwent the whole immunohistochemical staining without primary antibodies.

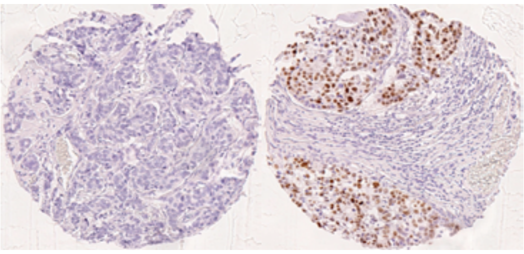
Evaluation of immunostaining

Two independent observers performed quantification of p53-, active caspase-3- and Ki67-positive stained cells in a blinded manner. For p53 the percentage positive stained nuclei of tumor cells were microscopically assessed by determining the mean percentage in all three punches of the TMA. Categorization was made by dividing the mean percentage scores into: wildtype (≤50% positive nuclei in the tumor material) and mutant pattern of staining (>50% expression of tumor nuclei stained positive for p53 (figure 1A)) [8].

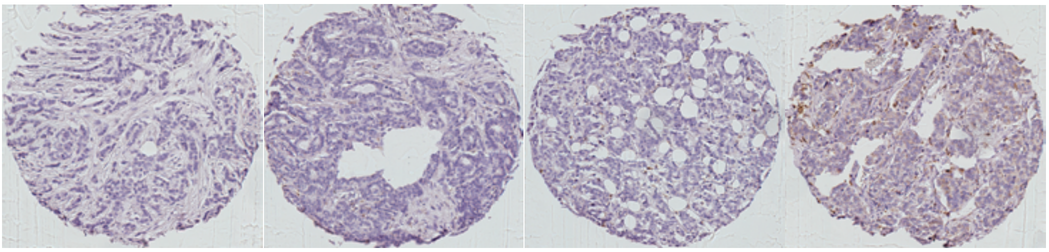
For active caspase-3 the mean expression grade of positively stained cells in the TMA was defined: absent (expression grade: 0-0.49 positive cells), low (expression grade: 0.5-1.49 positive cells), intermediate (expression grade: 1.5-2.49 positive cells) and high scores, corresponding with a mean expression of >2.5 positive cells in the tumor material (figure 1B).

Ki67 expression was divided into absent (0%) and present (>1%) positively stained nuclei, based on the mean percentage of all three-tumor punches per patient (figure 1C).

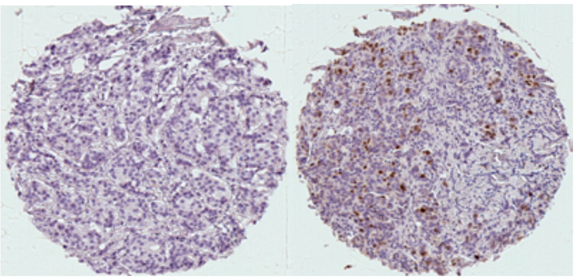
Figure 1:



A: Immunohistochemical p53 staining; left: wildtype staining pattern (≤ 50% of nuclei); right: mutant staining pattern (>50% of the tumor nuclei).



B: Immunohistochemical active caspase-3 staining; from left to right: negative (<0.49 positive cells), low (0.5-1.49 positive cells), intermediate (1.5-2.49 positive cells) and high (>2.49 positive cells) expression in human breast tumor (cut-off points: mean expression of active caspase-3 in three breast cancer tissue cores).



C: Immunohistochemical Ki67 staining; left: absent (0%) and right: present (>1%) staining in human breast tumor.

C2P® risk prediction score assay

C2P® risk prediction scores (C2P®-RS) is a proliferation assay developed by Sysmex Corporation which is based on cyclin-dependent kinase (CDK) 1 and CDK2, both playing a pivotal role in cell cycle regulation [9]. Risk prediction scores are based not only on CDK1 and CDK2 presence in the tumor material but also on the enzyme activity rate [9]. CDK1 and CDK2 assays were performed using frozen tissue samples. Subsequently, the C2P®-RS was calculated using a predetermined formula, after which the tumors were divided into three categories (high, intermediate and low RS groups) [9]. For a detailed assay protocol see manuscript by Kim *et al* [9].

Active caspase-3 assay

Biochemical quantification of active caspase-3 was determined in 18% of the BC patients (126/714). The enzymatic activity of caspase-3 was obtained by lysing ten 10μm thick cryostat sections per sample in 500μL lysis buffer containing 10mM HEPES, pH7.0, 40mM β-glycerophosphate, 50mM NaCl, 2mM MgCl2 and 5mM EGTA, followed by 10 minutes of homogenization using a Polytron homogenisator (PT-MR 2100, Kinematica, Luzern, Switzerland) and four freeze-thaw cycles before storing it at -80 degrees Celsius. Protein concentration was determined using the Bradford method [10]. For measurements of caspase-3 enzyme activity, 50 μL of each sample was incubated with 5 μL of 1mM substrate Ac-DEVD-AFC (A0466-1MG, Sigma Aldrich, USA) in a 100mM HEPES buffer, pH 7.25, containing 10% sucrose, 0.1% (v/v) Nonidet-P40 and 10mM dithiothreitol (DTT; D0632, Sigma Aldrich, USA) for two hours at 37˚C. During incubation at 37˚C, fluorescent AFC was cleaved off by active caspase-3, corresponding with the level of caspase-3-activity per sample. Fluorescent AFC absorbance was monitored in a fluorometer equipped with a 400-nm excitation filter and 505-nm emission filter at time-point: 00.00 hours and again at time-point: 02.00 hours. Calibration curves were prepared by plotting the values of free-AFC standard absorbance versus concentration in nmol/L.

Caspase-3 activity was indicated in pmolAFC/min/mg protein.

Statistical analysis

Statistical analyses were performed using the statistical package SPSS (version 20.0 IBM SPSS Statistics). Patients with missing data, mostly due to material handling were excluded from statistical analysis. Cohen's kappa coefficient was used to assess the inter-observer agreement in quantification of p53, active Caspase-3 and Ki67 expression. The χ^2 test was used to evaluate associations between various clinicopathological parameters and apoptotic and proliferative markers in the tumor material. The clinical endpoints examined were Relapse-Free Period (RFP), defined as the time from surgery until an event (locoregional recurrence and/or a distant recurrence, whichever came first) and Overall Survival (OS), defined as the time from surgery until death by any reason. The Kaplan–Meier method was used for survival plotting and log-rank test for comparison of RFP and OS curves. Cox proportional hazard analysis was used for univariate and multivariable analysis for RFP and OS. Variables with a p-value of < 0.1 in univariate analysis were entered in multivariable analysis.

In order to compare the agreement of the different techniques used for caspase-3 (IHC and biochemical assay) estimation, a Spearman's Rho correlation test was performed.

RESULTS

Patient and tumor characteristics

Perioperative tumor material was available of 87% (714/822) of the patients. The median age of this cohort was 58 years (range= 23-96 years) with a median follow-up of 10 years (range= 0.02-22years) (clinicopathological characteristics: table 1A and 1B). Good inter-observer agreement was seen (≥0.6) using the Cohen's kappa coefficient for quantification of immune-stained markers.

p53 expression

Immunohistochemical data for p53 expression was available for 80% (574/714) of the patients. Mutant p53 was significantly present in patients with more advanced pathological tumor stages (p<0.001), more advanced TNM stage (p=0.033), higher tumor grades (p<0.001) and ductal tumors (p=0.017) (table 1A). Tumors with adverse hormonal characteristics: Estrogen Receptor (ER) negative *(-)*, Progesterone Receptor (PGR) negative *(-)* and Human Epidermal Growth Factor Receptor-2 (HER-2) positive *(+)* are significantly associated with mutant p53 protein (ER: p=0.013; PGR: p=0.004 and HER2: p<0.001) (table 1A).

Analysis of the OS showed a statistical significant association between mutant p53 and survival outcome of patients (p<0.001, Hazard Ratio (HR): 2.150, 95% Confidence Interval (CI): 1.549-2.983; table 2A), also remaining an independent prognostic marker in multivariable analysis (p=0.009, HR: 1.776, 95%CI: 1.158-2.726). The explanation hereof lies in the fact that mutated p53 protein cannot be cleared away in the tumor cell leading to high amounts of inactive p53 stacking which is often seen to a greater extent in more aggressive tumor types since no apoptosis is induced [11]. For relapse free period (RFP) a significant relation was seen for mutant p53 in the univariate analysis only (p=0.002, HR: 1.838, 95%CI: 1.255-2.692) (figure 2A and table 2B).

Active caspase-3 expression

Data of active caspase-3 IHC was available for 80% (575/714) of the BC patients. Tumors in which determination of both active caspase-3 IHC expression and caspase-3 biochemical enzymatic activity was performed (N=106), comparison analyses showed excellent agreement (p=0.011). There was significant association between active caspase-3 expression in IHC and higher pathological tumor stage (p<0.001), more advanced TNM stage (p<0.001), higher tumor grade (p<0.001), ductal tumor histology (p<0.001), and a statistical trend was seen for lymph node involvement (p=0.065) (table 1A). ER negative, PGR negative and HER2 over-expressing tumors are related to high caspase-3 expression with *p*-values of <0.001, p=0.002 and p=0.002 respectively (table 1A). Additional analyses showed a close relationship between caspase-3 expression and Ki67 expression

Table 1A: Clinicopathologic characteristics of the patient population stratified for the tumor suppressor p53 protein and the apoptotic marker active caspase-3

	p53 wildtype		p53 mutant		*p-value*	caspase-3 Negative		caspase-3 Low		caspase-3 Intermediate		caspase-3 High		*p-value*
	N	%	N	%		N	%	N	%	N	%	N	%	
Total	522	100	52	100		177	100	177	100	121	100	100	100	
Age (y)														
<45	106	20.3	8	15.4	0.298	36	20.3	31	17.5	28	23.1	19	19.0	0.617
45-55	128	24.5	10	19.2		51	28.8	39	22.0	23	19.0	24	24.0	
55-65	113	21.6	17	32.7		38	21.5	45	25.4	26	21.5	20	20.0	
>65	175	33.5	17	32.7		52	29.4	62	35.0	44	36.4	37	37.0	
Missing	0		0			0		0		0		0		
Histological type														
Ductal	465	90.1	52	100	**0.017**	146	83.9	164	94.3	112	93.3	97	98.0	**<0.001**
Lobular	51	9.9	0	0		28	16.1	10	5.7	8	6.7	2	2.0	
Missing	6		0			3		3		1		1		
Grade														
I	87	16.9	2	3.8	**<0.001**	51	29.3	25	14.4	10	8.3	3	3.0	**<0.001**
II	266	51.8	10	19.2		92	52.9	98	56.3	53	44.2	31	31.3	
III	161	31.3	40	76.9		31	17.8	51	29.3	57	47.5	65	65.7	
Missing	8		0			3		3		1		1		
Tumor stage														
pT1	216	42.3	11	21.6	**<0.001**	90	52.0	85	48.6	33	27.7	20	20.8	**<0.001**
pT2	245	47.9	26	51.0		69	39.9	76	43.4	73	61.3	55	57.3	
pT3/4	50	9.8	14	27.5		14	8.1	14	8.0	13	10.9	21	21.9	
Missing	11		1			4		2		2		4		
Nodal stage														
pN0	271	53.0	22	44.9	0.276	108	62.1	98	56.6	51	43.2	44	45.8	0.065
pN+	240	47.0	27	55.1		66	37.9	75	43.4	67	56.8	52	54.2	
Missing	11		3			3		4		3		4		
TNM stage														
Stage 0	0	0	0	0	**0.033**	0	0	0	0	0	0	0	0	**<0.001**
Stage I	135	28.7	8	17.0		67	41.4	52	32.7	22	19.6	8	9.4	
Stage IIA	158	33.6	12	25.5		52	32.1	53	33.3	29	25.9	34	40.0	
Stage IIB	112	23.8	13	27.7		25	15.4	35	22.0	42	37.5	23	27.1	
Stage IIIA	24	5.1	4	8.5		7	4.3	7	4.4	9	8.0	5	5.9	
Stage IIIB	15	3.2	5	10.6		2	1.2	6	3.8	5	4.5	6	7.1	
Stage IIIC	26	5.5	5	10.6		9	5.6	6	3.8	5	4.5	9	10.6	
Stage IV	0	0	0	0		0	0	0	0	0	0	0	0	
Missing	14		5			15		18		9		15		
ER receptor														
Negative	210	40.8	30	58.8	**0.013**	54	31.2	55	32.2	57	47.5	63	63.6	**<0.001**
Positive	305	59.2	21	41.2		119	68.8	116	67.8	63	52.5	36	36.4	
Missing	7		1			4		6		1		1		
PGR receptor														
Negative	231	45.7	34	66.7	**0.004**	75	43.6	64	38.1	60	50.0	61	61.6	**0.002**
Positive	275	54.3	17	33.3		97	56.4	104	61.9	60	50.0	38	38.4	
Missing	16		1			5		9		1		1		
HER-2 overexpression														
No overexpression	419	91.3	33	73.3	**<0.001**	129	91.5	147	96.1	95	85.6	77	82.8	**0.002**
Overexpression	40	7.9	12	26.7		12	8.5	6	3.9	16	14.4	16	17.2	
Missing	63		7			36		24		10		7		

Table 1B: Clinicopathologic characteristics of the patient population stratified for the proliferative Ki67 marker and proliferative C2P assay

	Ki67 Low		**Ki67 High**		***p-value***	**C2P® Low**		**C2P® intermediate**		**C2P® High**		***p-value***
	N	**%**	**N**	**%**		**N**	**%**	**N**	**%**	**N**	**%**	
Total	299	100	257	100		69	100	22	100	83	100	
Age (y)												
<45	52	17.4	53	20.6	0.523	8	11.6	3	13.6	24	28.9	0.064
45-55	71	23.7	60	23.3		11	15.9	5	22.7	19	22.9	
55-65	66	22.1	63	24.5		21	30.4	4	18.2	17	20.5	
>65	110	36.8	81	31.5		29	42.0	10	45.5	23	27.7	
Missing	0		0			0		0		0		
Grade												
I	70	24.0	17	6.6	**<0.001**	8	11.6	2	9.1	9	10.8	**0.004**
II	166	56.8	99	38.7		38	55.1	15	68.2	31	37.4	
III	56	19.2	140	54.7		23	33.3	5	22.7	43	51.8	
Missing	7		1			0		0		0		
Histologic type												
Ductal	251	85.7	245	95.7	**<0.001**	62	89.9	18	81.8	77	92.8	0.326
Lobular	42	14.3	11	4.3		7	10.1	4	18.2	6	7.2	
Missing	6		1				0.0		0.0		0.0	
Tumor stage												
pT1	129	44.2	83	32.8	0.088	19	27.9	7	31.8	21	25.3	0.121
pT2	129	44.2	136	53.8		41	60.3	13	59.1	45	54.2	
pT3/4	34	11.6	34	13.4		8	11.8	2	9.1	17	20.5	
Missing	7		4			1		0		0		
Nodal stage												
pN0	167	57.4	114	45.6	0.102	36	53.7	12	54.5	31	37.8	0.242
pN+	124	42.6	136	54.4		31	46.3	10	45.5	51	62.2	
Missing	8		7			2		0		1		
TNM stage												
Stage 0	0	0	0	0	0.066	0	0	0	0	0	0	0.052
Stage I	80	30.4	53	22.2		12	19.0	4	19.0	11	14.1	
Stage IIA	93	35.4	75	31.4		26	41.3	11	52.4	23	29.5	
Stage IIB	58	22.1	63	26.4		14	22.2	3	14.3	25	32.1	
Stage IIIA	12	4.6	18	7.5		6	9.5	1	4.8	7	9.0	
Stage IIIB	7	2.7	14	5.9		3	4.8	1	4.8	10	12.8	
Stage IIIC	13	4.9	16	6.7		2	3.2	1	4.8	2	2.6	
Stage IV	0	0	0	0		0	0	0	0	0	0	
Missing	36					6		1		5		
ER receptor												
Negative	120	40.5	117	47.2	0.120	29	42.6	6	28.6	44	57.1	**0.049**
Positive	176	59.5	131	52.8		39	57.4	15	71.4	33	42.9	
Missing	3		9			1		1		6		
PGR receptor												
Negative	126	43.2	134	54.3	**0.010**	33	49.3	12	57.1	47	60.3	0.364
Positive	166	56.8	113	45.7		34	50.7	9	42.9	31	39.7	
Missing	7		10			2		1		5		
HER-2 overexpression												
No overexpression	246	92.8	190	86.4	**0.019**	52	85.2	18	94.7	58	84.1	0.251
Overexpression	19	7.2	30	13.6		9	14.8	1	5.3	11	15.9	
Missing	34		37			8		3		14		

in the same tumor material (p=0.001, data not shown), indicating that proliferation and apoptosis are closely linked within the tumor and thus should be accounted for if one seeks optimal prognostic-predictive value determination. Survival analysis showed that a higher caspase-3 expression is significantly associated with worse OS (p<0.001, HR: 1.908, 95%CI: 1.407-2.588, table 2A), however not remaining an independent prognostic factor after multivariate correction (p=0.414). For RFP a significant relation was found for high caspase-3 expression and relapse rate (p<0.001, HR: 1.943, 95%CI: 1.356-2.783, figure 2B), again not maintaining individual prognostic value in the multivariate correction (p=0.366), (table 2B).

Ki67 expression

Ki67 expression data were available for 78% (556/714) of the patients. No relation was seen for Ki67 expression in the tumor and tumor stage or nodal involvement (table 1B). However, for high tumor grades and tumors of ductal histology (both p<0.001), PGR negative (p=0.01) and HER2 over-expressing tumors (p=0.019) a significant association was found with high Ki67 expression, corresponding with a high proliferative rate (table 1B). A statistical trend was seen for TNM stage and high Ki67 expression (p=0.066).

Patients with high Ki67 tumor expression had worse OS (p=0.007, HR: 1.348, 95%CI: 1.086-1.673), however losing its significance in the multivariate correction (p=0.564) (table 2A). A significantly higher relapse rate was noted for high Ki67 expression compared to low proliferation rate in the tumor material (p=0.021, HR: 1.339, 95%CI: 1.045-1.716, figure 2C). High Ki67 did not remain significantly associated with a higher relapse rate in the multivariate correction (p=0.269, table 2B).

C2P® risk prediction score

Data previously published by our group already described the C2P® risk prediction score as a promising prognostic marker in early BC patients [12]. Using the same cohort, 43% (304/714) of the patients had tumor material available for C2P® analyses. Significance was found for high C2P® risk score and tumor grade III scores (p=0.004), young age (<55years of age, p=0.020) and ER positive tumors (p=0.049) (table 1B). A statistical trend was seen for TNM stage (p=0.052).

No statistical relation was seen for C2P® and OS (p=0.263) (table 2A). High C2P® risk scores were significantly associated with higher relapse rates (p=0.026, HR: 1.953, 95%CI: 1.199-3.181), however not remaining its significance in the multivariate correction (figure 2D and table 2B).

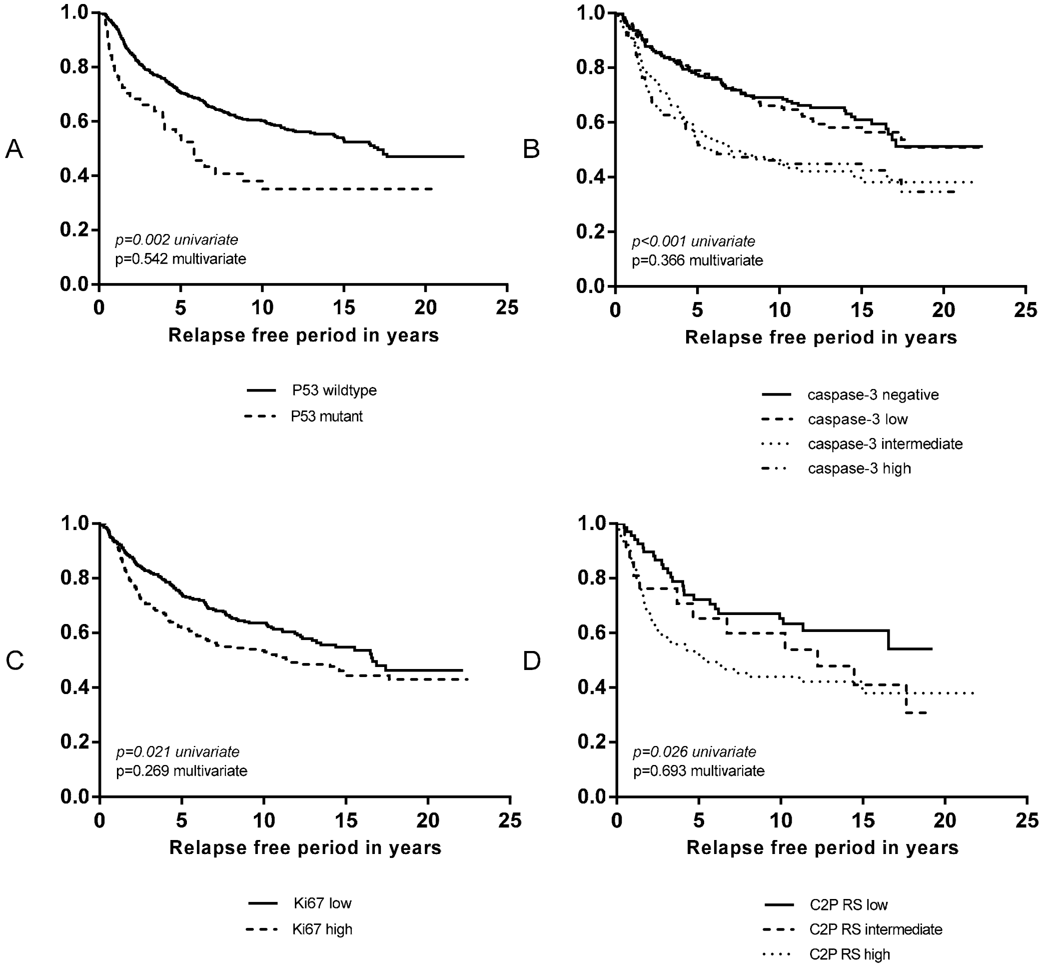


Figure 2: Relapse Free Period (RFP) curves for; **A)** tumor suppressor p53 expression **B)** active caspase-3 expression **C)** proliferative Ki67 expression and **D)** C2P®-Risk Score proliferation assay.

Combined IHC data

P53 - Ki67

From 72% (516/714) of the patients immunohistochemical data was available for both p53 and Ki67, making them eligible for the determination of the prognostic value of a combined p53-Ki67 marker. Significance was found in relation with OS and RFP, where high Ki67 combined with mutant p53 expression had the worse clinical outcome (OS: p<0.001, HR: 2.458, 95%CI: 1.654-3.655 (table 3A) and RFP: p=0.003, HR: 2.307, 95%CI: 1.479-3.598 (table 3B)) compared to a HR of 1.00 in low Ki67 combined with wildtype p53 protein expression. All other combinations of p53 and Ki67 data showed hazard ratios ranging between: >1.00 and <2.396 for the OS and >1.00 and <1.327 for the RFP. However, in the multivariate analysis for OS only the combination low Ki67 expression and mutant p53 remained significant (OS: p=0.037, table 3A).

Table 2A: Multivariable analyses for single apoptotic and proliferative markers in relation to overall survival

Characteristic	Overall Survival							
	Univariate analysis					Multivariable analysis		
	N	%	HR	95% CI	*P*	HR	95% CI	*P*
Age								
<45	137	19.2	1.00		**<0.001**	1.00		**<0.001**
45-55	175	24.5	0.789	0.559-0.115		0.696	0.446-1.084	
55-65	157	22.0	1.469	1.062-2.032		1.374	0.910-2.072	
>65	245	34.3	1.914	1.914-3.395		2.185	1.499-3.185	
Grade								
I	116	16.5	1.00		**<0.001**	1.00		0.718
II	342	48.7	1.380	1.012-1.879		1.057	0.679-1.645	
III	244	34.8	1.844	1.345-2.527		1.184	0.721-1.943	
Histological type								
Ductal	638	90.6	1.00		0.125			
Lobular	66	9.4	0.778	0.565-1.072				
Tumor stage								
pT1	289	41.6	1.00		**<0.001**	1.00		**0.003**
pT2	328	47.3	1.836	1.471-2.292		1.354	0.984-1.864	
pT3	44	6.3	2.072	1.390-3.089		1.696	0.986-2.915	
pT4	33	4.8	5.573	3.764-8.251		2.809	1.628-4.847	
Nodal stage								
Negative	381	54.9	1.00		**<0.001**	1.00		**<0.001**
Positive	313	45.1	2.105	1.725-2.568		1.783	1.360-2.338	
ER status								
Negative	288	42.3	1.00		0.266			
Positive	393	57.7	0.892	0.730-1.091				
PGR status								
Negative	316	47.4	1.00		**0.049**	1.00		0.948
Positive	351	52.6	0.818	0.670-0.999		1.009	0.768-1.327	
HER-2 status								
Negative	520	89.8	1.00		**<0.001**	1.00		**0.047**
Positive	59	10.2	1.861	1.359-2.548		1.511	1.006-2.269	
P53								
Wildtype	522	90.9	1.00		**<0.001**	1.00		**0.009**
Mutant	52	9.1	2.150	1.549-2.983		1.776	1.158-2.726	
C2P®								
Low	69	39.7	1.00		0.263			
Intermediate	22	12.6	0.951	0.498-1.816				
High	83	47.7	1.355	0.901-2.037				
Caspase3								
Absent	177	30.8	1.00		**<0.001**	1.00		0.414
Low	177	30.8	0.975	0.727-1.306		0.760	0.529-1.091	
Intermediate	121	21.0	1.575	1.167-2.128		0.957	0.668-1.370	
High	100	17.4	1.908	1.407-2.588		0.984	0.669-1.447	
Ki67								
Low	299	53.7	1.00		**0.007**	1.00		0.564
High	257	46.3	1.348	1.086-1.673		1.089	0.816-1.453	

Table 2B: Multivariable analyses for single apoptotic and proliferative markers in relation to relapse free period

Characteristic	**Relapse Free Period**							
	Univariate analysis					**Multivariable analysis**		
	N	**%**	**HR**	**95% CI**	***P***	**HR**	**95% CI**	***P***
Age								
<45	137	19.2	1.00		0.357			
45-55	175	24.5	0.755	0.547-1.042				
55-65	157	22.0	0.898	0.648-1.246				
>65	245	34.3	0.824	0.605-1.122				
Grade								
I	116	16.5	1.00		**<0.001**	1.00		0.845
II	342	48.7	1.460	1.013-2.106		0.927	0.454-1.894	
III	244	34.8	2.158	1.490-3.125		0.816	0.373-1.783	
Histological type								
Ductal	638	90.6	1.00		0.209			
Lobular	66	9.4	1.265	0.877-1.824				
Tumor stage								
pT1	289	41.6	1.00		**<0.001**	1.00		**0.046**
pT2	328	47.3	1.716	1.336-2.203		1.227	0.723-2.081	
pT3	44	6.3	1.955	1.242-3.078		0.767	0.277-2.127	
pT4	33	4.8	4.011	2.476-6.499		3.634	1.521-8.680	
Nodal stage								
Negative	381	54.9	1.00		**<0.001**	1.00		**<0.001**
Positive	313	45.1	2.964	2.349-3.739		2.462	1.519-3.991	
ER status								
Negative	288	42.3	1.00		0.377			
Positive	393	57.7	0.901	0.716-1.135				
PGR status								
Negative	316	47.4	1.00		0.235			
Positive	351	52.6	0.870	0.691-1.095				
HER-2 status								
Negative	520	89.9	1.00		**0.002**	1.00		0.811
Positive	59	10.1	1.772	1.229-2.555		0.909	0.417-1.981	
P53								
Wildtype	522	90.9	1.00		**0.002**	1.00		0.542
Mutant	52	9.1	1.838	1.255-2.692		1.288	0.571-2.906	
C2P®								
Low	69	39.7	1.00		**0.026**	1.00		0.693
Intermediate	22	12.6	1.638	0.822-3.264		0.807	0.443-1.468	
High	83	47.7	1.953	1.199-3.181		1.363	0.550-3.377	
Caspase3								
Absent	177	30.8	1.00		**<0.001**	1.00		0.366
Low	177	30.8	1.060	0.754-1.489		1.208	0.613-2.381	
Intermediate	121	21.0	1.860	1.323-2.615		1.564	0.815-3.004	
High	100	17.4	1.943	1.356-2.783		1.865	0.849-4.099	
Ki67								
Low	299	53.7	1.00		**0.021**	1.00		0.269
High	257	46.3	1.339	1.045-1.716		1.304	0.815-2.087	

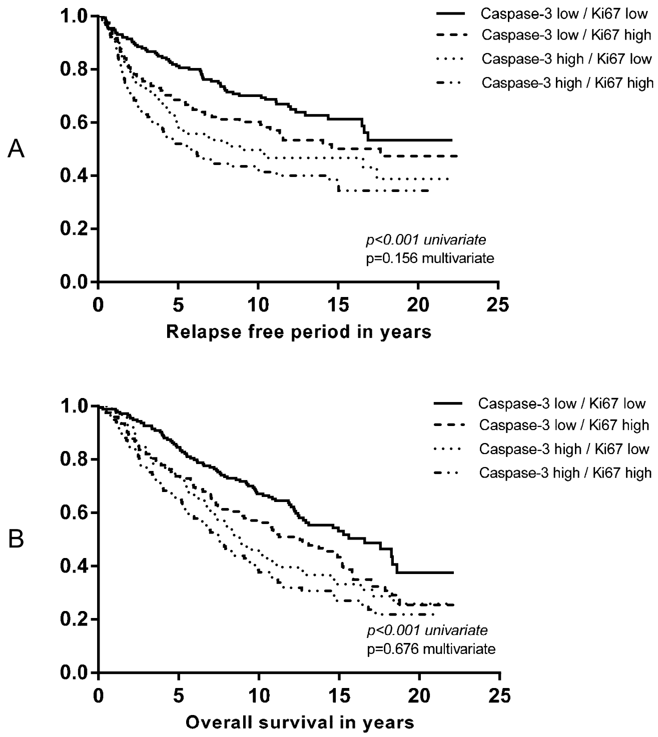


Figure 3: A) Relapse Free Period (RFP) curves for combined analysis of active caspase-3 and the proliferative marker Ki67. Both single markers were grouped into low or high expression in the tumor tissue (for active caspase-3 the division was made based on the RFP curve seen in Figure 2B) after which they were combined. **B)** The same was done for the Overall Survival (OS) curves for this combined marker.

When we compared the highest hazard ratios of the single markers for p53 (OS HR: 2.150 and RFP HR: 1.838) and Ki67 (OS HR: 1.348 and RFP HR: 1.339), we concluded that by combining these two markers in one combination (p53-Ki67) we induce additive strength to his prognostic-predictive marker, leading to a higher hazard ratio (OS HR: 2.458 and RFP HR: 2.307) than the single biomarker hazard ratios (table 4A and 4B).

P53 - active caspase-3

Seventy four percent (529/714) of the patients had both p53 and active caspase-3 IHC data available. Again for both OS and RFP significance was found with the combined p53-caspase-3 biomarker. Mutant p53 protein expression combined with high active caspase-3 expression resulted in the highest HR for death in OS ($p<0.001$, HR: 3.012, 95%CI: 2.044-4.439, table 3A) and the RFP ($p<0.001$, HR: 2.673, 95%CI: 1.703-4.195, table 3B). For the OS this remained an independent prognostic biomarker after multivariate correction ($p=0.037$, HR: 2.008, 95%CI: 1.241-3.249, table 3A).

Again a higher hazard ratio (HR OS: 3.012 and HR RFP: 2.673) was seen when patients with the clinically most adverse expression pattern of single markers p53 (OS HR: 2.150 and RFP HR: 1.838) and active caspase-3 (OS HR: 1.908 and RFP HR: 1.943) were compared to the HR of the combined p53-caspase-3 marker, indicating the probability of an additive quality (table 4A and 4B).

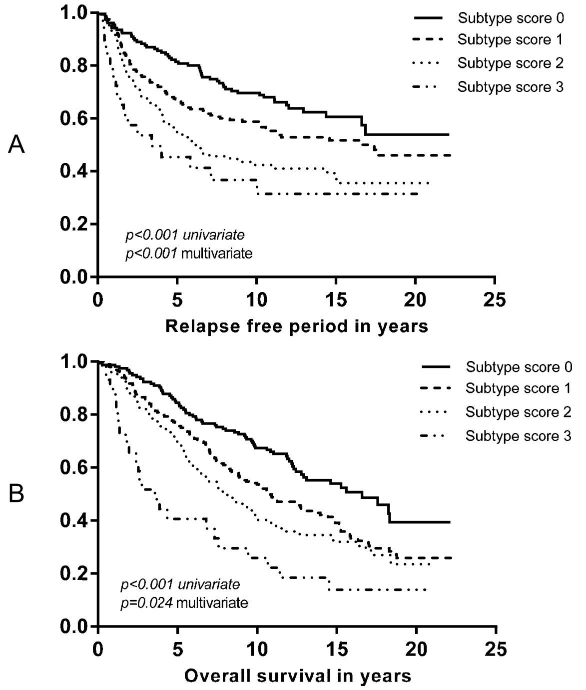


Figure 4: Apoptotic-proliferative tumor subtypes: all curves and the univariate p-values are based on the entire patient population in whom all markers (p53, active caspase-3 and Ki67) are known. *Multivariate p-values are based on only grade I breast tumors from this cohort.

Ki67 - active caspase-3

Data of both Ki67 and active capase-3 expression was available from 33% (239/714) of the patients of this cohort. Both high expression of Ki67 and active caspase-3 had a significant worse OS (p<0.001, HR: 3.012, 95%CI: 2.044-4.439 (table 3A)) and RFP (p<0.001, HR: 2.258, 95%CI: 1.599-3.189 (table 3B)) compared to low Ki67 with low Caspase-3 expression (figure 3A and 3B). In the multivariate analyses, neither the RFP (p=0.156) nor the OS (p=0.676) remained an individual prognostic marker. Again additive properties were seen for the combined biomarker: Ki67-active-caspase3 (OS HR: 2.137 and RFP HR: 2.258), compared to the single biomarkers (Ki67**:** HR-OS: 1.348 and HR-RFP: 1.339; Caspase-3: HR-OS: 1.908 and HR-RFP: 1.943) (table 4A and 4B).

C2P® in combination with p53 or active caspase-3 or Ki67

Neither p53 (20% (142/714)), active caspase-3 (21% (147/714)) nor Ki67 (21% (150/714)) combined with C2P®-RS showed a statistical significant relation with outcome.

Apoptotic - proliferative tumor subtype

Due to the supporting outcome of the combined markers, we constructed a prognostic model based on the expression pattern of the three risk contributing markers: p53, active caspase-3 and Ki67(488/714, 68%). C2P® was not included in this model due to the limited number of patients in whom this marker was determined (frozen tumor tissue was needed), leading to lack of power in the combined analysis. Expression scores of

Table 3A: Multivariable analyses for combined apoptotic and proliferative markers in relation to overall survival

Characteristic	Overall Survival combination(s)							
	Univariate analysis					Multivariable analysis		
	N	%	HR	95% CI	*P*	HR	95% CI	*P*
Ki67 - p53 *								
Low-wildtype	259	50.2	1.00		**<0.001**	1.00		**0.037**
Low-mutant	12	2.3	2.396	1.259-4.561		2.377	1.113-5.079	
High-wildtype	207	40.1	1.296	1.019-1.646		1.081	0.813-1.437	
High-mutant	38	7.4	2.458	1.654-3.655		1.717	1.033-2.852	
Ki67 - caspase3*								
low-negative	86	36.0	1.00		**<0.001**	1.00		0.676
high-negative	56	23.4	1.492	1.094-2.035		1.111	0.752-1.643	
low-positive	40	16.7	1.737	1.249-2.415		1.160	0.782-1.720	
high-positive	57	23.9	2.137	1.575-2.899		1.282	0.855-1.923	
Caspase3 - p53*								
negative-wildtype	300	56.7	1.00		**<0.001**	1.00		**0.037**
negative-mutant	12	2.3	1.694	0.831-3.451		1.480	0.594-3.689	
positive-wildtype	179	33.8	1.580	1.242-2.009		1.095	0.834-1.439	
positive-mutant	38	7.2	3.012	2.044-4.439		2.008	1.241-3.249	
C2P® - p53								
Low-wildtype	49	34.5	1.00		0.313			
Low-mutant	8	5.6	1.865	0.765-4.548				
Intermed-wildtype	14	9.9	1.122	0.508-2.479				
Intermed-mutant	2	1.4	0.944	0.128-6.963				
High-wildtype	65	45.8	1.338	0.820-2.185				
High-mutant	4	2.8	3.612	1.089-11.984				
Caspase3 - C2P®								
negative-low	30	20.4	1.0		0.697			
negative-intermediate	15	10.2	1.267	0.579-2.772				
negative-high	32	21.8	1.096	0.568-2.112				
positive-low	29	19.7	0.995	0.507-1.950				
positive-intermediate	4	2.7	0.371	0.049-2.791				
positive-high	37	25.2	1.395	0.753-2.584				
Ki67 - C2P®								
low-low	29	19.3	1.00		0.280			
high-low	30	20.0	1.679	0.834-3.381				
low-intermediate	13	8.7	1.573	0.652-3.796				
high-intermediate	7	4.7	0.571	0.128-2.536				
low-high	32	21.3	1.801	0.901-3.601				
high-high	39	26.0	1.947	0.999-3.793				

Table 3A: (continued)

	Overall Survival combination(s)							
Characteristic	**Univariate analysis**					**Multivariable analysis**		
	N	**%**	**HR**	**95% CI**	***P***	**HR**	**95% CI**	***P***
CDK1 - caspase3*								
<median of the ratio	54	50.5	1.00		**0.014**	1.00		**0.015**
>median of the ratio	53	49.5	1.877	1.134-3.108		2.137	1.161-3.934	
CDK2 - caspase3								
<median of the ratio	58	50.4	1.00		0.179			
>median of the ratio	57	49.6	1.407	0.856-2.313				
CDK1&2 - caspase3								
<median of the ratio	50	47.6	1.00		0.124			
>median of the ratio	55	52.4	1.504	0.894-2.530				
Subtype**								
Score 0	46	65.7	1.00		**0.050**	1.00		**0.024**
Score 1	20	28.6	1.964	0.879-4.387		0.903	0.277-2.947	
Score 2	4	5.7	3.529	1.156-10.772		7.344	1.538-35.066	
Score 3	0	0.0	-	-		-	-	
Subtype***								
Score 0	52	45.2	1.00		**0.002**	1.00		**0.056**
Score 1	47	40.9	1.606	0.815-3.165		0.986	0.460-2.111	
Score 2	13	11.3	1.238	0.444-3.454		0.802	0.234-2.751	
Score 3	3	2.6	11.711	3.271-41.925		8.107	1.694-38.805	
Subtype****								
Score 0	46	32.1	1.00	0.955-2.697	**0.043**	1.00		0.255
Score 1	60	42.0	1.605	0.926-3.119		1.064	0.610-1.858	
Score 2	30	21.0	1.700	1.384-8.512		1.058	0.555-2.018	
Score 3	7	4.9	3.433			2.670	0.992-7.187	

*All adjusted for age, grade, pathological tumor stage, nodal stage, PGR and HER-2

****Subtypes** only for grade I tumors, adjusted for age, pathological tumor stage, nodal stage, PGR and HER-2

*** **Subtypes** only for TNM stage I patients, adjusted for age, PGR and HER-2

**** **Subtype** only for TNM stage IIA patients, adjusted for age, PGR and HER-2

All combinations were tested in separate models

these markers were dichotomized. For all patients one point was allocated for each marker expressed, indicating one risk factor present; resulting in a score of zero for patients without expression of any marker and a score of three for patients with all markers highly expressed. The apoptotic-proliferative subtype model was significantly associated with the molecular subtype of the tumor, in which higher apoptotic-proliferative scores were related to more aggressive molecular tumor subtypes (HER2+ type and Basal

Table 3B: Multivariable analyses for combined apoptotic and proliferative markers in relation to relapse free period

Characteristic	**Relapse Free Period Combination(s)**							
	Univariate analysis					**Multivariable analysis**		
	N	**%**	**HR**	**95% CI**	***P***	**HR**	**95% CI**	***P***
Ki67 - p53*								
Low-wildtype	259	50.2	1.00		**0.003**	1.00		0.538
Low-mutant	12	2.3	1.327	0.541-3.256		1.503	0.591-3.820	
High-wildtype	207	40.1	1.257	0.954-1.657		0.963	0.700-1.326	
High-mutant	38	7.4	2.307	1.479-3.598		1.356	0.760-2.419	
Ki67 - caspase3*								
low-negative	86	36.0	1.00		**<0.001**	1.00		0.156
high-negative	56	23.4	1.437	0.998-2.069		2.363	1.049-5.325	
low-positive	40	16.7	1.804	1.238-2.628		1.283	0.506-3.253	
high-positive	57	23.9	2.258	1.599-3.189		1.942	0.890-4.240	
Caspase3 - p53*								
negative-wildtype	300	56.7	1.00		**<0.001**	1.00		0.075
negative-mutant	12	2.3	1.653	0.726-3.762		1.304	0.405-4.195	
positive-wildtype	179	33.8	1.811	1.379-2.378		1.353	0.992-1.844	
positive-mutant	38	7.2	2.673	1.703-4.195		1.943	1.121-3.368	
C2P® - p53								
Low-wildtype	49	34.5	1.00		0.331			
Low-mutant	8	5.6	1.698	0.572-5.039				
Intermed-wildtype	14	9.9	1.758	0.764-4.045				
Intermed-mutant	2	1.4	-					
High-wildtype	65	45.8	1.755	0.992-3.104				
High-mutant	4	2.8	3.828	0.883-16.592				
Caspase3 - C2P®								
Negative-low	30	20.4	1.00		0.226			
Negative-intermediate	15	10.2	1.297	0.510-3.299				
Negative-high	32	21.8	1.269	0.593-2.716				
Positive-low	29	19.7	0.791	0.341-1.831				
Positive-intermediate	4	2.7	1.670	0.471-5.927				
Positive-high	37	25.2	1.935	0.957-3.915				
Ki67 - C2P®*								
low-low	29	19.3	1.00		0.069	1.00		0.202
high-low	30	20.0	3.704	1.366-10.045		4.257	1.413-12.822	
low-intermediate	13	8.7	3.431	1.047-11.249		3.919	1.062-14.459	
high-intermediate	7	4.7	2.973	0.794-11.127		3.627	0.692-18.993	
low-high	32	21.3	3.991	1.471-10.831		3.098	1.083-8.865	
high-high	39	26.0	4.770	1.804-12.614		3.130	1.043-9.398	

Table 3B: (continued)

Characteristic	Relapse Free Period Combination(s)							
	Univariate analysis					Multivariable analysis		
	N	**%**	**HR**	**95% CI**	***P***	**HR**	**95% CI**	***P***
CDK1 - caspase3*								
<median of the ratio	54	50.5	1.00		**0.016**	1.00		**0.009**
>median of the ratio	53	49.5	2.071	1.144-3.748		2.460	1.248-4.849	
CDK2 - caspase3*								
<median of the ratio	58	50.4	1.00		**0.003**	1.00		**0.012**
>median of the ratio	57	49.6	2.560	1.385-4.731		2.501	1.228-5.096	
CDK1&2 - caspase3*								
<median of the ratio	50	47.6	1.00		**0.049**	1.00		0.121
>median of the ratio	55	52.4	1.842	1.003-3.383		1.818	0.854-3.869	
Subtype**								
Score 0	46	65.7	1.00		0.125	1.00		**0.001**
Score 1	20	28.6	1.573	0.626-3.958		1.119	0.316-3.964	
Score 2	4	5.7	3.609	1.016-12.820		21.396	4.111-111.351	
Score 3	0	0.0	-	-		-	-	
Subtype***								
Score 0	52	45.2	1.00		0.059			
Score 1	47	40.9	1.796	0.805-4.007				
Score 2	13	11.3	1.485	0.463-4.767				
Score 3	3	2.6	7.956	1.717-36.863				
Subtype****								
Score 0	46	32.1	1.00		0.259			
Score 1	60	42.0	1.671	0.913-3.057				
Score 2	30	21.0	1.513	0.738-3.101				
Score 3	7	4.9	2.503	0.836-7.499				

*all adjusted for grade, pathological tumor stage, nodal stage and HER-2
****Subtypes** only for grade I tumors, adjusted for pathological tumor stage, nodal stage and HER-2
*** **Subtypes** only for TNM stage I patients, adjusted for age and HER-2
**** **Subtype** only for TNM stage IIA patients, adjusted for age and HER-2
All combinations were tested in separate models

like) and negative to low apoptotic-proliferative scores to the less aggressive Luminal A and Luminal B molecular tumor subtypes (p<0.001).

For the OS (p<0.001, *score 1*: HR 1.569 (95%CI: 1.171-2.103); *score 2*: HR 1.922, 95%CI: 1.386-2.667); *score 3*: HR 3.657 (95%CI: 2.297-5.822)) and RFP (p<0.001, *score 1*: HR 1.468 (95%CI: 1.046-2.061); *score 2*: HR 2.122 (95%CI: 1.473-3.059); *score 3*: HR 3.058 (95%CI: 1.792-5.218) significant univariate association was found (figure 4). When the cohort was split on tumor grade, we found a significant association in the multivariate corrected analyses for both the OS (p=0.024) and RFP (p=0.001) for only grade I tumors (figure 4 and table 3A and B). When the cohort was split on TNM stage, we found that only stage I and IIA patients had a significant outcome in the univariate OS analysis for the apoptotic-proliferative subtype model. This remained borderline significant in the multivariate corrected analysis for OS in TNM stage I patients (p=0.056, table 3A).

Biochemical assay active caspase-3

Eighteen percent (126/714) of the patients had frozen material available for a biochemical caspase-3 assay. For analysis, outcomes were converted into a categorical parameter (< and > the median value (2.74 pmol AFC/min/mg protein)). In the univariate analyses, neither for OS (p=0.7) or RFP (p=0.5) a significant relation was found herewith.

When caspase-3 assay data were combined with the C2P® data (75/714,10.5%), a significant association was found for the C2P® risk prediction and the dichotomized biochemical caspase-3 expression (low/high). Results showed that high C2P® was significantly associated with high biochemical caspase-3 expression. However, there was no significant relation regarding OS (p=0.670) or RFP (p=0.628) for this combination (data not shown).

Next, we calculated the ratio between CDK-1activity, a crucial contributor of the C2P® biomarker, and biochemical caspase-3 (107/714,15%). The ratio was transformed in a dichotomous variable by use of the median value due to a skewed distribution. Significant associations, in the favor of weaker proliferative characteristics of the tumor, were seen in the RFP (p=0.016) and OS (p=0.014), both maintaining their significance in the multivariable analyses (RFP: p=0.009, HR 2.460, 95%CI: 1.248-4.849 and OS: p=0.015, HR 2.137, HR1.161-3.934 (table 3A and 3B respectively). Combined CDK-2 and biochemical

Table 4A: Single marker and combined marker hazard ratios for overall survival

Marker-1	HR	*p-value*	Marker-2	HR	*p-value*	Combined	HR	*p-value*	95% CI
P53	2.2	<0.001	**Ki67**	1.3	0.007	**P53-Ki67**	2.5	<0.001	1.7-3.7
P53	2.2	<0.001	**Caspase-3**	1.9	<0.001	**P53-caspase-3**	3.0	<0.001	2.0-4.4
Ki67	1.3	0.007	**Caspase-3**	1.9	<0.001	**Ki67-caspase-3**	2.1	<0.001	1.6-2.9

An overview of single (Marker 1 and 2) and combined marker hazard ratios (HR), as seen in tables 3B and 4B. All hazard ratios and p-values shown in this table are univariate results.

Table 4B: Single marker and combined marker hazard ratios for relapse free period

Marker-1	HR	*p-value*	Marker-2	HR	*p-value*	Combined	HR	*p-value*	95% CI
P53	1.8	0.002	**Ki67**	1.3	0.021	**P53-Ki67**	2.3	0.003	1.5-3.6
P53	1.8	0.002	**Caspase-3**	1.9	<0.001	**P53-caspase-3**	2.7	<0.001	1.7-4.2
Ki67	1.3	0.021	**Caspase-3**	1.9	<0.001	**Ki67-caspase-3**	2.3	<0.001	1.6-3.2

An overview of single (Marker 1 and 2) and combined marker hazard ratios (HR), as seen in tables 3A and 4A. All hazard ratios and p-values shown in this table are univariate results.

caspase-3 (115/174, 16.1%) only showed a significant association in the RFP (p=0.003) in favor of a higher apoptotic rate, remaining an independent factor after multivariate correction with p-value=0.012, HR 2.501, 95%CI: 1.228-5.096 (table 3B).

DISCUSSION

Over the last few years the impact of single apoptotic and proliferative markers on tumor progression and patient outcome in BC was thoroughly investigated but often showed contradictory results [13-15]. An explanation could be the misinterpretation that emanates from single apoptotic and proliferative marker expression due to the fact that they do not reflect the interaction with one another. In this manuscript we circumvented this shortcoming by combining dual markers and constructed a apoptotic-proliferative subtype model, in which all important markers were incorporated to prevent misinterpretation of these closely linked pathways.

It is hypothesized that imbalanced presence of apoptosis and proliferation is a hallmark for tumor aggressiveness. Consequently, this apoptotic-proliferative misbalance results in either progression or inhibition of tumor growth, depending on the direction of the outcome of the balance.

For both single and combined markers, independent of being a proliferative or apoptotic marker, high expression rates are associated with higher hazard ratios, in which the majority of combined markers have an additive effect on one another leading to higher hazard ratios.

For active caspase-3 our data showed counter intuitive worse clinical outcome when highly expressed, thus corresponding with a high apoptotic rate in the tumor [2]. Combined analyses demonstrated that this poor outcome was associated with high proliferative Ki67 presence in the breast tumor, being a good example of how single marker experiments can be misinterpreted. It should be clear that the high proliferative Ki67 marker apparently dominates the clinical outcome of these high active caspase-3 expressing tumors. It could be considered that the apoptotic marker can merely keep up with the high proliferation rate of the tumor, resulting in excess proliferation, consequently leading to progression of the BC. Nevertheless, this difference in apoptosis induction in tumors expressing high levels of Ki67 is also a tumor characteristic worthy of observation and serves as an excellent marker for more accurate prognostication. The combined high apoptosis - high proliferation relation seen in this study was also seen in work done by Parton *et al* [16].

Biochemical assay data retrieved from this study strengthens the conclusion found in IHC focusing on combined marker analyses. Our assay results are supported by data from Zeestraten *et al.* whom also showed the high prognostic value of CDK1 in stage II colon cancer patients [17].

By constructing an apoptotic-proliferative tumor subtyping model, we demonstrated that the combination of the expression rates of all relevant apoptotic and proliferative markers leads to a valuable prognostic indicator in grade I breast tumors. To the best of our knowledge, we are the first group providing such detailed insight in the tumor apoptosis and proliferation ratio in BC, showing that this cell proliferative and death ratio is of crucial value compared to single marker interpretation in the control of tumor progression and therefore in determining patient prognosis. Results of this study lead to assume that apoptotic-proliferative subtyping in grade I tumors could be of crucial importance in identifying patients with a low tumor grade with an increased risk of poor prognosis, being those containing the most detrimental apoptotic-proliferative marker combination. With the increased tendency of earlier diagnosis due to better BC awareness and the introduction of population based screening, it comes as no surprise that the BC incidence has tilted to more early stage, low grade breast tumors [18]. Introducing our newly designed apoptotic-proliferative tumor subtyping model will lead to targeted selection of the grade I BC patients that would truly benefit of an aggressive therapeutic regime due to an adverse apoptotic-proliferative balance. In the current state of affairs, where over- and under- treatment leads to considerable debate in clinical practice, identification of patient groups for implementation of personalized therapy will become increasingly important.

This cohort consisted of BC patients diagnosed and treated between 1985 and 1996, this time frame also marking the beginning of adjuvant hormonal therapy which led to less protocolled regimes and documentation hereof. Also, the chemotherapy given at that time point clearly does not meet today's standards and therefore no clinical consequence could be deduced. Despite these shortcomings, this study clearly states high prognostic value. Further research should validate our findings and focus on the predictive value in light of today's therapeutic standards.

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