

Featured Article

Trastuzumab Administration Can Effectively Target Chemotherapy-Resistant Cytokeratin-19 Messenger RNA-Positive Tumor Cells in the Peripheral Blood and Bone Marrow of Patients With Breast Cancer

Vassiliki Bozionellou,¹ Dimitris Mavroudis,¹ Maria Perraki,³ Savvas Papadopoulos,⁴ Stella Apostolaki,³ Efstathios Stathopoulos,² Aliko Stathopoulou,⁵ Evi Lianidou,⁵ and Vassilis Georgoulis¹

Departments of ¹Medical Oncology, and ²Pathology, University General Hospital of Heraklion, and ³Laboratory of Tumor Cell Biology, School of Medicine, University of Crete, Crete; and ⁴Department of Pathology, Hygeia Hospital of Athens, and ⁵Laboratory of Analytical Chemistry, Department of Chemistry, University of Athens, Athens, Greece

ABSTRACT

Purpose: The detection of disseminated occult breast cancer cells in peripheral blood and bone marrow is associated with poor prognosis. Since a high proportion of these cells express the HER-2 receptor, we evaluated the effectiveness of the anti-HER-2 antibody trastuzumab (Herceptin) administration to eliminate them.

Experimental Design: Thirty patients with prior chemotherapy exposure were recruited to the study on the basis of having detectable cytokeratin-19 (CK-19) mRNA transcripts by nested reverse transcription (RT)-PCR in the peripheral blood and/or bone marrow. There were 13 patients with stage I, II, or III breast cancer and 17 with stage IV disease. They were treated in two cohorts with either 4 to 8 weekly infusions of trastuzumab at 2 mg/kg (4 mg/kg loading dose; 20 patients) or 2 to 3 infusions every 3 weeks at 6 mg/kg (8 mg/kg loading dose; 10 patients). All of the patients' samples were also analyzed for HER-2 by nested RT-PCR, but detectable HER-2 messenger RNA (mRNA) was not required for inclusion in the study. After trastuzumab infusions, patients were closely monitored by nested

RT-PCR and real-time RT-PCR for the detection of CK-19 mRNA-positive cells.

Results: Before trastuzumab infusions, CK-19 mRNA-positive cells were detected in the peripheral blood ($n = 10$), bone marrow ($n = 14$), or both ($n = 6$). In 25 of 30 patients (83%), HER-2 mRNA expression was detected by nested RT-PCR in the pretrastuzumab CK-19-positive sample. After trastuzumab infusions, overall, 28 of 30 (93%) patients became CK-19 mRNA negative by nested RT-PCR and 20 of 30 (67%) by real-time RT-PCR. After a median follow-up of 6 months (range 2 to 22+), the median duration of CK-19 mRNA negativity by nested RT-PCR was 9, 12, and 6 months for stage I/II, III, and IV disease, respectively.

Conclusions: Therapy-resistant CK-19 mRNA-positive cells in the peripheral blood and bone marrow can be effectively targeted by trastuzumab administration. Further studies are needed to evaluate the prognostic significance of the disappearance of these cells.

INTRODUCTION

The development of metastases is because of the migration of tumor cells from the original tumor to distant organs. This phenomenon probably occurs early during the evolution of the disease and, in some cases, even before the surgical excision of the primary tumor. Many investigators have shown that by using either monoclonal antibodies against molecules expressed on epithelial but not on mesenchymal cells, or molecular biology techniques, occult tumor cells can be detected in the bone marrow and/or the peripheral blood of patients with early and metastatic breast cancer (1–3). Moreover, prospective studies in patients with early stage breast cancer have shown that the detection of disseminated occult tumor cells in the bone marrow or the peripheral blood is an independent adverse prognostic factor associated with decreased disease-free interval and overall survival (4, 5).

In many patients, these disseminated occult tumor cells are not killed by the administration of chemotherapeutic agents, probably because only a minority of these cells are in the proliferation phase of the cell cycle (6). Indeed, most of the cells are dormant, resting in the G₀ phase of the cell cycle, as shown by the absence of Ki-67 positivity (7). However, under certain conditions, these cells can grow and give rise to overt metastases. Although disseminated tumor cells have heterogeneous proliferative potential, their *in vitro* growth characteristics correlate well with prognosis and to a large extent determine the final clinical outcome (8).

The malignant phenotype of these cells is suggested by

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Requests for reprints: Dimitris Mavroudis, Department of Medical Oncology, University General Hospital of Heraklion, P.O. Box 1352, 71110 Heraklion, Crete, Greece. Phone: 30-2810-392823; Fax: 30-2810-392802; E-mail: mavrudis@med.uoc.gr.

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many lines of evidence. These cells usually harbor multiple chromosomal aberrations characteristic of neoplastic cells and were found to be clonogenic in cell cultures (8, 9). Furthermore, the detection of these cells after the administration of chemotherapy is associated with an increased risk of relapse and reduced survival (10).

Nonetheless, not all of the disseminated occult tumor cells have the same metastatic potential. Being a heterogeneous population, some of these cells exhibit a more aggressive behavior by generating early metastases. The HER-2 receptor, product of the human *erbB2* proto-oncogene, has been proposed as a prognostic marker for the fate of these cells. Although HER-2 is overexpressed in 25 to 30% of the primary breast carcinomas, it is detected in 67.6% of patients with disseminated occult tumor cells in the bone marrow (7). Moreover, Braun *et al.* (11) detected HER-2 expression in 60% of patients with bone marrow micrometastases and independently of HER-2 expression on the primary tumor. In multivariate analysis, the expression of HER-2 on the disseminated occult tumor cells was an independent prognostic factor associated with increased risk of relapse and reduced survival (11). Therefore, the expression of HER-2 receptor could represent a putative marker for the malignant potential of these cells, and at the same time, a suitable target for their elimination.

In this pilot study, we investigated the effectiveness of the recombinant human anti-HER-2 monoclonal antibody (rhuMAB-HER2, trastuzumab, Herceptin) to target the chemoresistant occult tumor cells in the peripheral blood and bone marrow of patients with breast cancer. For the detection and follow-up of these cells, we used a nested reverse transcription (RT)-PCR and a real-time RT-PCR assay specific for cytokeratin-19 (CK-19) messenger (mRNA). In our previous experience, the nested RT-PCR assay was capable of detecting one tumor cell among one million normal peripheral blood mononuclear cells, and in two large cohorts of patients, positive results obtained before or after chemotherapy had significant prognostic implications (5, 12). Therefore, it was very important to determine if trastuzumab could successfully be used against the CK-19 mRNA-positive cells that escape the cytotoxic effect of standard chemotherapy.

PATIENTS AND METHODS

Patient Population. Eligible patients for this study were women with either locoregional or metastatic breast cancer who tested CK-19 mRNA positive by nested RT-PCR in the peripheral blood and/or the bone marrow after the administration of chemotherapy and/or hormone treatment. Demonstration of HER-2 overexpression or gene amplification in the primary tumor was not required for study entry. All of the patients should have received at least one type of standard chemotherapy, and if eligible, hormonal therapy, before positivity for CK-19 mRNA was determined. No prior trastuzumab therapy was allowed. All of the previous therapies should have been discontinued at least 1 month before study entry. No other concomitant anticancer therapy was allowed after entering the study. Patients with clinically progressive disease requiring prompt administration of other anticancer therapy were excluded from the study. Patients should also have no significant

cardiac history and a normal left ventricular ejection fraction on Technetium-99 multiple gated acquisition scan or echocardiogram. CK-19 mRNA investigations were done in the peripheral blood and bone marrow according to the following schedule: (a) as screening for the detection of resistant tumor cells after completion of standard chemotherapy and hormonal therapy; (b) positive results were confirmed by repeat testing before trastuzumab administration; (c) after trastuzumab infusions to assess the efficacy; and (d) every 3 months thereafter during the follow-up until CK-19 mRNA positivity reappeared. Blood samples (20 mL) in EDTA were obtained at the mid of vein puncture after the first 5 mL of blood were discarded. This precaution was undertaken to avoid contamination of blood with epidermal (epithelial) cells during sample collection. Bone marrow (4 mL) in EDTA was aspirated from the posterior iliac crest, under local anesthesia. All of the blood sample collections, bone marrow aspirations, and antibody infusions were done at the Department of Medical Oncology, University General Hospital of Heraklion (Crete, Greece). All of the patients including those who received immunoglobulin IgG gave their written informed consent to participate in the study, which was approved by the Ethics and Scientific Committees of our Institution.

Treatment. Two different and clinically relevant administration schedules of trastuzumab (Herceptin, Roche, Basel, Switzerland) were investigated. The first 20 eligible patients received trastuzumab i.v. at a loading dose of 4 mg/kg of body weight followed by 2 mg/kg once a week for 4 to 8 consecutive weeks. The antibody infusion was prepared in sterile glass bottles containing 250 mL of 0.9% NaCl and infused over a time period of 30 minutes. Patients were evaluated by nested RT-PCR after 4 weekly trastuzumab infusions. If no CK-19 mRNA signal was detectable in both peripheral blood and bone marrow, the trastuzumab administration was stopped. If the CK-19 mRNA signal was still present in either the peripheral blood or bone marrow, trastuzumab was given for an additional 4 weeks, and then repeat testing by RT-PCR was done. If still positive, no additional treatment was given. A second cohort of 10 eligible patients received trastuzumab i.v. at a loading dose of 8 mg/kg of body weight followed by 6 mg/kg administered every 3 weeks for 2 to 3 times. If after the first two infusions the patient tested negative for CK-19 mRNA in both peripheral blood and bone marrow, then trastuzumab administration was stopped. If either peripheral blood or bone marrow were still positive for CK-19 mRNA, one more trastuzumab infusion at 6 mg/kg was given, and then repeat testing by RT-PCR was done. If still positive, no additional treatment was administered. To assess the possibility of a nonspecific effect from the antibody administration, in a control group of 5 CK-19 mRNA-positive patients who fulfilled all of the predefined eligibility criteria of the study, treatment consisted of immunoglobulin IgG (Sandoglobulin, Novartis Pharma AG, Basel, Switzerland) administered i.v. at the dose of 500 mg/kg given once at the recommended infusion rate. In those patients, repeat testing by RT-PCR for CK-19 mRNA was done 1 week after the immunoglobulin infusion.

Follow-up. To assess the efficacy of this approach, we monitored the detection of disseminated tumor cells by RT-PCR for CK-19 mRNA in the follow-up peripheral blood and bone marrow samples every 3 months after the completion of trastu-

Table 1 Oligonucleotide primers for HER-2 PCR

PCR	Primer	Sequence 5'–3'*	Product (bp)
1st	Sense (Her-2-A)	5'TCC TCC TCG CCCTCTGC 3'	330
	Antisense (Her-2-B)	5'GCGGGTCTCCATTGTCTA 3'	
2nd	Sense (Her-2-C)	5'AGCCGCGAGCACCCAAGT 3'	147
	Antisense (Her-2-D)	5'ACCTGCTGAACTGGTGTATGCA 3'	

* GenBank accession no. M11730.

zumab administration. The duration of CK-19 negativity was measured from the time that the first CK-19 mRNA-negative results were obtained in both the peripheral blood and bone marrow, after the completion of trastuzumab therapy, until the first CK-19 mRNA-positive results were detected again in either the peripheral blood and/or bone marrow or until death. All of the patients were closely monitored for possible toxicities throughout the study. Especially cardiotoxicity was evaluated by repeating the assessment of left ventricular ejection fraction on MUGA scan or echocardiogram after the completion of trastuzumab infusions.

Sample Preparation. The procedure for the separation of peripheral blood mononuclear cells from peripheral blood and bone marrow samples as well as the RNA extraction was done exactly as described previously (5, 12). All of the preparations and handling steps of RNA took place in a laminar flow hood, under RNase-free conditions. The isolated RNA was dissolved in diethyl pyrocarbonate-treated water and stored at -80°C until used. RNA integrity was tested by PCR amplification of the β -actin housekeeping gene. Only samples with β -actin expression were additionally tested to avoid false negative results. As positive and negative controls for CK-19 mRNA expression, RNA samples were also prepared from the breast carcinoma cell line MCF-7 and the plasmacytoma cell line ARH-77, respectively.

Reverse Transcription-PCR for Cytokeratin-19. RT of RNA was carried out as described previously (5, 12). Two different PCR reactions, with the respective negative controls, were done with each sample to amplify fragments of CK-19 and β -actin. The sequences of primers used for CK-19 and β -actin as well as the PCR conditions were exactly the same as in our previous publications (5, 12). The corresponding sizes of PCR products were 745 bp for CK-19 and 154 bp for β -actin. The primers extend across at least an intron, thus an eventual DNA contamination would not pose a significant problem. All of the RT-PCR results were confirmed as positive or negative by repeating the assay three times for each individual sample. On repeat testing the results of the RT-PCR assay were highly reproducible. The sensitivity of CK-19 mRNA detection by nested RT-PCR was evaluated in a previous study and was found that this assay was capable of consistently detecting 1 MCF-7 cell among 10^6 normal hematopoietic cells (5). In the same study, the specificity of the detection was evaluated with peripheral blood mononuclear cells from 54 healthy female blood donors and was found that only 2 (3.7%) samples were positive, whereas 52 (96.3%) samples were negative (5).

Reverse Transcription-PCR for HER-2. The expression of HER-2 mRNA in the CK-19-positive samples before trastuzumab administration was investigated by doing nested

RT-PCR. The cDNA was synthesized from 5 μg total RNA with the ThermoScript RT-PCR system (Invitrogen Ltd., Paisley, United Kingdom). To amplify cDNA, 5 μL were subjected to first PCR in 50 μL of PCR buffer [10 mmol/L of HCL, buffer (pH 8.3), 50 mmol/L KCl, and 2.5 mmol/L MgCl_2] containing 1 mmol/L deoxynucleotide triphosphate, 0.5 mmol/L of primers (Her-2A and Her-2B), and 2.5 units platinum TaqDNA polymerase (Invitrogen). For the second round of amplification (nested PCR), a 2- μL aliquot of the first PCR product was added to the same PCR buffer with 1 mmol/L deoxynucleotide triphosphate, 0.5 mmol/L of primers (Her-2C and Her-2D), and 2.5 units platinum TaqDNA polymerase. The primer sequences and the PCR protocols for the first and nested PCR are presented in Tables 1 and 2, respectively. The nested PCR was done with a modified touchdown program as described previously (13). All of the PCR products were resolved by agarose 2% gel stained with ethidium bromide and photographed under UV conditions. To determine the sensitivity of the assay, total RNA decreasing dilutions of MCF7 and T47D HER-2-positive breast carcinoma cell lines were tested. In these experiments, the nested RT-PCR assay was consistently capable of detecting the HER-2 mRNA signal of 10 or more MCF7 or T47D cells, although the dilution of one cell was detected in only 1 of 5 experiments (Fig. 1).

Real-time Reverse Transcription-PCR for Cytokeratin-19 mRNA. In addition to the nested RT-PCR, the CK-19 mRNA signal was investigated by quantitative real-time RT-PCR with the LightCycler system (Roche Diagnostics) as de-

Table 2 PCR protocols for HER-2

PCR step	HER-2 1st PCR	No of cycles	HER-2 2nd PCR	No of cycles
Denaturation	6 min/94°C	1	3 min/94°C	1
	50 s/94°C	1	30 s/94°C	5
Amplification		35	30 s/72°C	5
			30 s/94°C	
			30 s/70°C	
			30 s/72°C	
			30 s/94°C	
			30 s/68°C	
			30 s/72°C	
			30 s/94°C	
			30 s/66°C	
			30 s/72°C	
			30 s/94°C	
			30 s/64°C	
	30 s/72°C			
	30 s/94°C			
	30 s/62°C			
	30 s/72°C			
Final extension	10 min/72°C	1	7 min/72°C	1

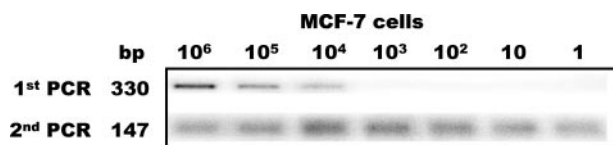


Fig. 1 Nested RT-PCR of total RNA decreasing dilutions of the MCF-7 breast carcinoma cell line showing amplification of the HER-2 mRNA signal in the second PCR down to the dilution of one tumor cell (1 of 5 experiments).

scribed previously (14). The primers and the hybridization probes used for CK-19 were designed and synthesized by TIB MOLBIOL (Berlin, Germany; ref. 14). To ensure that amplifiable material was present in all of the specimens, and to avoid false negative results, real-time amplification of glyceraldehyde-3-phosphate dehydrogenase and porphobilinogen deaminase housekeeping genes was done for all of the samples (14). Real-time PCR was done in a total volume of 20 μ L in the LightCycler glass capillaries. For the PCR, 2 μ L of cDNA were placed into a 18- μ L reaction volume containing 1 μ L of the sense primer CK19-for (3 μ mol/L), 1 μ L of the antisense primer CK19-do (3 μ mol/L), 2.4 μ L of the LightCycler Fast Start DNA Master Hybridization Probes reagent (10 \times concentration), 1 μ L of the probe CK19-FL (3 μ mol/L), 1 μ L of the probe CK19-LC (3 μ mol/L), and diethyl pyrocarbonate-H₂O was added to the final volume. PCR reaction was initiated with a 10-minute denaturation at 95°C and terminated with a 30-second cooling step at 40°C. The cycling protocol consisted of denaturation step at 95°C for 10 seconds, annealing at 60°C for 10 seconds, and extension at 72°C for 20 seconds and repeated for 50 times. Fluorescence detection was done at the end of each annealing step for 0 seconds.

For quantification, an external calibration curve was obtained by using external standard cDNAs. Total RNA was prepared from 1 \times 10⁶ MCF-7 cells (as verified by a hemocytometer). Serial dilutions of this RNA preparation in diethyl pyrocarbonate-treated water, corresponding to 1 to 10,000 MCF-7 cells, were used for cDNA synthesis. These cDNAs were kept in aliquots at -20°C and used throughout the study as external standards. This calibration curve was created by plotting the number of MCF-7 cells corresponding to each external standard cDNA *versus* the value of its crossing point. The number of circulating CK-19 mRNA-positive cells for all of the tested samples was expressed as MCF-7 cell equivalents/5 μ g of total-RNA, as determined by LightCycler software 3.1, according to the external standard calibration curve.

Fluorescence *In situ* Hybridization for HER-2 and Aneusomy. Mononuclear cells were separated by density centrifugation of peripheral blood and bone marrow samples through Ficoll-Hypaque, and cytopspins were prepared (5 \times 10⁵ mononuclear cells/slide) and stored at -70°C until used. Then frozen slides were left at room temperature for 15 minutes, immersed in 70% acetic acid for 30 seconds, washed and dehydrated in ETOH, immersed in cold acetone for 2 minutes, and air dried. Thereafter, they were placed in 2 \times SSC solution (pH 5.3) for 2 minutes at 73°C. Proteolysis with pepsin was done for 5 minutes at 37°C, and then slides were placed in 2 \times SSC solution for 1 minute at room temperature with post fixation in

formalin buffer solution for 5 minutes and in 2 \times SSC for 1 minute at room temperature and dehydrated in ETOH. For hybridization, the PathVysion HER-2 DNA probe and Breast Aneusomy probe set (Vysis, Downers Grove, IL) were used at 85°C for 2 minutes and overnight at 37°C. The next morning slides were placed in the posthybridization wash buffer for 2 minutes at 72°C, 10 μ L of 4',6-diamidino-2-phenylindole (DAPI) were added, and the covered slides were stored at -20°C.

The microscopic examination was done with an Axioplane 2 Nikon microscope with Vysis filters, and the images were processed with the Metasystems' ISIS fluorescence *in situ* hybridization (FISH) imaging system (Altlusheim, Germany). For HER-2/neu probe, we used single-band filters for orange, green, and DAPI and for Breast Aneusomy probe set single band filters for red, gold, green, aqua, and DAPI. We considered FISH HER-2/neu positive the cells with >4 orange signals or a ratio of HER-2/neu orange signals to CEP17 green signals \geq 2 in cases of aneuploidy. In a normal diploid cell, there are two copies of each of the following: LSI 1 (two gold signals), CEP 8 (two red signals), CEP 11 (two green signals), and CEP 17 (two aqua signals). Copy numbers of more or less than two signals of any probe indicates chromosome gain or loss, respectively.

Immunohistochemistry for HER-2 Overexpression in the Primary Tumor. Immunohistochemistry with the monoclonal antibody CB11 (NCL-CB11, Novocastra Laboratories, Newcastle upon Tyne, United Kingdom), at a dilution of 1 of 50 with incubation period of 60 minutes, was done with the OPTIMAX automated system (with the Super Sensitive Link-Label Detection System RTU Multilink AP/Fast Red, QA2000XE purchased from BioGenex Laboratories, San Ramon, CA), after antigen retrieval by microwave pretreatment at 500 watts for 3 \times 5 minutes in citrate buffer (0.01 mol/L, pH 6). Scoring was based on the criteria recommended by DAKO A/S for the HercepTest (DAKO A/S, Glostrup, Denmark). Only membrane staining intensity and pattern was scored with the 0 to 3+ scale: scores of 0 to 1+ were considered negative, score 2+ was considered weak positive, and score 3+ was considered (strongly) positive.

RESULTS

Patient Characteristics. Between March 2001 and November 2002 we screened 115 breast cancer patients (42 with stage I/II, 18 with stage III, and 55 with stage IV disease) to enroll 30 patients and five controls in this study. Table 3 shows the pretreatment patient characteristics. The median age was 53 years, and 13 (43%) patients were premenopausal. Seventeen (57%) patients had metastatic disease, 4 (13%) patients had stage III, and 9 patients had (30%) stage I to II. Eleven (37%) patients had primary tumors lacking estrogen and progesterone receptor expression, although HER-2 overexpression (2+ or 3+) on the primary tumor was present in 10 (33%) patients. Prior treatment included neoadjuvant and/or adjuvant chemotherapy and hormonal therapy for the 13 patients with nonmetastatic disease; most patients with metastases had already received multiple chemotherapy regimens. The median number of prior chemotherapy regimens for the entire group was 1 (range

Table 3 Patient characteristics

Number of patients	30
Age, median (range)	53 (31–75)
Menopausal status	
Premenopausal	13 (43%)
Postmenopausal	17 (57%)
Disease stage	
I	3 (10%)
II	6 (20%)
III	4 (13%)
IV	17 (57%)
Hormone receptor expression of primary tumor	
ER+PR+	6 (20%)
ER+PR–	7 (23%)
ER–PR+	4 (13%)
ER–PR–	11 (37%)
Unknown	2 (7%)
HER-2 expression of primary tumor	
3+	6 (20%)
2+	4 (13%)
1+	5 (17%)
0	14 (47%)
Unknown	1 (3%)
Prior treatment	
Surgery	30 (100%)
Radiation	18 (60%)
Hormonal therapy	12 (40%)
Chemotherapy	30 (100%)
adjuvant	22 (73%)
neoadjuvant	7 (23%)
1st line metastatic	17 (57%)
≥2nd line metastatic	10 (33%)

1 to 4). No patient had previously received trastuzumab treatment. Table 4 shows the disease stage at study enrollment and time elapsed from prior systemic therapies for 30 patients treated with trastuzumab infusions and 5 “control” patients treated with immunoglobulin IgG. All of the patients had CK-19 mRNA-positive cells detected by nested RT-PCR in the peripheral blood and/or bone marrow after the completion of chemotherapy and/or hormonal therapy and before study entry. In 25 of 30 patients (83%), HER-2 mRNA expression was detected by nested RT-PCR in the pretrastuzumab treatment CK-19–positive peripheral blood and/or bone marrow sample. In 5 of those patients for whom mononuclear cells cytopins on slides had been prepared from the pretrastuzumab treatment CK-19–positive peripheral blood and/or bone marrow sample, we found cells presenting FISH amplification of the HER-2/neu gene with >10 signals and a HER-2/neu to CEP17 ratio >4 in all of the five cases and aneusomy cells as detected by LSI 1, CEP 8, CEP 11, and CEP 17 with chromosomal gain or loss in two of the cases (Fig. 2).

Response to Trastuzumab Administration by Nested Reverse Transcription-PCR for Cytokeratin-19 mRNA. The first 20 patients received trastuzumab 2 mg/kg weekly for 4 to 8 consecutive weeks. As shown in Table 5, before trastuzumab treatment, 12 (60%) patients had CK-19 mRNA detected only in the bone marrow, 2 (10%) only in the peripheral blood, and 5 (25%) both in peripheral blood and bone marrow. In 1 patient (number 19), only peripheral blood CK-19 mRNA was done, which was positive. After 4 weekly trastuzumab infusions, 15 (75%) patients had become negative for the CK-19 mRNA

transcripts and discontinued additional treatment. The other 5 patients who remained CK-19 mRNA positive received 4 additional weekly trastuzumab infusions (total of 8 weeks), after which all but 1 patient became negative for CK-19 mRNA. So overall, after 4 or 8 weekly infusions, 19 of 20 (95%) patients had turned CK-19 mRNA negative, and only 1 (5%) patient remained positive despite trastuzumab administration (patient number 4 on Table 5).

The next 10 patients received trastuzumab 6 mg/kg every 3 weeks for 6 to 9 consecutive weeks. As shown in Table 5, before treatment, 2 (20%) patients had CK-19 mRNA detected only in the bone marrow, 1 (10%) in both peripheral blood and bone marrow, and for the remaining 7 (70%) patients, only the peripheral blood was tested, and it was positive. After 6 weeks (2 trastuzumab infusions), 6 (60%) patients had become negative for CK-19 mRNA transcripts, and additional treatment was discontinued. The other 4 patients who remained CK-19 mRNA positive received one additional trastuzumab infusion (total of 9 weeks), after which all but 1 patient became negative for CK-19 mRNA. So overall, 9 of 10 (90%) patients had turned CK-19 mRNA negative with 2 to 3 trastuzumab infusions given every 3 weeks. Only 1 (10%) patient remained positive (patient number 13 on Table 5).

After a median follow-up of 6 (range 2 to 22+) months, the median duration of CK-19 mRNA negativity for the whole group of 30 patients was 6 months (range 0 to 21+), and 13 (43%) patients had become CK-19 mRNA positive. The median duration of CK-19 mRNA negativity according to the stage of disease at study entry was as follows: 9 months (range 9 to 18) for stage I, 9 months (range 3 to 9+) for stage II, 12 months (range 3 to 21+) for stage III, and 6 months (range 3 to 15+) for stage IV [$P = 0.07$ for the comparison of stage IV (metastatic) versus stage I to III (nonmetastatic) by the log-rank test]. Two patients were retreated with trastuzumab when they became CK-19 mRNA positive, and both turned negative again after 4 weekly infusions of 2 mg/kg. At this time, 18 of 30 (60%) patients have discontinued follow-up because of the following reasons: no response to trastuzumab ($n = 2$); reappearance of CK-19 mRNA-positive cells ($n = 13$); and disease progression and death without detectable CK-19 mRNA ($n = 3$). Twelve patients are still CK-19 mRNA negative on follow-up.

Response to Trastuzumab Administration by Real-Time Reverse Transcription-PCR for Cytokeratin-19 mRNA. To verify the absence of CK-19 mRNA transcripts, all of the post-trastuzumab treatment peripheral blood and bone marrow samples were also analyzed by real-time RT-PCR, and 10 of 30 (33%) patients had detectable CK-19 mRNA. More specifically, for 20 patients, both the nested and the real-time RT-PCR of the post-trastuzumab samples were negative, for 2 patients both were positive, and for 8 patients the real-time RT-PCR was positive, whereas the nested RT-PCR was negative; the concordance rate for positivity and negativity was 73.3% (22 of 30 patients). The discordant results were obtained in bone marrow samples ($n = 4$), peripheral blood samples ($n = 2$), or both ($n = 2$). All of these patients in whom discordant results were obtained with the two methods had metastatic disease at study entry. The positive samples contained a mean of 3.15 (range 0.6 to 14.3) MCF-7 cell equivalents/5 μ g of total-

Table 4 Disease stage at study enrollment and prior treatment of 30 patients treated with trastuzumab infusions and 5 control patients treated with immunoglobulin IgG

Patient number	Disease stage at study enrollment	Number of prior chemotherapy regimens	Number of prior hormonal therapies	Patient enrollment on the study and time elapsed from previous systemic therapy
1	I	1	1	After adjuvant hormonal therapy
2	I	1	1	After adjuvant hormonal therapy
3	I	1	1	After adjuvant hormonal therapy
4	IIA	1	1	After adjuvant hormonal therapy
5	IIA	1	1	After adjuvant hormonal therapy
6	IIA	1	1	After adjuvant hormonal therapy
7	IIA	1	1	After adjuvant hormonal therapy
8	IIB	1	1	After adjuvant hormonal therapy
9	IIB	1	1	After adjuvant hormonal therapy
10	IIB	1	1	After adjuvant hormonal therapy
11	IIIA	1	1	After adjuvant hormonal therapy
12	IIIB	1	1	After hormonal therapy
13	IIIB	1	1	After hormonal therapy
14	IIIB	1	1	After hormonal therapy
15	IV	3	2	After 2nd line hormonal therapy
16	IV	2	1	3 mo from 2nd line chemotherapy
17	IV	4	3	After 4th line chemotherapy
18	IV	2	2	After 2nd line hormonal therapy
19	IV	2	2	After 2nd line hormonal therapy
20	IV	1	1	2 mo from 1st line hormonal therapy
21	IV	2	2	2 mo from 2nd line chemotherapy
22	IV	1	1	After 1st line hormonal therapy
23	IV	3	2	After 2nd line hormonal therapy
24	IV	2	2	After 2nd line hormonal therapy
25	IV	3	2	After 3rd line chemotherapy
26	IV	2		After 2nd line chemotherapy
27	IV	2		3 mo from 2nd line chemotherapy
28	IV	3	1	After 1st line hormonal therapy
29	IV	1	1	After 1st line hormonal therapy
30	IV	2		5 mo from 2nd line chemotherapy
31	IV	2		1 mo from 2nd line chemotherapy
32	IV	1		After 1st line chemotherapy
33	IV	1	2	6 mo from 2nd line hormonal therapy
34	IV	2	1	3 mo from 2nd line chemotherapy
35	IV	1	2	After 2nd line hormonal therapy

RNA. For 16 patients who had both pre- and post-trastuzumab treatment peripheral blood or bone marrow samples analyzed by real-time RT-PCR for CK-19 mRNA, there was a reduction in the mean number of MCF-7 cell equivalents/5 μ g of total-RNA from 3.84 (pretrastuzumab) to 0.36 (post-trastuzumab; Wilcoxon paired *t* test, *P* < 0.001; Fig. 3).

Trastuzumab Toxicity. Trastuzumab-related toxicity consisted primarily of a mild allergic reaction during the infusion with low grade fever and chills in 8 (27%) patients. All of the patients were able to complete the scheduled treatments. No hematologic or biochemical toxicity was observed. In no patient was the post-treatment left ventricular ejection fraction decreased by >10% of baseline value, and no one developed symptomatic congestive heart failure.

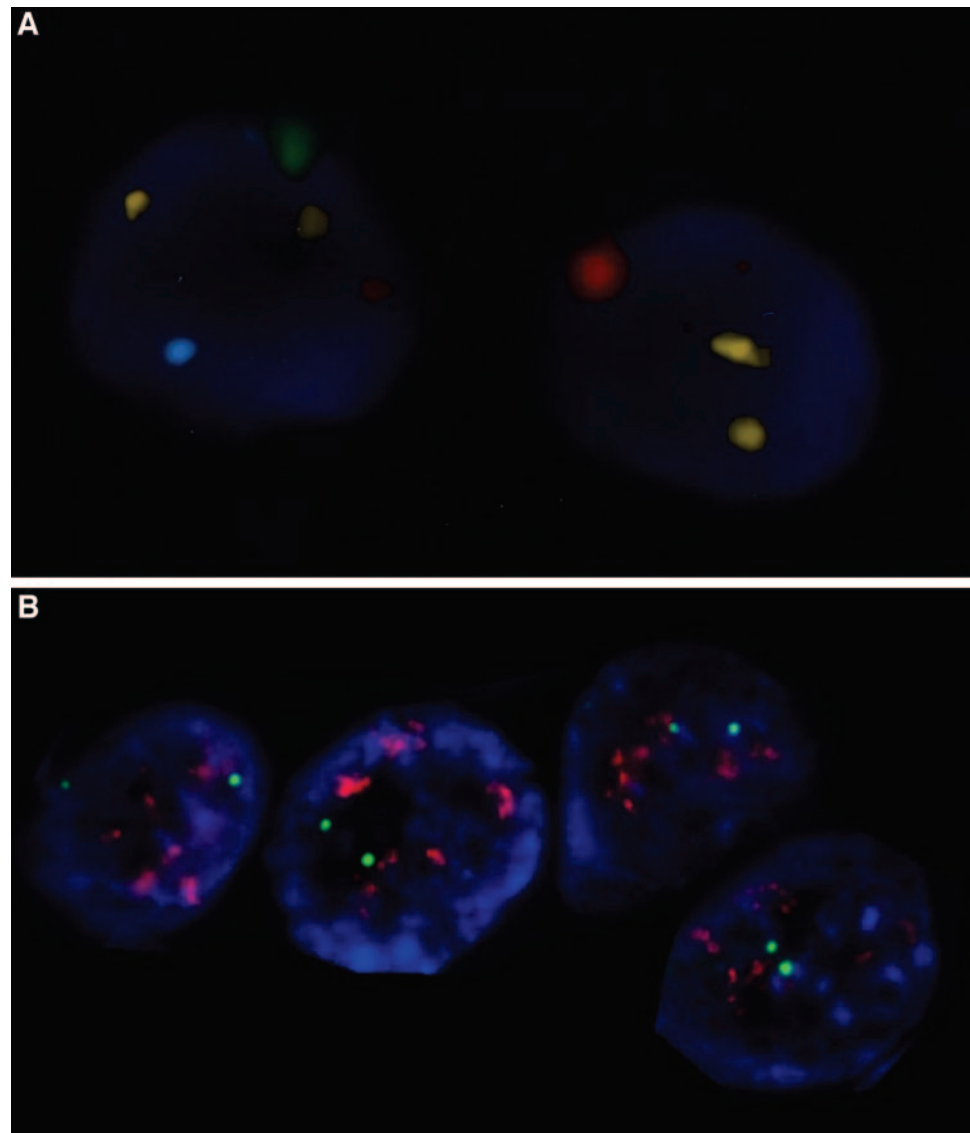
Immunoglobulin Administration. To investigate the possibility of a nonspecific effect of trastuzumab infusions on the elimination of CK-19 mRNA-positive cells, we administered immunoglobulin IgG (Sandoglobulin) 500 mg/kg i.v. to 5 patients who fulfilled all of the eligibility criteria for this study (all of the patients were CK-19 mRNA positive in the peripheral blood despite prior chemotherapy). The disease stage at study enrollment and prior treatments received for these 5 patients are

shown in Table 4 (patient numbers 7, 32 to 35). None of these patients became CK-19 mRNA negative after the immunoglobulin administration.

DISCUSSION

In this pilot study, we used the anti-HER-2 monoclonal antibody trastuzumab to target the chemotherapy-resistant CK-19 mRNA-positive breast cancer cells in the peripheral blood and bone marrow. Our group, as well as other investigators, has previously used the detection of CK-19 mRNA as a marker for the presence of breast cancer cells in the peripheral blood and bone marrow (15–17, reviewed in 18). Although the CK-19 mRNA is not an ideal marker for breast cancer cell detection, mainly because of a low rate of false positives in normal females and patients with hematologic malignancies (5), it is the most sensitive assay with good specificity under optimized conditions (19). The malignant nature of cytokeratin-positive cells has been shown with genomic analysis and specialized cell culture assays (8, 9, 20, 21). In this study, we were able to cross-validate in a limited number of patients the malignant nature of these cytokeratin-positive cells by doing FISH

Fig. 2 *A.* Four breast cancer cells with HER-2/neu amplification (>10 orange signals per cell) in a FISH preparation with PathVysion HER-2 DNA probe. *B.* Two breast cancer cells with aneusomy in chromosomes 8 (red signal), 11 (green signal), and 17 (aqua signal) in a FISH preparation with Breast Aneusomy probe set from Vysis.



analysis for HER-2/neu gene amplification and aneusomy detection for chromosomes 1, 8, 11, and 17. Other investigators have also used FISH analysis for these markers to detect breast cancer cells in cytological specimens (22–24). Furthermore, in a previous study we have shown that in patients with early breast cancer, the detection of CK-19 mRNA-positive cells in the peripheral blood before the administration of adjuvant chemotherapy was an independent prognostic marker of reduced disease-free interval ($P = 0.0007$) and overall survival ($P = 0.01$; ref. 5). We as well as other investigators have reported that chemotherapy might fail to eliminate occult tumor cells in up to 50% of breast cancer patients, and that the persistence of these chemotherapy-resistant cells could have adverse prognostic implications (12, 25, 26). Therefore, it is important to determine if novel biological agents can be used effectively to target these chemoresistant cells.

HER-2 is a member of a family of receptors that interact

with each other and various ligands to stimulate mitogenic signal transduction pathways leading to increased cell proliferation (27). This perhaps explains why HER-2 overexpression is an indicator of poor prognosis in breast tumors (28). On the contrary, HER-2 overexpression is predictive of response to trastuzumab therapy (29). Taking advantage of the reported high expression of HER-2 on micrometastatic breast cancer cells (7, 11), we used the humanized anti-HER-2 monoclonal antibody trastuzumab to target the disseminated CK-19 mRNA-positive cells. This is the first study to show that a short course of trastuzumab infusions can very effectively reduce to undetectable levels the chemotherapy-resistant disseminated tumor cells in the blood and bone marrow of breast cancer patients.

For this study, we selected the patients only on the basis of having detectable CK-19 mRNA-positive cells in the peripheral blood and/or bone marrow after the administration of chemotherapy. Most patients with metastatic disease had previously

Table 5 Results of CK-19 mRNA detection by nested RT-PCR for the 30 patients treated with trastuzumab and the 5 control patients

Patient number	Tumor HER-2 status	HER-2 mRNA expression on pretreatment CK-19-positive sample	Type of treatment	CK-19 mRNA Before treatment (BM/PB)	CK-19 mRNA after treatment (BM/PB)		Follow-up post-trastuzumab treatment (BM/PB)							Duration of CK-19 mRNA negativity (mo)
					4-6 wks	8-9 wks	3 mo	6 mo	9 mo	12 mo	15 mo	18 mo	21 mo	
1	0	+	weekly	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	nd/+	18
2	0	+	3-weekly	nd/+	nd/+	nd/-	nd/-	nd/-	nd/-					9+
3	0	-	3-weekly	nd/+	nd/-	+/-	nd/+							9
4	+1	+	weekly	+/-	+/-									0
5	0	+	weekly	nd/+	nd/-		nd/+							3
6	0	+	3-weekly	nd/+	nd/-		nd/-	nd/-	nd/-					9+
7	+2	nd	control	nd/+	nd/+									
8	+1	+	weekly	+/+	+/-		nd/+							3
9	0	+	3-weekly	nd/+	nd/-		nd/-	nd/-	nd/-					9+
10	0	+	3-weekly	nd/+	nd/-		nd/-	-/-	-/-					9+
11	+3	+	weekly	+/-	+/-		-/-	-/-	-/-	-/-	-/-	-/-	-/-	21+
12	+3	-	weekly	+/-	-/-		-/-	-/-	-/-	-/-				12+
13	+1	-	3-weekly	+/-	+/+	+/+								0
14	0	+	3-weekly	nd/+	nd/+	nd/-	nd/-							3+
15	+3	+	weekly	+/-	+/-	-/-	-/-	-/-	+/+					9
16	+3	+	weekly	+/-	-/-		-/-	-/-	-/-	-/-	+/+			15
17	0	+	weekly	+/+	-/-	-/-	+/-							3
18	+2	+	weekly	+/+	+/-		-/-	dead						3
19	+2	+	weekly	+/-	-/-		-/-	-/-	-/-	-/-	+/+			15
20	+2	+	weekly	+/+	-/-		+/+							3
21	0	+	weekly	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-			15+
22	+3	+	weekly	-/+	-/-		-/-	-/-	-/-	-/-	+/+			15
23	+2	+	weekly	+/+	-/-		-/-	dead						3
24	0	-	weekly	+/-	-/-		-/-	-/-	-/-	-/-	-/-			15+
25	0	+	weekly	+/-	-/-		-/-	-/-	dead					6
26	+1	+	weekly	+/-	-/-		+/-							3
27	0	+	weekly	-/+	-/-		nd/+							3
28	UK	+	weekly	+/-	-/-		+/+							3
29	+1	+	3-weekly	+/-	-/-		-/-	-/-	-/-					9+
30	0	-	3-weekly	nd/+	nd/+	nd/-	nd/-	nd/-						6+
31	+3	+	3-weekly	+/+	-/-		-/-							3+
32	+3	nd	Control	nd/+	nd/+									
33	0	nd	Control	nd/+	nd/+									
34	+1	nd	Control	nd/+	nd/+									
35	+1	nd	Control	nd/+	nd/+									

Abbreviations: BM, bone marrow; PB, peripheral blood; nd, not done; UK, unknown.

failed multiple different chemotherapy regimens, although patients with hormone-receptor positive tumors had also received hormonal treatment. Patients with operable breast cancer had completed the administration of adjuvant chemotherapy and those with hormone-receptor positive tumors had also received adjuvant hormonal therapy. So the detected CK-19 mRNA-positive cells were truly resistant to "standard" therapies. We did not select our patients based on HER-2 overexpression in the primary tumor, because HER-2 overexpression in the micrometastatic cells is much more common and has been reported at two-fold the frequency of detection than in the primary tumors (7, 11). Furthermore, extrapolation for HER-2 positivity of the micrometastatic cells from the detection on the primary tumor has been shown to be unreliable (11). Nevertheless, in 28 of 30 (93%) patients, the CK-19 mRNA signal as measured by nested RT-PCR disappeared after 4 to 8 weekly or 2 to 3 3-weekly infusions of trastuzumab, indicating that CK-19 mRNA-positive cells were susceptible to trastuzumab therapy most likely because of HER-2 receptor expression. We retrospectively analyzed HER-2 mRNA expression in the pretrastuzumab CK-19-

positive peripheral blood and bone marrow samples by RT-PCR, and we found them positive in 25 of 30 patients (83%). Furthermore, in 5 patients for whom pretreatment peripheral blood or bone marrow mononuclear cells cytopspins were available (cytopspins not available for the remaining 25 patients), we found cells with FISH amplification of the HER-2/neu gene in all of the cases. These samples confirmed by FISH to have cells with HER-2/neu gene amplification also tested positive for HER-2 mRNA expression. This frequency of HER-2 expression in the micrometastatic cells is higher than the 60 to 67% reported in the literature with an immunohistochemical method (7, 11). As the elimination rate of the CK-19 mRNA signal by nested RT-PCR exceeds that of HER-2 mRNA expression in the pretrastuzumab samples, it is possible that HER-2 expression was below the detection limit of the RT-PCR assay in some patients with a low number of CK-19-positive cells. Indeed, in the decreasing dilution experiments with MCF-7 cells, the nested RT-PCR for HER-2 mRNA reliably detected the signal of 10 or more tumor cells.

Furthermore, the results of the quantitative real-time RT-

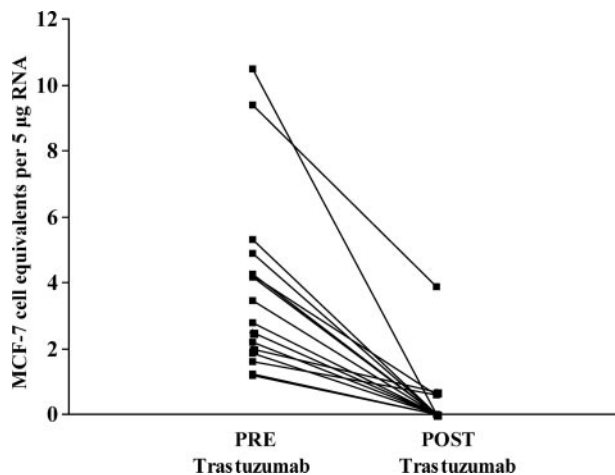


Fig. 3 Pre- and post-trastuzumab CK-19 mRNA detection by real-time RT-PCR in peripheral blood or bone marrow of 16 patients with breast cancer (Wilcoxon paired *t* test, $P < 0.001$). Quantitative results are expressed as MCF-7 cell equivalents/5 μg RNA.

PCR assay confirmed those of nested RT-PCR, because no CK-19 mRNA transcripts were detectable in 20 of 30 patients (67%) after the trastuzumab infusions. This quantitative RT-PCR assay for CK-19 mRNA detection with the LightCycler system has been developed and compared with nested RT-PCR in a many samples with a good concordance in respect to positivity and negativity (14). Interestingly, in 16 patients who had both pre- and post-trastuzumab treatment peripheral blood or bone marrow samples available for analysis by real-time RT-PCR for CK-19 mRNA, there was a 10-fold (1 log) reduction in the mean number of circulating tumor cells. Therefore, our results on the efficacy of trastuzumab should be interpreted with caution, because the lack of detection of the CK-19 mRNA signal after the infusions might represent a reduction of circulating tumor cells below the detection limit of the assay and not necessarily the elimination of all of the tumor cells.

To investigate the possibility of a nonspecific cytotoxic effect of trastuzumab, we administered immunoglobulin IgG to a control group of 5 CK-19 mRNA-positive patients who fulfilled all of the eligibility criteria of the study and according to their characteristics were no different from the study population, and none of them turned negative with the immunoglobulin infusions. Moreover, to exclude an interference in the RT-PCR assay by the presence of trastuzumab in the samples we exposed dilutions of MCF-7 cells to increasing concentrations of trastuzumab (at 2, 100, and 200 nmol/L) for 24 to 48 hours. CK-19 mRNA transcripts were still detectable by the nested RT-PCR assay down to the dilution of 1 MCF-7 cell among 10^6 normal hematopoietic cells (data not shown).

With close monitoring by RT-PCR of the peripheral blood and bone marrow during the follow-up, we observed that the disappearance of CK-19 mRNA signal was not short-lived as it lasted for several months even in patients with metastatic disease. This is especially important because overt metastases could easily "seed" the circulating blood with new tumor cells. Perhaps this lasting effect is because of the long half-life of

trastuzumab in the body, which is estimated to be several months after multiple infusions (30). Interestingly, trastuzumab was still effective in 2 patients who were retreated when the CK-19 mRNA signal reappeared during the follow-up. Therefore, it might be possible with repeated trastuzumab administrations, given either periodically or on demand when the RT-PCR assay becomes positive, to prolong the time that circulating CK-19 mRNA-positive cells are not detectable.

Whereas the efficacy of trastuzumab in HER-2 overexpressing breast tumors is proven (29, 31), so is the risk of cardiac toxicity estimated to be 3 to 7% when trastuzumab monotherapy is used (32). In our study, we observed no cardiac or other significant toxicity with the short courses of trastuzumab except of the well-described hypersensitivity reaction, which occurs during the infusion. Considering the observed high efficacy of trastuzumab in reducing to undetectable levels the chemoresistant occult tumor cells in peripheral blood and bone marrow, our results additionally strengthen the rationale for using trastuzumab in the adjuvant treatment of breast cancer. However, the efficacy and safety of administering trastuzumab in the adjuvant setting is still under clinical investigation (33). Moreover, the prognostic significance of the disappearance of these occult tumor cells in terms of reduction in the risk of relapse and death remains to be proven.

Other investigators have also used monoclonal antibody-based treatments to target the disseminated occult tumor cells with variable success. Schlimok *et al.* (34) used a monoclonal Lewis Y antibody, and observed eradication or significant reduction of CK-positive/Lewis Y-positive cells, in 5 of 10 breast cancer patients presenting with micrometastatic tumor cells in the bone marrow. Braun *et al.* (35) used the murine monoclonal antibody 17-1A (Edrecolomab) directed against the epithelial adhesion molecule EpCAM and showed a marked reduction, and in 4 cases complete elimination, of EpCAM⁺/CK⁺ cells from the bone marrow of 10 patients with advanced breast cancer. More recently, Kirchner *et al.* (36) used the same monoclonal antibody (Edrecolomab) in nine patients with early breast cancer, who had bone marrow micrometastases after adjuvant chemotherapy, and observed complete elimination in seven and significant reduction in the remaining 2 patients.

Considering that HER-2 overexpression in the primary tumor (28) or the micrometastatic tumor cells (11) is a poor prognostic marker, our approach of targeting HER-2-positive tumor cells has the comparative advantage, over other antibody-based treatments, of aiming at the elimination of malignant cells with an aggressive biological phenotype. However, until a meaningful clinical benefit from the disappearance of these cells is clearly shown, this experimental approach cannot be recommended. Future studies will need to evaluate the immunophenotypic and genomic characteristics of disseminated occult tumor cells as well as the potential clinical benefit from their elimination.

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Clinical Cancer Research

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Vassiliki Bozionellou, Dimitris Mavroudis, Maria Perraki, et al.

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