CEA-, Her2/neu-, BCRP- and Hsp27-positive Microparticles in Breast Cancer Patients*

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Abstract. *Background: This is the first prospective case-control study that evaluates the expression of tumour-specific antigens on circulating microparticles (MP) in breast cancer patients and in women with benign breast tumour. Materials and Methods: MP were determined by flow cytometry in patients with breast cancer (n=34; T1 (n=19) and T2 (n=15)) and women with benign breast tumour (n=19). Results: Patients with lymph node metastases (N1, n=9) showed significantly higher numbers of annexin V$^+$ MP (p=0.042), CD66$^+$ MP (p=0.045), BCRP1$^+$ MP (breast cancer resistance protein) (p=0.025) and Hsp27$^+$ MP (p=0.034) than controls. Furthermore, T1 patients had significantly higher levels of annexin V$^+$ MP (p=0.004), CD66$^+$ MP (p=0.025), BCRP1$^+$ MP (p=0.008) and Hsp27$^+$ MP (p=0.02) than controls. Conclusion: Significant differences are present between breast cancer patients with lymph node metastases and controls concerning annexin V-, CD66-, BCRP1- and Hsp27-positive MP. To specify the role of these MP subpopulations in breast cancer progression, further studies enrolling larger patient groups are part of ongoing research.*

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So far, the only serum tumour markers for breast cancer are cancer antigen (CA) 15-3 and carcinoembryonic antigen (CEA). Neither marker is used in diagnosis of primary breast cancer but only to diagnose recurrent disease and to evaluate response to chemotherapy (1). Prognostic factors for breast cancer are the status of axillary lymph nodes, age, tumour load and histological parameters (2), urokinase-type plasminogen activator (uPA) and its inhibitor (PAI-1) (3), and the presence of bone marrow micrometastases (4).

Several investigators have demonstrated elevated concentrations of circulating microparticles (MPs) in breast cancer patients (5-7). MPs are cellular membrane vesicles of a size between 0.1 and 1.0 μm, which are shed into the plasma during cell activation and apoptosis. (8) MPs are present in the plasma of healthy humans, where they originate mainly from platelets, followed by endothelial cells, granulocytes and erythrocytes. (9) Increased plasma concentrations of MPs have been reported in vascular diseases, diabetes and severe trauma (10) and in several types of cancer including gastric (11), lung (12), and colorectal cancer (13), leukaemia (14), myeloproliferative disorders (15), pancreatic (5) and breast cancer (5-7). In patients with prostate cancer, high platelet-derived MP (PMP) is correlated with short overall survival. (16) MP promote tumour growth, spreading and tumour-induced neovascularization (11).

There are several antigens which have been identified on breast cancer tissue: cluster of differentiation 66 (CD66), human epidermal growth factor receptor 2 (Her2/neu), breast cancer resistance protein (BRCP) and heat-shock protein 27 (Hsp27). CD66 is known as the CEA (carcinoembryonic antigen) family of molecules (1) and plays a role in cell

adhesion and rejection processes and therefore in tumour growth and spreading (1, 17). Elevated levels of CEA occur in patients with colorectal carcinoma (18), but also in patients with breast, lung, ovarian, prostate, hepatocellular and pancreatic cancer (19). Her2/neu, a transmembrane glycoprotein from the epidermal growth factor receptor family (EGFR) (20, 21), is expressed by various epithelial tissues. Her2/neu is overexpressed in 10-31% of invasive breast cancers, where it is a marker of poor prognosis (22-24). BCRP, a 72 kDa plasma membrane protein from the ABC (ATP binding cassette) transporter family (25), was first discovered in doxorubicin-resistant breast cancer cells (26). Endogenous BCRP expression is found in placenta, liver, bowel, ovary, blood vessels and in the ductuli and lobuli of the breast (27). Heat-shock proteins mediate protein transport and folding and are cytoprotective. (28) Hsp27 belongs to the family of small Hsps (15-30 kDa) (28). An overexpression of Hsp27 is found in various carcinomas like breast, ovarian, colorectal, hepatocellular, kidney, lung and prostate carcinoma (29).

The aim of our prospective case–control study was to detect tumour-specific MPs in women with breast cancer. Therefore, MP-expressing CD66, Her2/neu, BCRP and Hsp27 were evaluated in breast cancer patients using women with benign breast tumours well as controls.

Patients and Methods

Study population. Newly diagnosed patients with histologically proven breast cancer (n=34) were included in the study before surgical treatment or neoadjuvant chemotherapy and classified according to the TNM system: 19 patients had tumours <2 cm (T1) and 15 had tumours of a size between 2 and 4 cm (T2). 23 patients had negative axillary lymph nodes (N0), 9 had positive lymph nodes (N1) and in 2 patients the nodal state was unknown (Nx). Women with a benign breast tumour (n=19) were included in the control group before core biopsy of breast tumours which had been detected during routine mammography. Neither patients nor controls smoked or were taking oral contraceptives or hormonal treatment. Patients with the confounding factors hypertension, history of cancer or thrombosis, aspirin or low molecular weight heparin (LMWH) treatment were excluded. All patients were Caucasians. Signed informed consent was obtained from all participants, allowing analysis of all clinical and laboratory data mentioned in this paper. The Human Investigation Review Board of the Ludwig Maximilians University, Munich, Germany, approved the study.

Blood sampling and measurements. All blood samples were collected between 09.00-12.00 am by a single experienced medical student by puncture of the antecubital vein without tourniquet through a 20-gauge needle.

For MP analysis, platelet-poor plasma was prepared by centrifugation at 1550×g for 20 minutes within 15 minutes after collection. The plasma was then snap frozen in liquid nitrogen for 15 minutes and stored at –80˚C until assayed.

Reagents for microparticle analysis. Fluorescein isothiocyanate (FITC)-labelled annexin V, phycoerythrin (PE)-labelled annexin V, and IgG-PE were from Immuno Quality Products (Groningen, Netherlands) and IgG-FITC from Immunotech (Marseille, France). Anti-CD66-PE (detecting CD66c, e) and anti-Her2/neu-PE were from BD Biosciences Pharmingen (San Jose, USA), anti-BCRP1-FITC from Chemicon International (Billerica, USA) and anti-Hsp27-FITC from Stressgen (Victoria, Canada). All antibodies and annexin V were diluted with phosphate-buffered saline (PBS; 154 mmol/l NaCl, 1.4 mmol/l phosphate, pH 7.4). Final dilutions were: annexin V-FITC 1:100 (v/v), annexin V-PE 1:200, IgG-PE 1:10, IgG-FITC 1:10, anti-Her2/neu-PE 1:2, BCRP1-FITC 1:5, Hsp27-FITC 1:5, anti-CD66-PE was used undiluted.

Isolation and identification of MP were performed as described by Nieuwland (30). In brief, frozen plasma (250 μl) was thawed slowly on melting ice for approximately one hour. After centrifugation at 17570×g and 20˚C for 30 minutes, 225 μl of MP-free supernatant was taken off. The remaining MP pellet was diluted with 225 μl of PBS containing 10.9 mmol/l trisodium citrate (PBS/citrate buffer), re-suspended and centrifuged again for 30 minutes at 17570×g and 20˚C. Afterwards the supernatant (225 μl) was removed, 75 μl of PBS/citrate buffer was added, and the MP pellet was resuspended again. Five μl of the MP suspension was diluted in 35 μl $CaCl_2$ (2.5 mmol/l)-containing PBS. For MP staining, 5 μl APC-labelled annexin V and 5 μl of a cell-specific monoclonal antibody or isotype-matched control antibodies were added to the specimen. For detection of tumour-specific MP, antibodies against Her2/neu, BCRP, CD66 and Hsp27 were used. Samples were incubated in the dark for 15 minutes at room temperature. The reaction in all samples was stopped with 900 μl calcium buffer (2.5 mmol/l), except for the annexin V control, where citrate-containing PBS (900 μl) was added.

MP were analysed in a FACScan flow cytometer (Becton Dickinson, Heidelberg, Germany) using Cell Quest Software (Becton Dickinson, San Jose, CA, USA). Forward scatter (FSC) and side scatter (SSC) were set at a logarithmic gain. MPs were identified on basis of their size and density and their capacity to bind to a cell-specific monoclonal antibody and annexin V. Cell-specific labelling with monoclonal antibodies was corrected for identical concentrations of isotype-matched control antibodies and annexin V measurements were corrected for autofluorescence. The concentration of MPs per litre of plasma was estimated according to Berckmans (9).

Statistics. Parametrically distributed data were expressed as mean±standard deviation (SD) (minimum-maximum). All other data were presented as the median (Q1-Q3=interquartile range). Independent variables were analyzed by Mann-Whitney-*U*-test, all others by using Chi-square and Fisher's exact test. *P*-values <0.05 were regarded as statistically significant. Data were examined with SPSS (SPSS, Chicago, IL, USA) 16.0 for Windows.

Results

Study population. Patients and controls did not differ significantly concerning age, body mass index, haemoglobin level, platelet count, leucocyte count, CEA and CA15-3 levels (Table I).

Microparticle analysis. In brief, it was possible to detect different subpopulations of MPs ($CD66^+$, $Her2/neu^+$, $BCRP1^+$, $Hsp27^+$ MP). The $Hsp27^+$ subgroup represented

Table I. *Study population*.

	Age (years)	BMI (kg/m^2)	Hb (g/dl)	Platelets ($\times 10^6$/l)	Leukocytes ($\times 10^6$/l)	CEA (ng/ml)	CA15-3 (U/l)
Control	51.3±16.2	23.4±4.2	13.7±0.9	281.8±49.4	7.6±2.5	1.9±1.2	18.9±7.0
(n=19)	(19-80)	(18.6-30.8)	(12.3-15.4)	(199-366)	(4.9-16.0)	(0.7-.3.8)	(7.5-27.7)
Patients	59.2±13.8	25.4±3.8	13.3±1.2	281.9±61.6	6.7±1.3	4.1±10.6	18.6±9.4
(n=34)	(26-83)	(17.2-33.6)	(11.3-15.7)	(178-426)	(4.5-9.0)	(0.4-56.1)	(5.6-58.0)

Demographic patient data are shown. Parametrically distributed data are presented as mean±standard deviation (minimum-maximum).

Table IIa. *Annexin V$^+$ MPs, CD66$^+$ MPs, Her2/neu$^+$ MPs, BCRP$^+$ MPs and Hsp27$^+$ MPs ($\times 10^6$/l) in the study population*.

	Annexin V	CD66	Her-2/neu	BCRP	Hsp27
Control	5468	835	26	600	2344
(n=19)	(1900-9834)	(219-1507)	(6-106)	(251-1720)	(608-5060)
	(4306-6624)	(610-1066)	(20-43)	(453-715)	(1763-3258)
Patients	7296	998	30	728	2825
(n=34)	(1717-17455)	(367-2662)	(10-229)	(246-2012)	(891-8447)
	(4316-7296)	(732-1621)	(19-67)	(562-1299)	(1869-4741)
T1	9353	1167	34	769	3585
(n=19)	(1717-17455)	(506-2628)	(10-207)	(407-1399)	(891-8447)
	(6563-11240)	(848-1656)	(22-90)	(639-1301)	(2702-5119)
T2	5707	790	23	651	2267
(n=15)	(1904-13643)	(367-2662)	(11-229)	(246-2012)	(1072-6227)
	(2565-8701)	(606-1461)	(16-52)	(371-1280)	(1572-3541)

Data are presented as median value, (minimum-maximum), (interquartile range).

Table IIb. *Annexin V$^+$ MPs, CD66$^+$ MPs, Her2/neu$^+$ MPs, BCRP$^+$ MPs and Hsp27+ MPs ($\times 10^6$/l) in nodal-positive and nodal-negative breast cancer patients*.

	Annexin V	CD66	Her-2/neu	BCRP	Hsp27
N0	7161	859	27	700	2634
(N=23)	(1717-17455)	(435-2662)	(11-125)	(329-2012)	(891-8447)
	(2565-9787)	(726-1534)	(16-66)	(433-997)	(1665-2634)
N1	8707	1301	34	796	3943
(n=9)	(3191-14056)	(367-2628)	(10-229)	(246-1797)	(1626-6381)
	(5502-12565)	(997-1453)	(22-163)	(689-1481)	(2571-5486)

Data are presented as median value, (minimum-maximum), (interquartile range) in patients with positive (N1) or negative (N0) axillary lymph node metastases as compared to controls.

38.7% of the total number of MPs in patients and 42.9% in controls. A total of 13.7% (patients) and 15.3% (controls) of annexin$^+$ MPs were CD66$^+$; 10.0% (patients) and 11.0% (controls) of annexin V$^+$ MPs were BCRP$^+$; 0.4% (patients) and 0.5% (controls) of annexin V$^+$ MPs were Her-2/neu$^+$.

Patients with smaller tumour size (T1) had significantly higher concentrations of annexin V$^+$ MPs (p=0.004), CD66$^+$ MPs (p=0.025), BCRP$^+$ MPs (p=0.008) and Hsp27$^+$ MPs (p=0.02) than controls (Table IIa). However, MP levels did not differ significantly between T2 patients and controls.

Considering nodal status and MP count, significantly higher MP levels were found in patients with lymph node metastases (N1) compared to the control group (p=0.042) (Table IIb, Figure 1). There were also significant differences between N1 patients and controls with regard to CD66$^+$ MPs (p=0.045), BCRP$^+$ MPs (p=0.025) and Hsp27$^+$ MPs (p=0.034). The concentration of Her2/neu$^+$ MPs was elevated in N1 patients compared to controls without reaching significance (Table IIb, Figure 1).

Thus, the total number of MPs as well as three of the four measured subpopulations were significantly elevated in N1 patients compared to controls. With regard to N1 patients, no

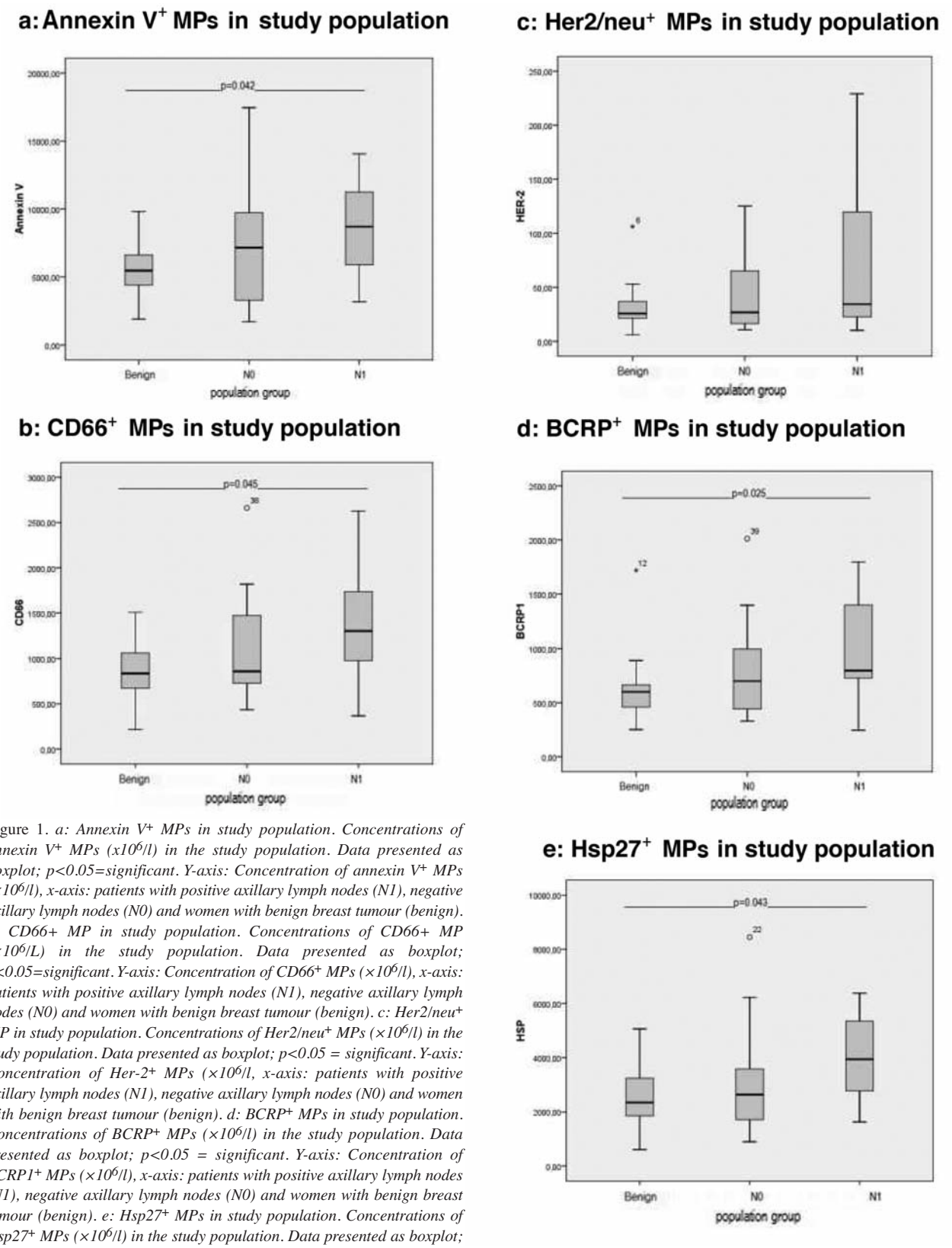

Figure 1. *a: Annexin V+ MPs in study population. Concentrations of annexin V+ MPs (x10⁶/l) in the study population. Data presented as boxplot; p<0.05=significant. Y-axis: Concentration of annexin V+ MPs (×10⁶/l), x-axis: patients with positive axillary lymph nodes (N1), negative axillary lymph nodes (N0) and women with benign breast tumour (benign). b: CD66+ MP in study population. Concentrations of CD66+ MP (×10⁶/L) in the study population. Data presented as boxplot; p<0.05=significant. Y-axis: Concentration of CD66+ MPs (×10⁶/l), x-axis: patients with positive axillary lymph nodes (N1), negative axillary lymph nodes (N0) and women with benign breast tumour (benign). c: Her2/neu+ MP in study population. Concentrations of Her2/neu+ MPs (×10⁶/l) in the study population. Data presented as boxplot; p<0.05 = significant. Y-axis: Concentration of Her-2+ MPs (×10⁶/l, x-axis: patients with positive axillary lymph nodes (N1), negative axillary lymph nodes (N0) and women with benign breast tumour (benign). d: BCRP+ MPs in study population. Concentrations of BCRP+ MPs (×10⁶/l) in the study population. Data presented as boxplot; p<0.05 = significant. Y-axis: Concentration of BCRP1+ MPs (×10⁶/l), x-axis: patients with positive axillary lymph nodes (N1), negative axillary lymph nodes (N0) and women with benign breast tumour (benign). e: Hsp27+ MPs in study population. Concentrations of Hsp27+ MPs (×10⁶/l) in the study population. Data presented as boxplot; p=0.05=significant. Y-axis: Concentration of Hsp27+ MPs (×10⁶/l), x-axis: patients with positive axillary lymph nodes (N1), negative axillary lymph nodes (N0) and women with benign breast tumour (benign).*

significant differences were present concerning the concentrations of annexin V^+ MPs as well as all subpopulations in comparison to N0 patients (Table IIb).

Discussion

This prospective case–control study is the first report addresing the exposure of cancer-related markers on circulating MPs in breast cancer patients and women with benign breast tumour. The total numbers of MPs as well as three of the four measured subpopulations ($CD66^+$, $BCRP^+$, $Hsp27^+$) of MPs were elevated in patients with lymph node metastases compared to women with benign breast tumour. Since MPs from cancer cells have been implicated to contribute to tumour growth and spreading *via* various mechanism, elevated levels of circulating MP expressing cancer-related markers may reflect cancer activity in these patients.

Only few studies are currently reported concerning MP concentrations in breast cancer patients (5-7). In our former studies, PMP, leucocyte-derived MP (LMP) and endothelial-cell-derived MP (EMP) were investigated by flow cytometry and electron microscopy in breast cancer patients (6, 7) The total number of MP as well as the number of PMP were significantly higher in patients than in controls, increasing with augmenting tumour size, and they were highest in patients with distant metastases (6). In breast cancer patients, 82.3% of MP originated from platelets, 14.6% from activated endothelial cells and 0.3% from leucocytes (6). Furthermore, significant differences in LMP concentrations were present between breast cancer patients and controls, which may reflect the disease stage in these patients (7). The sensitivity-specificity profile of LMP was equal to that of the established tumour marker CA15-3 (7).

Since MPs may expose surface proteins, indicating the cell from which they originate (10), it is hypothesised that MP shed by cancer cells would be detectable in plasma samples from breast cancer patients by using specific antibodies directed against cancer- or epithelial-specific structures. Our present study shows significantly elevated concentrations of MP expressing cancer-related antigens in patients with lymph node metastases compared to controls. However, these markers are not specific for breast cancer but they are also expressed in healthy breast tissue as well as in various other carcinomas. In particular, Hsp27 is expressed in various tissues, which may explain the high percentage of $Hsp27^+$ MPs.

MPs could possibly be of use as a prognostic factor. Established prognostic factors in breast cancer are currently the status of axillary lymph nodes, age and tumour load as well as histological parameters (2). New markers of breast cancer are urokinase-type plasminogen activator (uPA) and its inhibitor (PAI-1), which contribute to invasiveness and dissemination potential of tumour cells (3) as well as the presence of bone marrow micrometastases (4).

Most prognostic parameters in breast cancer require extraction of tumour tissue, lymph nodes or bone marrow all of which are invasive methods. The detection of tumour markers in peripheral blood would be of value for patients and health care professionals. Attempts have been made to detect disseminated tumour cells in the peripheral blood using several markers, among others CEA and Her2/neu (31). Our preliminary data suggest differences in the expression of cancer-related MP between breast cancer patients and controls. Future investigations on MP concentrations in breast cancer patients should focus on follow-up and survival rates. In particular, the possible role of MPs as a prognostic factor should be highlighted in the presence of a larger patient group, including patients with distant metastases.

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