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ORIGINAL REPORT

Different Prognostic Value of Cytokeratin-19 mRNA–Positive Circulating Tumor Cells According to Estrogen Receptor and HER2 Status in Early-Stage Breast Cancer

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ABSTRACT

Purpose
To examine the prognostic value of cytokeratin-19 (*CK-19*) mRNA–positive circulating tumor cells (CTCs) in early-stage breast cancer patients focusing on clinically relevant subgroups based on estrogen receptor (ER) and HER2 expression.

Patients and Methods
CK-19 mRNA–positive CTCs were detected by real-time reverse transcriptase polymerase chain reaction in the blood of 444 consecutive, stage I-III, breast cancer patients before initiation of adjuvant chemotherapy. The association between detection of *CK-19* mRNA–positive CTCs and clinical outcome was analyzed for patients with ER-positive, ER-negative, triple-negative, HER2-positive, and ER-positive/HER2-negative tumors.

Results
CK-19 mRNA–positive CTCs were detected in 181 (40.8%) of 444 patients; 109 (41.9%) of 260 patients with ER-positive tumors; 71 (40.6%) of 175 patients with ER-negative tumors; 27 (35%) of 77 patients with triple-negative tumors; 35 (39.8%) of 88 patients with HER2-positive tumors; and 82 (44.1%) of 186 patients with ER-positive/HER2-negative tumors. After a median follow-up of 53.5 months, patients with *CK-19* mRNA–positive CTCs experienced reduced disease-free survival (DFS; $P < .001$) and overall survival (OS; $P < .001$); this was mainly observed in patients with ER-negative ($P < .001$ and $P < .001$, respectively) but not ER-positive tumors ($P = .172$ and $P = .425$, respectively) and in patients with triple-negative ($P = .008$ and $P = .001$, respectively) and HER2-positive ($P = .023$ and $P = .040$, respectively) but not ER-positive/HER2-negative tumors ($P = .210$ and $P = .578$, respectively). In multivariate analysis, the interaction between *CK-19* mRNA–positive CTCs and ER status was the strongest independent prognostic factor for reduced DFS (hazard ratio [HR], 3.808; 95% CI, 2.415 to 6.003; $P < .001$) and OS (HR, 4.172; 95% CI, 2.477 to 9.161; $P < .001$).

Conclusion
Detection of *CK-19* mRNA–positive CTCs before adjuvant chemotherapy predicts poor clinical outcome mainly in patients with ER-negative, triple-negative, and HER2-positive early-stage breast cancer.

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INTRODUCTION

Different markers have been used for the molecular detection of circulating tumor cells (CTCs). Among them, cytokeratin-19 (*CK-19*), a cytoskeletal protein expressed on epithelial but not on mesenchymal cells, has been the most extensively studied.[1] Although studies using reverse transcriptase polymerase chain reaction (RT-PCR) of *CK-19* mRNA as a biomarker for CTCs, have been flawed by problems of illegitimate expression and pseudogene amplification, our group has optimized the assay to improve its sensitivity and specificity.[2,3] Thus, using a highly sensitive and specific real-time RT-PCR assay,[3] we demonstrated that the detection of *CK-19* mRNA–positive CTCs before the initiation of adjuvant chemotherapy was an independent prognostic factor for disease recurrence and decreased survival in patients with node-negative breast cancer.[4]

Until now the prognostic value of micrometastatic disease has been studied without considering the heterogeneity of breast cancer.[4-9] Primary tumor gene expression profiling studies

using unsupervised hierarchical clustering analysis have shown that breast tumors are grouped into two main clusters: predominately estrogen receptor (ER) negative and ER positive.[10-15] Moreover, from these studies, at least three stable molecular subtypes have been consistently identified, namely the ER-negative/HER2-negative or basal-like, the HER2-positive , and the ER-positive/HER2-negative or luminal subtypes.[16]

Apart from differences in gene expression profiles, ER-negative and ER-positive tumors differ in their response to treatment and clinical course.[17] The last Early Breast Cancer Trialists' Collaborative Group meta-analysis demonstrated that women with ER-negative early-stage breast cancer experienced relapse more often than those with ER-positive tumors during the first 5 years, while the opposite was true for the period of 5 to 15 years after diagnosis.[17] Similarly, the breast cancer molecular subtypes have been reported to differ in their response to treatment and clinical outcome.[11,15,16,18]

In this study we sought to validate our previous results regarding the prognostic value of peripheral blood *CK-19* mRNA–positive cells in an extended cohort of 444 patients with stage I-III breast cancer. Furthermore, considering the heterogeneity of the disease, we investigated the prognostic value of *CK-19* mRNA–positive cells, in the ER-negative and ER-positive subgroups and in the three molecular subtypes—namely the ER-negative/HER2–negative/progesterone receptor (PR)-negative or triple-negative, the HER2-positive, and the ER-positive/HER2-negative subtypes.

PATIENTS AND METHODS

Patients

From 1997 until 2004, a total of 444 consecutive patients who had all received adjuvant chemotherapy for stage I-III breast cancer at the Department of Medical Oncology of the University Hospital of Heraklion (Crete, Greece) and who had sufficient follow-up (at least 10 months) were included in this study. For every patient enrolled, a complete diagnostic evaluation to exclude the presence of distant metastasis was performed consisting of chest x-rays, mammography, ultrasound of the liver, and a whole-body bone scan. Computed tomography scans and/or magnetic resonance imaging studies were performed if clinically indicated (patients with symptoms, physical signs, or abnormal findings on chest x-ray, ultrasound of the liver, and bone scan). The administration of adjuvant chemotherapy and hormone treatment was decided independently of the *CK-19* mRNA detection. All patients included in this study received adjuvant chemotherapy and most of them were treated in the context of research protocols of the Hellenic Oncology Research Group. Adjuvant chemotherapy regimens consisted of either FEC (fluorouracil 700 mg/m^2 day 1 plus epirubicin 75 mg/m^2 day 1 plus cyclophosphamide 700 mg/m^2 day 1 every 3 weeks for six cycles) or EC-T (epirubicin 75 mg/m^2 day 1 plus cyclophosphamide 700 mg/m^2 day 1 every 3 weeks for four cycles followed by docetaxel 100 mg/m^2 day 1 every 3 weeks for four additional cycles) or classical CMF (cyclophosphamide 100 mg/m^2 orally days 1 through 14, methotrexate 40 mg/m^2 days 1 and 8, and fluorouracil 600 mg/m^2 days 1 and 8 every 4 weeks for six cycles). All patients with ER- and/or PR-positive tumors received tamoxifen 20 mg daily for 5 years; premenopausal women also received luteinizing hormone-releasing hormone (LHRH) analogs for 2 years. There were no subgroups of patients who received hormone therapy only or no systemic therapy at all. Patients with HER2-positive tumors did not receive adjuvant trastuzumab because all patients were enrolled before the positive results from the adjuvant trastuzumab trials were reported.[19,20] Patient follow-up consisted of clinical examination with laboratory and imaging studies every 3 months for the first 2 years, every 6 months for the next 3 years, and yearly thereafter. All patients signed an informed consent to participate in the study which was approved by the ethics and scientific committees of our institution.

Clinical Samples, Real-Time RT-PCR Assay for CK-19 mRNA

Peripheral blood (20 mL in EDTA) was obtained from every patient, 3 to 4 weeks after primary surgery and before the initiation of any adjuvant treatment. To avoid contamination with epithelial cells from the skin, all blood samples were obtained at the middle of vein puncture after the first 5 mL of blood were discarded.

The procedures of RNA extraction and cDNA synthesis have already been described elsewhere.[3,4] The real-time RT-PCR assay for *CK-19* mRNA–positive CTCs and the primers used, have been previously described in detail and were used in this study without any modification.[3,4] According to the analytic detection limit of our assay, the presence of $\geq$ 0.6 MCF-7 equivalents/5 μg of total RNA was a positive result. Using the above cutoff, only two of 89 healthy female donors were positive (2.2%).[3] Furthermore, none of nine women with benign breast disease had positive blood samples.

Immunohistochemistry for HER2, ER, and PR

HER2 expression of the primary tumors was detected by immunohistochemistry (IHC) with the monoclonal antibody CB11 (Novacastra, Newcastle on Tyne, United Kingdom), using the OPTIMAX automated system (BioGenex Laboratories, San Ramon, CA). Scoring was based on the criteria recommended by DAKO A/S for the HercepTest (DAKO Corporation, Carpinteria, CA). Fluorescence in situ hybridization was not performed for tumors HER2 2+ by IHC.

ER and PR expression of the primary tumors was detected by IHC with monoclonal antibodies to ER and PR (DakoCytomation, Denmark, A/S), respectively, using the same automated system as above. All carcinoma cells in three hot spots (areas with the highest density of ER-positive or PR-positive carcinoma cell nuclei) per slide were evaluated independently by two pathologists (M.K., E.N.S.), and the mean of the two independent counts was considered the final value for each field and hot spot. The final immunoreactivity index (score) was calculated as the mean percentage of ER-positive or PR-positive carcinoma cell nuclei in the three hot spots. Specimens were interpreted as positive for ER or PR if at least 10% of the cells demonstrated nuclear staining of any intensity of reactivity, from 1+ to 3+. Staining intensity was graded as negative (0), weak (1+), intermediate (2+), or strong (3+), and reported separately.

Molecular Subtypes

The expression of ER, PR, and HER2 by IHC was used to define the three stable molecular subtypes. The triple-negative or basal-like tumors were defined as ER-negative/PR-negative/HER2 negative (0, 1+ by IHC), the HER2 positive as HER2 3+ by IHC, and the luminal as ER-positive/HER2 negative (0, 1+ by IHC).

Statistical Analysis

Summary descriptive statistics were expressed as mean (standard deviation [SD]) or percent, as appropriate. Continuous variables were compared between different groups with unpaired *t*-test or Kruskal-Wallis test as appropriate, and categoric data with Fisher's exact test. The time from study entry until the day of the first evidence of disease recurrence either locoregional or distant (disease-free survival [DFS]) and the time from study entry to death (overall survival [OS]) were the main dependent variables of the study. DFS and OS curves for subgroups of patients were constructed using the Kaplan-Meier product limit estimate method[21] and were compared by the log-rank test in order to provide a univariate assessment of the prognostic value of selected clinical risk factors, measured at study entry. Clinicopathological factors known to be associated with prognosis like menopausal status (premenopausal *v* postmenopausal), tumor size (T2-3 *v* T1), nodal infiltration (yes *v* no), histology grade (III *v* I-II), ER status (negative *v* positive), PR status (negative *v* positive), HER2 status (positive *v* negative), triple-negative status (yes *v* no), chemotherapy regimen (FEC/EC-T *v* CMF) and additionally the detection of *CK-19* mRNA–positive CTCs (yes *v* no) were tested in univariate analysis. Variables that were found to be significant at the univariate screen were then entered in a stepwise multivariate Cox proportional hazards regression model with and without the interaction effect between *CK-19* and ER

status, in order to identify those with independent prognostic information.[22] Entry into and removal from the model were set at 5% and 10%, respectively. All statistical tests were performed at the 5% level of significance. SPSS, version 13 (SPSS Inc, Chicago, IL), statistical software was used for the analysis.

RESULTS

Patient Characteristics

The characteristics of the 444 patients are presented in Table 1. The patients' median age was 54 years (range, 26 to 78 years). Primary tumor size was less than or equal to 2 cm, absence of axillary lymph node infiltration and ER-positive disease were observed in 35.4%, 36.7%, and 58.6% of the patients, respectively. Overall, *CK-19* mRNA–positive CTCs were detected in 181 patients (40.8%) and detection of CTCs was not significantly associated with any of the known clinicopathological characteristics (Table 1). There was no significant difference in the proportion of patients with detectable *CK-19* mRNA–positive CTCs in the ER-negative and ER-positive subgroups (40.6% and 41.9%, respectively; $P = .779$) as well as in the three molecular subtypes namely the triple negative, the HER2 positive, and the ER positive/HER2 negative (35%, 39.8%, and 44.1%, respectively; $P = .385$).

Table 1. Patient Characteristics

Characteristic	All		Patients *CK-19* mRNA+		*CK-19* mRNA−		*P*
	No	%	No	%	No	%	
Patients enrolled	444	100	181	40.8	263	59.2	
Age, years							
Median	54		54		55		
Range	26-78		26-74		30-78		.752
Menopausal status							.075
Premenopausal	191	43	87	45.5	104	54.5	
Postmenopausal	253	57	94	37.2	159	62.8	
Tumor size							.648
T1	157	35.4	61	38.9	96	61.1	
T2	251	56.5	103	41	148	59	
T3	36	8.1	17	47.2	19	52.8	
Histology grade							.316
I/II	204	46	87	42.6	117	57.4	
III	191	43	72	37.7	119	62.3	
Unknown	49	11	22		27		
Infiltrated axillary lymph nodes							.538
0	163	36.7	61	37.4	102	62.6	
1-3	122	27.5	53	43.5	69	56.5	
≥ 4	159	35.8	67	42.1	92	57.9	
ER							.779
Negative	175	39.4	71	40.6	104	59.4	
Positive	260	58.6	109	41.9	151	58.1	
Unknown	9	2	1		8		
PR							.126
Negative	234	52.7	89	38	145	62	
Positive	201	45.3	91	45.3	110	54.7	
Unknown	9	2	1		8		
HER2							.897
0, 1+	290	65.3	122	42.1	168	57.9	
2+	53	11.9	21	39.6	32	60.4	
3+ by IHC	88	19.8	35	39.8	53	60.2	
Unknown	13	3	3		10		
Adjuvant chemotherapy							.425
CMF	43	9.7	14	32.6	29	67.4	
FEC	209	47.1	84	40.2	125	59.8	
EC-T	192	43.2	83	43.2	109	56.8	
Surgery							.478
L	310	69.8	123	39.7	187	60.3	
M	134	30.2	58	43.3	76	56.7	
Radiotherapy							.799
No	81	18.2	32	39.5	49	60.5	
Yes	363	81.8	149	41	214	59	

Abbreviations: *CK-19*, cytokeratin-19; ER, estrogen receptor; PR, progesterone receptor; L, lumpectomy; M, mastectomy; IHC, immunohistochemistry; FEC, fluorouracil, epirubicin, cyclophosphamide; EC-T, epirubicin, cyclophosphamide, docetaxel; CMF, cyclophosphamide, methotrexate, fluorouracil.

Similarly, there was no significant difference in the distribution (median, range) of *CK-19* mRNA values between the ER-negative and ER-positive subgroups ($P = .559$) and between the three molecular subtypes ($P = .185$; Appendix Table A1, online only).

Detection of CK-19 mRNA–Positive CTCs and Clinical Outcome

We first investigated the association between detection of *CK-19* mRNA–positive CTCs and clinical outcome of the whole population. The median follow-up was 53.5 months (range, 10 to 106 months). During this period, 94 (21%) of 444 patients relapsed (18 local and 76 distant) and 42 patients (9%) died due to disease progression. Median follow-up for alive patients was 56.4 months (range, 10 to 96 months) and 45% of alive patients had follow-up for longer than 5 years. Relapse and death were more frequent in patients with *CK-19* mRNA–positive CTCs ($P < .001$ and $P = .001$; Table 2). The median DFS and OS of patients with and without *CK-19* mRNA–positive CTCs cannot, as of yet, be estimated; however, patients with *CK-19* mRNA–positive CTCs had significantly shorter DFS ($P < .001$; Fig 1A) and OS ($P < .001$; Fig 2A).

Because axillary nodal status is very important in tumor staging, we sought to examine the prognostic value of the detection of *CK-19* mRNA–positive CTCs in patients' groups based on nodal infiltration. The detection of *CK-19* mRNA–positive CTCs was associated with worse DFS and OS both in the node-negative (log-rank test, $P = .003$ and $P = .001$, respectively) and in the node-positive subgroup (log-rank test, $P = .001$ and $P = .046$, respectively).

Subgroups Based on ER Status

ER-negative patients. During the follow-up period, patients with ER-negative tumors relapsed more frequently than patients with ER-positive tumors (28% *v* 16.5%; $P = .004$). In the ER-negative subgroup (n = 175), disease relapse and death were more common in patients with *CK-19* mRNA–positive CTCs ($P < .001$ and $P = .001$, respectively; Table 2). The estimated median DFS for patients with *CK-19* mRNA–positive CTCs was 62 months (95% CI, 46.8 to 77.1), whereas it has not yet been reached for patients without *CK-19* mRNA–positive CTCs ($P < .001$; Fig 1B). The median OS for patients with and without *CK-19* mRNA–positive CTCs has not yet been reached; however, patients with *CK-19* mRNA–positive CTCs had significantly shorter OS ($P < .001$; Fig 2B).

ER-positive patients. In the ER-positive subgroup (n = 260), relapses and deaths did not differ significantly for patients with detectable *CK-19* mRNA–positive CTCs and those without ($P = .315$ and $P = .499$, respectively; Table 2). Although no significant differences in DFS and OS were observed, with longer follow-up there was a nonsignificant trend for worse DFS in ER-positive patients with detectable *CK-19* mRNA–positive CTCs, ($P = .172$; Fig 1C and $P = .425$; Fig 2C, respectively). DFS and OS of ER-positive/*CK-19*–negative, ER-positive/*CK-19*–positive, ER-negative/*CK-19*–negative, ER-negative/*CK-19*–positive patients is depicted in Appendix Figure A1 (online only).

Subgroups Based on Molecular Subtypes

Triple-negative patients. In the triple-negative subgroup (n = 77), relapses and deaths were significantly more frequent in patients with *CK-19* mRNA–positive CTCs ($P = .030$ and $P = .001$, respectively; Table 2). Patients with *CK-19* mRNA–positive CTCs had significantly shorter DFS and OS ($P = .008$, Fig 1D; and $P = .001$, Fig 2D, respectively).

HER2-positive patients. In the HER2-positive subgroup (n = 88), relapses and deaths were observed with higher frequency in patients with *CK-19* mRNA–positive CTCs ($P = .033$ and $P = .038$, respectively; Table 2). Patients with *CK-19* mRNA–positive CTCs had

Table 2. Incidence of Relapses and Deaths in Different Groups of Patients With Early-Stage Breast Cancer According to the Presence of *CK-19* mRNA-Positive CTCs

Variable	No. of Patients	Relapse			Death		
		No.	%	*P*	No.	%	*P*
Patient group							
Entire population	444						
CK-19 positive	181	54	29.8	< .001	27	14.9	.001
CK-19 negative	263	40	15.2		15	5.7	
ER negative	175						
CK-19 positive	71	32	45.1	< .001	18	25.3	.001
CK-19 negative	104	17	16.3		7	6.7	
ER positive	260						
CK-19 positive	109	21	19.2	.315	8	7.3	.499
CK-19 negative	151	22	14.6		8	5.3	
Triple negative	77						
CK-19 positive	27	11	40.7	.030	8	29.6	.001
CK-19 negative	50	9	18		2	4	
HER2 positive	88						
CK-19 positive	35	13	37.1	.033	7	20	.038
CK-19 negative	53	9	16.9		3	5.6	
ER positive/HER2 negative	186						
CK-19 positive	82	13	15.8	.392	4	4.8	.731
CK-19 negative	104	12	11.5		4	3.8	

Abbreviations: *CK-19*, cytokeratin-19; ER, estrogen receptor.

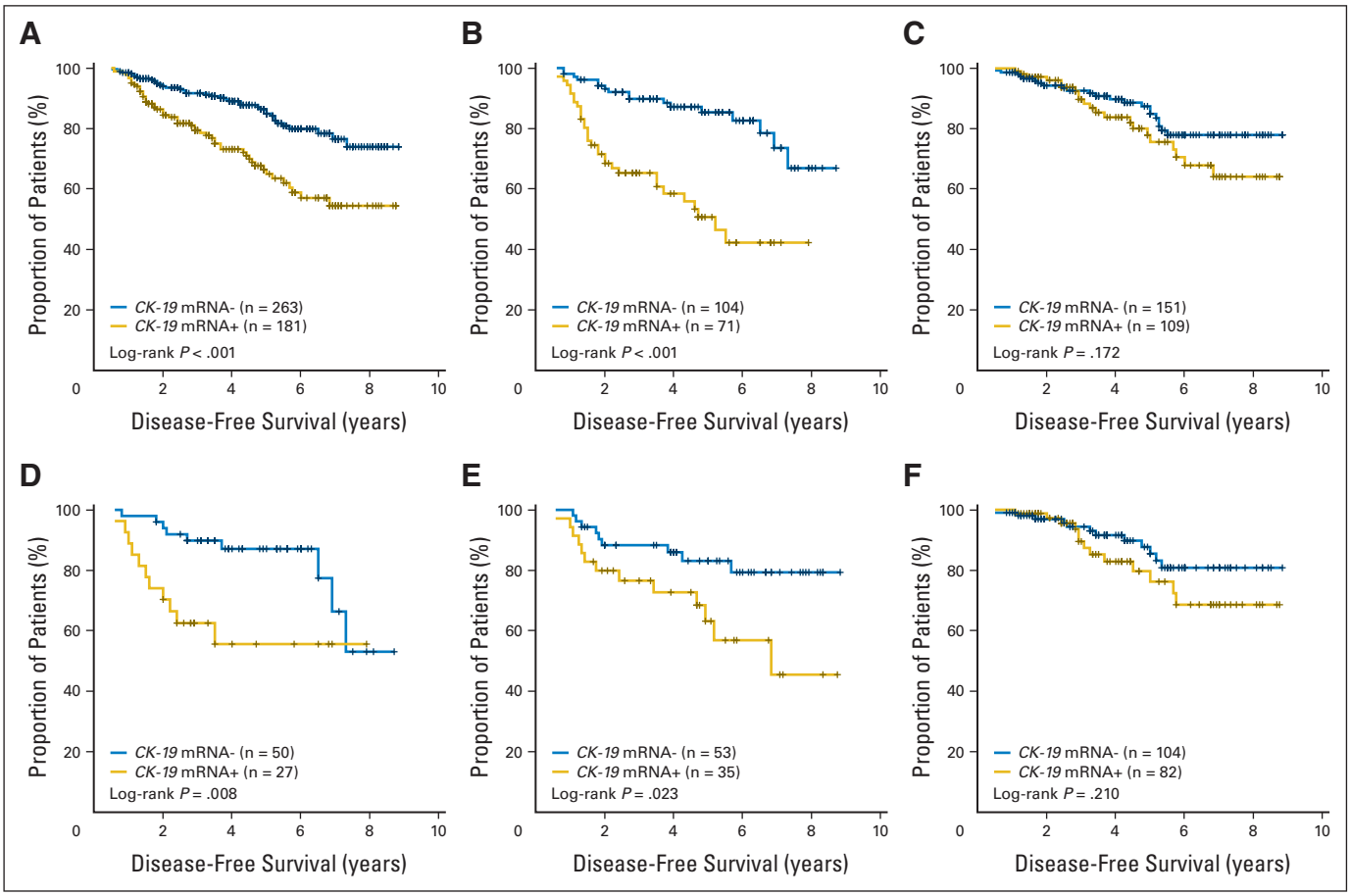

Fig 1. Disease-free survival in patients with and without cytokeratin-19 (*CK-19*) mRNA–positive circulating tumor cells (CTCs): (A) entire patient population, (B) estrogen receptor (ER)-negative, (C) ER-positive, (D) triple-negative, (E) HER2-positive, and (F) ER-positive/HER2-negative subgroups.

also significantly shorter DFS and OS ($P = .023$, Fig 1E; and $P = .040$, Fig 2E, respectively).

ER-positive/HER2-negative patients. Among the ER-positive/HER2-negative subgroup (n = 186), disease recurrences and deaths were not significantly different in patients with detectable *CK-19* mRNA–positive CTCs versus those without ($P = .392$ and $P = .731$, respectively; Table 2). Although no significant differences in DFS and OS were observed, with longer follow-up there was a nonsignificant trend for worse DFS in ER-positive/HER2-negative patients with detectable *CK-19* mRNA–positive CTCs ($P = .210$; Fig 1F and $P = .578$; Fig 2F, respectively).

The 5-year DFS and OS for patients with or without *CK-19* mRNA–positive CTCs in the entire population, the subgroups based on ER status, and the three molecular subtypes are depicted in Appendix Table A2 (online only).

Univariate and Multivariate Analysis

In univariate analysis, tumor size larger than 2 cm, ER-negative tumors, histology grade III, as well as the detection of *CK-19* mRNA–positive CTCs were associated with significantly shorter DFS and OS in the entire patient cohort (Table 3). In multivariate analysis that included 387 patients, ER-negative tumors and the detection of *CK-19* mRNA–positive CTCs were independently associated with decreased DFS and OS (Table 4). Furthermore, when the interaction between *CK-19* mRNA–positive CTCs and ER status was included in the multivariate model, this interaction emerged as the strongest independent prognostic factor for reduced DFS and OS (Table 4).

DISCUSSION

In this study of an extended cohort of 444 node-negative and node-positive breast cancer patients, we confirmed our previous results[4] on the adverse, independent prognostic value of *CK-19* mRNA–positive CTCs in early-stage breast cancer. The node-negative patients included in this analysis were to a large extent different from the node-negative patients of our previous report.[4] In this study, we report for the first time (to our knowledge) that the presence of *CK-19* mRNA–positive CTCs predicted poor clinical outcome (relapse and death) in patients with ER-negative but not ER-positive early-stage breast cancer, despite the similar proportion of patients with detectable *CK-19* mRNA–positive CTCs in both subgroups. Similarly, the presence of *CK-19* mRNA–positive CTCs was associated with shorter DFS and OS in the triple-negative and HER2-positive , but not in the ER-positive/HER2-negative subgroups. In multivariate analysis, the interaction between *CK-19* mRNA–positive CTCs and ER status was the strongest independent prognostic factor for DFS and OS.

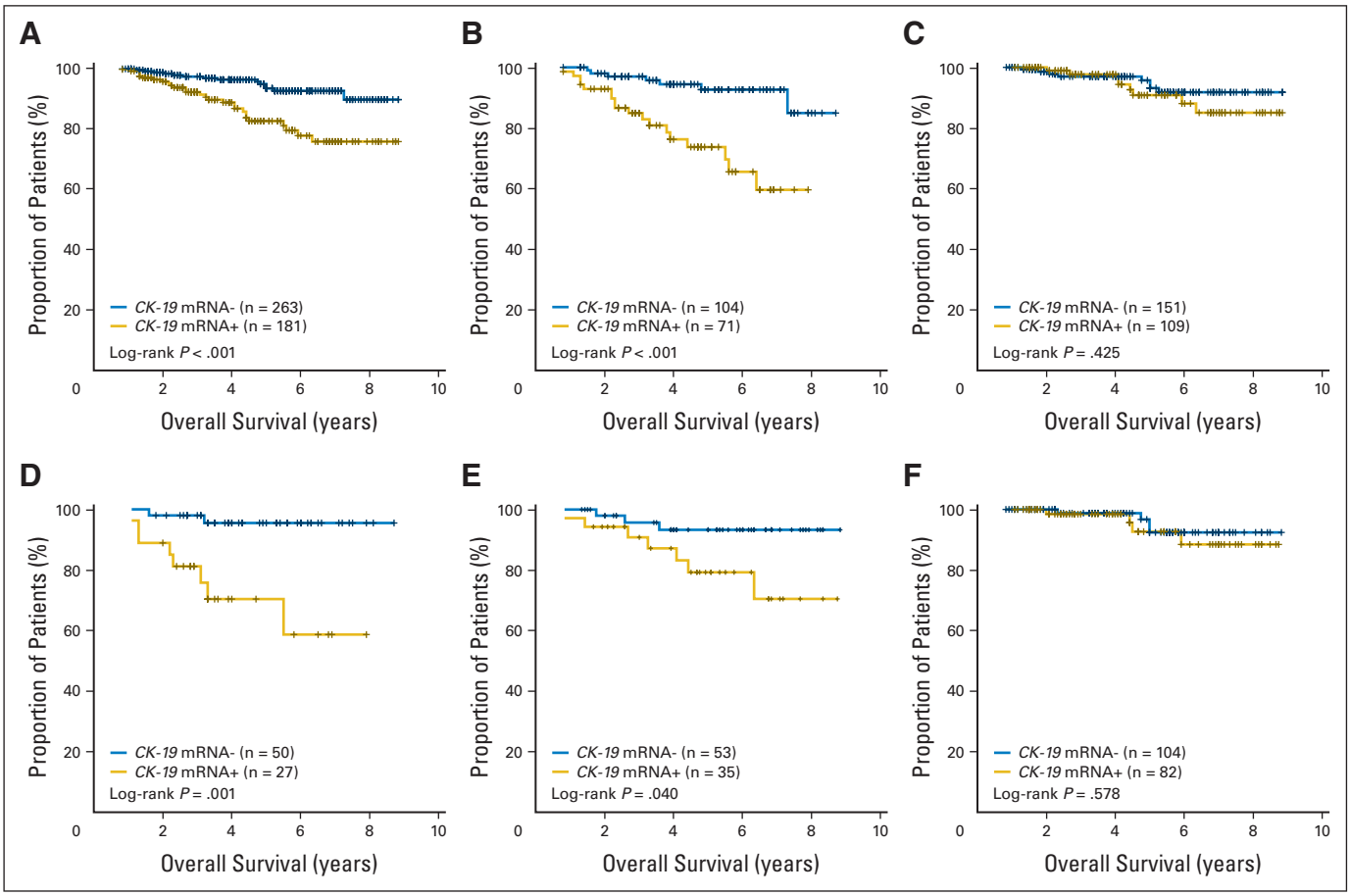

Fig 2. Overall survival in patients with and without cytokeratin-19 (*CK-19*) mRNA–positive circulating tumor cells (CTCs): (A) entire patient population, (B) estrogen receptor (ER)-negative, (C) ER-positive, (D) triple-negative, (E) HER2-positive, and (F) ER-positive/HER2-negative subgroups.

During the 53.5-month median follow-up, patients with ER-negative tumors relapsed more frequently than patients with ER-positive tumors (28% *v* 16.5%; $P = .004$). This is in accordance with the Oxford meta-analysis, where ER-negative patients relapsed more frequently than ER-positive patients during the first 5 years after diagnosis.[17] Therefore, *CK-19* mRNA–positive CTCs are correlated with the development of early metastasis within the first 5 years in patients with ER-negative but not in patients with ER-positive disease. A possible explanation for this is that in the ER-negative group, micrometastatic cells could be targeted only by adjuvant chemotherapy, whereas in the ER-positive subgroup they could be controlled by both adjuvant chemotherapy as well as hormone treatment. Nevertheless, it appears that the curves in the ER-positive group tend to separate after 5 years (Figs 1C and 2C), which would be consistent with the 5 years of tamoxifen received by ER-positive patients. Therefore, with longer follow-up, initial *CK-19* mRNA levels might also predict long-term outcome of ER-positive patients and thus might help identifying those who could benefit from extended adjuvant hormone therapy. Therefore, the 53.5-month median follow-up of this study is relatively short to draw definite conclusions for patients with ER-positive tumors. Furthermore, since all patients received adjuvant chemotherapy with or without hormone therapy, the information we derived from the detection of *CK-19* mRNA–positive cells is not purely prognostic. Validation of our results in a well-designed, prospective, multicenter trial, where therapeutic decision will be based on *CK-19* mRNA detection, is needed. Further development of this biomarker should take into consideration the problems related to tumor marker prognostic studies described by McShane et al.[23]

Based on our results, we could also hypothesize that *CK-19* mRNA–positive CTCs have different biologic behavior in patients with ER-negative (basal-like CTCs) and ER-positive (luminal-like CTCs) tumors. However, molecular and immunophenotypic characterization of *CK-19* mRNA–positive CTCs in patients with ER-negative and ER-positive disease is required to further validate this hypothesis. Furthermore, it could be argued that the presence of *CK-19* mRNA–positive CTCs in a given patient may reflect the biology of the primary tumor as determined by the ER status. According to Klein et al, [24] occult micrometastatic cells are heterogeneous in a given patient with early-stage breast cancer. After the identification of tumorigenic breast cancer cells,[25] it would be interesting to examine whether there are different subpopulations of micrometastatic cells with stem-cell/progenitor properties responsible for the development of metastasis in ER-negative and ER-positive patients.

Another important question is whether the study of *CK-19* mRNA–positive CTCs could provide additional prognostic information to currently developed gene expression signatures.[26-30] Interestingly, according to these signatures, the majority of ER-negative

Table 3. Univariate Analysis for DFS and OS for Patients With Early-Stage Breast Cancer

Parameter	Hazard Ratio	95% CI	*P*
DFS			
Menopausal status (pre *v* post)	0.703	0.460 to 1.074	.103
Tumor size (T2-3 *v* T1)	2.186	1.321 to 3.616	.002
Histology grade (III *v* I/II)	1.760	1.135 to 2.727	.011
Lymph nodes (pos *v* neg)	1.297	0.837 to 2.010	.244
ER (neg *v* pos)	1.838	1.220 to 2.769	.004
PR (neg *v* pos)	1.457	0.945 to 2.247	.089
HER2 (pos *v* neg)	1.132	0.699 to 1.832	.614
Triple negative (yes *v* no)	1.292	0.787 to 2.120	.311
Adjuvant chemotherapy (FEC/T-EC *v* CMF)	1.451	0.702 to 3.000	.315
CK-19 mRNA (pos *v* neg)	2.428	1.611 to 3.661	< .001
OS			
Menopausal status (pre *v* post)	0.916	0.494 to 1.697	.781
Tumor size (T2-3 *v* T1)	2.200	1.018 to 4.754	.045
Histology grade (III *v* I/II)	2.497	1.260 to 4.951	.009
Lymph nodes (pos *v* neg)	1.402	0.718 to 2.740	.322
ER (neg *v* pos)	2.382	1.271 to 4.465	.007
PR (neg *v* pos)	1.704	0.869 to 3.343	.121
HER2 (pos *v* neg)	1.161	0.565 to 2.383	.685
Triple negative (yes *v* no)	1.626	0.794 to 3.329	.184
Adjuvant chemotherapy (FEC/T-EC *v* CMF)	1.309	0.466 to 3.676	.609
CK-19 mRNA (pos *v* neg)	3.020	1.605 to 5.683	.001

Abbreviations: DFS, disease-free survival; OS, overall survival; pos, positive; neg, negative; ER, estrogen receptor; PR, progesterone receptor; HER2 positive, 3+ by immunohistochemistry; HER2 negative/equivocal, 0, 1+, 2+ by immunohistochemistry; FEC, fluorouracil, epirubicin, cyclophosphamide; EC-T, epirubicin, cyclophosphamide, docetaxel; CMF, cyclophosphamide, methotrexate, fluorouracil.

tumors are assigned to the poor outcome group, whereas ER-positive tumors comprise a mixture of poor and good prognosis tumors.[30-33] Therefore, gene expression signatures are more useful for predicting clinical outcome in ER-positive disease.[15,34] On the contrary, our results indicate that the detection of *CK-19* mRNA–positive CTCs could subdivide ER-negative and triple-negative as well as HER2-positive patients into better and worse prognosis groups. Therefore, it would be interesting to prospectively assess the hypothesis that by combining information from primary tumor gene expression profiling and the detection of micrometastatic cells, we could further improve prognosis in early-stage breast cancer.

Furthermore, the monitoring of *CK-19* mRNA–positive CTCs could be used to investigate the potential value of secondary adjuvant strategies. We have previously reported that a short

Table 4. Independent Prognostic Factors by Multivariate Analysis Without and With the Interaction *CK-19**ER for DFS and OS for Patients With Early-Stage Breast Cancer (n = 387)

Parameter	Hazard Ratio	95% CI	*P*
Without the interaction *CK-19**ER			
DFS			
Tumor size (T2-3 *v* T1)	2.116	1.236 to 3.622	.006
Histology grade (III *v* I/II)	1.337	0.837 to 2.137	.224
ER (neg *v* pos)	1.949	1.256 to 3.024	.003
CK-19 mRNA (pos *v* neg)	2.406	1.549 to 3.738	< .001
OS			
Tumor size (T2-3 *v* T1)	1.852	0.798 to 4.302	.152
Histology grade (III *v* I/II)	2.149	1.059 to 4.361	.034
ER (neg *v* pos)	2.242	1.131 to 4.446	.021
CK-19 mRNA (pos *v* neg)	2.482	1.286 to 4.791	.007
With the interaction *CK-19**ER			
DFS			
*CK-19**ER	3.808	2.415 to 6.003	< .001
Tumor size	2.071	1.211 to 3.541	.008
OS			
*CK-19**ER	4.172	2.477 to 9.161	< .001
Histology grade	2.040	1.015 to 4.117	.039

Abbreviations: *CK-19*, cytokeratin-19; DFS, disease-free survival; OS, overall survival; ER, estrogen receptor; pos, positive; neg, negative.

course of trastuzumab could eliminate chemotherapy-resistant *CK-19* mRNA– and HER2 mRNA–positive CTCs and bone marrow disseminated tumor cells for patients with breast cancer.[35] Thus, the identification of suitable targets for individualized adjuvant breast cancer treatment may need to take into account, not only the characteristics of the primary tumor, but also those of micrometastatic cells.

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The author(s) indicated no potential conflicts of interest.

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Appendix

The Appendix is included in the full-text version of this article, available online at www.jco.org. It is not included in the PDF version (via Adobe® Reader®).