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Original Research

Influence of adjuvant radiotherapy on circulating epithelial tumor cells and circulating cancer stem cells in primary non-metastatic breast cancer

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ABSTRACT

Background: : There is an unmet need to identify biomarkers that directly reflect response to adjuvant radiotherapy (RT). Circulating epithelial tumor cells (CETCs) represent the liquid component of solid tumors and are responsible for metastatic relapse. CETC subsets with cancer stem cell characteristics, circulating cancer stem cells (cCSCs), play a pivotal role in the metastatic cascade. Monitoring the most aggressive subpopulation of CETCs could reflect the aggressiveness of the remaining tumor burden. There is limited data on the detection and monitoring changes in CETC and cCSC numbers during RT in early breast cancer.

Methods: : CETC numbers were analyzed prior to, at midterm and at the end of RT in 52 primary non-metastatic breast cancer patients. Hormone receptor status was determined in CETCs prior to and at the end of RT. For the identification of cCSCs cell suspensions from the peripheral blood of patients were cultured *in vitro* under conditions favoring growth of tumorspheres.

Results: : Hormone receptor status in CETCs before RT was comparable to that in primary tumor tissue. Prior to RT numbers of CETCs correlated with aggressiveness of primary tumors. cCSCs could be successfully identified and monitored during RT. Prior to RT patients treated with neoadjuvant chemotherapy had significantly higher numbers of CETCs and tumorspheres compared to patients after adjuvant chemotherapy. During RT, the number of CETCs decreased continuously in patients after neoadjuvant chemotherapy but not after adjuvant chemotherapy.

Conclusion: : Monitoring the number of CETCs and the CETC subset with cancer stem cell properties during RT may provide additional clinically useful prognostic information.

Introduction

Breast cancer is the most commonly diagnosed malignancy in women and accounts for 30% of all cancers [1]. The management of breast cancer has changed significantly in the last few decades. It is often diagnosed at an early, potentially curable stage and treatment has become more effective [2]. Treatment of local disease includes surgery, often followed by adjuvant radiotherapy. Neoadjuvant or adjuvant systemic treatment options include chemotherapy, endocrine therapy, biologic therapy or a combination of these. Multimodal treatment is guided by TNM classification and risk stratification including histology, hormone receptor status, human epidermal growth factor receptor 2 (HER2) status, histologic grade and proliferation index. Recently, in addition to these clinical and pathological features, molecular characterization and in selected cases additional gene expression assays of the tumor are used to determine the potential benefit from systemic therapy [3]. Radiotherapy after breast conserving surgery (BCS) or mastectomy is an important component of therapy as it significantly reduces local recurrence rates and breast cancer mortality [4–8]. In contrast to chemotherapy, indication for radiotherapy mostly relies on clinical and pathological features. Since the implementation of radiotherapy only 2.5% of women with primary breast cancer experience locoregional, but 20% to 30% still experience distant relapse without previous local relapse whatever the treatment undertaken and patients may die from their disease [1,9,10]. In women with early-stage breast cancer, surgery is supposed to completely remove detectable macroscopic disease; however, microscopic tumor foci might still remain in the conserved breast or chest wall. Current tumor staging procedures as well as high resolution imaging technologies are not sensitive enough to detect micro-metastases or early tumor cell dissemination.

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Cells originating from primary tumors, locoregional recurrences, or metastases, which circulate in the peripheral blood and possess antigenic and genetic tumor-specific characteristics are so called circulating tumor cells [11]. The mechanism of metastasis formation from solid tumors is not fully understood, but recent evidence indicates that the key event in tumor progression is the dissemination of cells from the primary tumor at an early stage of the disease [11].

Circulating tumor cells represent the liquid component of solid tumors and indicate the presence of residual disease. This minimal residual disease could lead to locoregional recurrence or distant metastases, or both [12,13]. In metastatic disease they have been proposed to identify subpopulations of patients who are most likely to respond to a given therapy and for development of improved individualized therapies [14,15]. It was shown that the number of circulating tumor cells in metastatic breast cancer patients is an independent predictor for progression and overall survival [16,17]. Even in low-risk, non-metastatic patients circulating tumor cells may become prognostic of imminent metastasis and survival [18,19]. By means of maintrac®, a non-dissipative method avoiding cell loss [20], trajectories of circulating epithelial tumor cell (CETC) counts can be used as a marker for therapy response in the adjuvant situation allowing for continuous monitoring during treatment [21]. Monitoring the number of circulating tumor cells is superior to conventional imaging as a method for evaluating the response to different therapies, but has not yet been applied to assess the outcome of radiotherapy, especially in early breast cancer [18,22–28].

Solid tumors consist mainly of differentiated cells which have limited or no self-renewal abilities. Although cancer stem cells may be difficult to define definitively, growing evidence suggests that a tumor consists of a small subpopulation of undifferentiated cells within a tumor that have the ability to grow clonally and self-renew [9]. They are responsible for tumor initiation, tumor progression, metastasis and most importantly for recurrence after treatment, caused by resistance to conventional therapies [9,29]. Also circulating tumor cells seem to harbor a subpopulation of cancer stem cells, the so called circulating cancer stem cells (cCSCs), which are essential for metastatic spread [30,31]. We have shown that also a subpopulation of cells in the peripheral blood is capable to expand clonally and correlates strongly with the likelihood of the presence of metastasis in breast cancer patients [31,32].

So far, there is only limited data [33] about monitoring the number of CETCs and the number of cCSCs during radiotherapy, especially in early-stage breast cancer. We have now investigated the number of CETCs, and the number of cCSCs, prior to, midterm and at the end of adjuvant radiotherapy after breast conserving therapy. Hormone receptor and HER2 status of CETCs were also investigated and correlated to those of the primary tumor tissue.

Methods

The study is designed as a biology driven, translational research trial to investigate the feasibility of identifying and characterizing circulating epithelial tumor cells, as well as circulating cancer stem cells from whole blood samples prior to radiotherapy, at midterm of the radiotherapy series and at the end of radiotherapy using the maintrac® method.

Inclusion criteria

52 breast cancer patients treated at the University Hospital Jena were enrolled between April 2016 and March 2019. This study was conducted according to the Declaration of Helsinki and was approved by the local ethics committee (No. 0921-08/02) and registered in the German Clinical Trials Register (DRKS00011840). Breast cancer patients were enrolled after giving written informed consent if they met the following eligibility criteria: female patients aged ≥ 18 years with histologically proven primary non-metastatic invasive breast carcinoma (stages I-IIIA). Breast conserving surgery had to be completed at the University Hospital Jena with the curative-intended adjuvant radiotherapy of the breast/chest wall +/- lymphatic drainage had to be foreseen.
The allowed dose concepts were as follows:

1 hypofractionated therapy (5 × 2.66 Gy/week) to a total absorbed dose of max. 42.56 Gy or
2 normofractionated therapy (5 × 1.8 Gy or 5 × 2.0 Gy/week) to a total absorbed dose of 50.4 Gy or 50.0 Gy.

If indicated, an additional boost to the tumor bed was given within 5 × 2.0 Gy/week to total dose 16.0 Gy.
Exclusion criteria for the study were the presence of distant metastases, prior malignancies within 10 years of the breast cancer diagnosis or previous radiotherapy.

Study procedures

For CETC analysis, peripheral blood samples were collected (2 × 7.5 ml) into EDTA-tubes (ethylenediaminetetraacetic acid) as an anticoagulant and sent to the Laboratory in Bayreuth for analysis. Peripheral blood samples were obtained from all patients at three time points: at the same day of the first session of radiotherapy (pre RT), in the middle of the radiotherapy series (mid RT) (after 8 sessions for hypofractionated RT and after 14 sessions for normofractionated RT) and on the last day of the radiotherapy series (end RT). In addition to breast cancer patients, blood samples were collected from 10 healthy female donors aged 20-45 years for control.

Immunoflourescence assay for identification of CETCs (maintrac® method)

Samples were maintained at room temperature and processed within 72 h after blood collection. For counting and further characterization of CETCs, we used the maintrac® approach, as reported previously [34]. In short, after red blood cell lysis, an immunocytochemistry approach was used to identify CETCs. Cells in the remaining cell suspension were stained in a 1.5 ml reaction tube with a fluorescein-isothiocyanate (FITC)-conjugated anti-human epithelial cell adhesion molecule antibody (EpCAM) (clone HEA-125, Miltenyi Biotec GmbH, Germany) at a final concentration of up to 10^7 cells/100 µl. The corresponding isotypic control for EpCAM (Mouse IgG1K FITC, Miltenyi Biotec GmbH, Germany) was used at the same concentration to determine and subtract background levels of staining. The samples were subsequently transferred to wells of ELISA plates (Greiner Bio-one, USA) and stained additionally with propidium iodide (PI) (Sigma-Aldrich, USA) for the discrimination between live and dead cells. Analysis of red and green fluorescence of the cells was performed using a Fluorescence Scanning Microscope, ScanR, (Olympus, Hamburg, Germany), enabling detection and relocation of cells for visual examination of EpCAM positive cells. For data analysis, we used the ScanR Analysis software (Olympus, Hamburg, Germany). Vital CETCs were defined as EpCAM-positive cells with intact morphology, but lacking nuclear PI staining. Exclusively these cells were counted for the analysis (Fig. 1). We used fluorospheres (FlowCheck 770, Beckman Coulter) for daily verification of optical components and detectors of the microscope, which are required to ensure the consistent analysis of samples.

Secondary antibody staining

The secondary antibody stains of CETCs were performed as described previously in order to compare EpCAM positive cells with basal histological characteristics of the primary tumor [34,35]. Cell suspensions were stained as described above. EpCAM positive cells were additionally stained for estrogen (ER) and progesterone (PR) receptors. For ER receptor staining we used an anti-human ER PE-conjugated antibody (clone E115, abcam, Cambridge, USA) at a final cell concentration of up to 10^7 cells/100µl cell suspension (Fig. 2a). For the PR staining we used an Alexa Fluor®594 conjugated PR antibody (clone YR85, abcam,

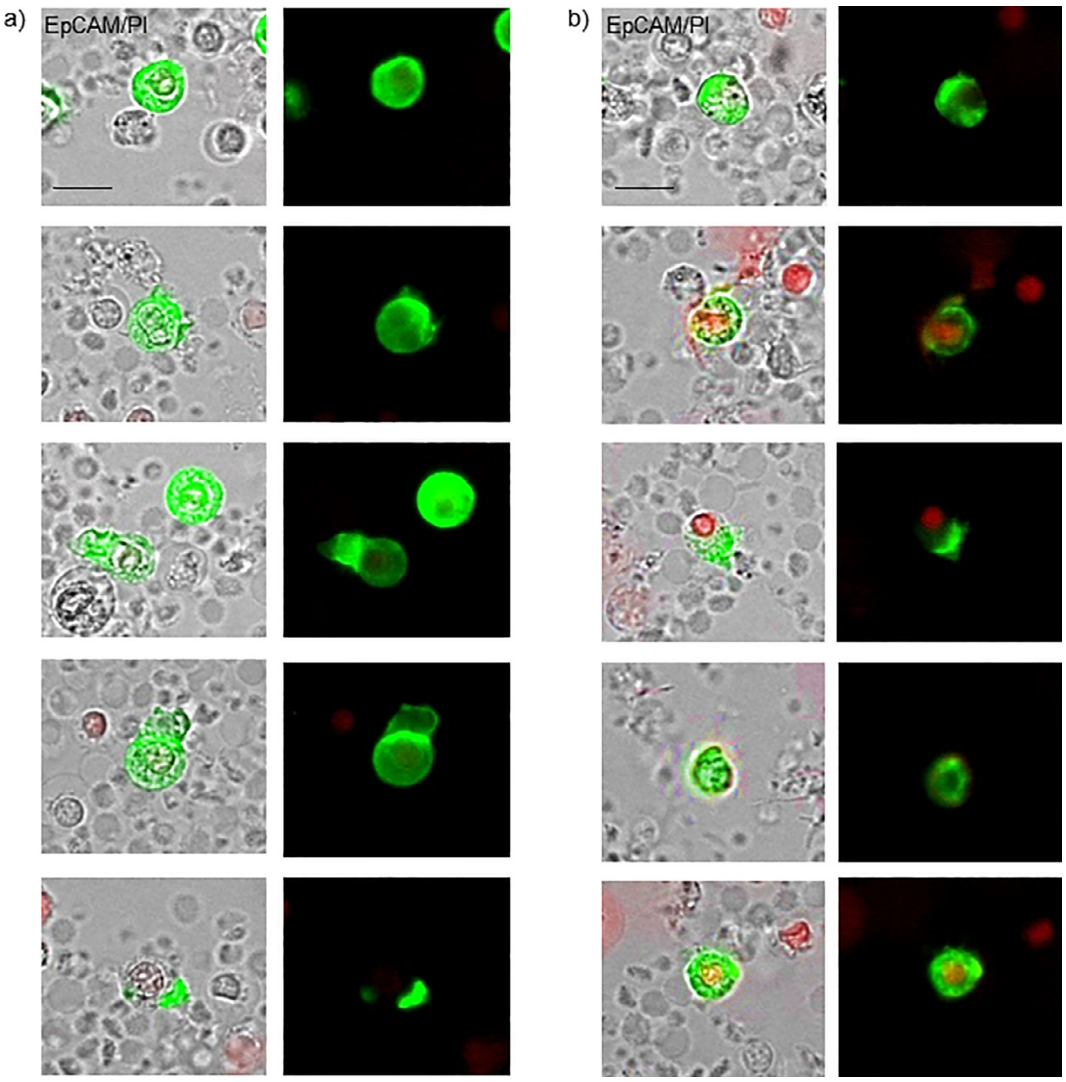

Fig. 1. Exemplary cell galleries of (a) vital and (b) dead CETCs from one patient. (a) vital CETCs are positive for EpCAM (green) and negative for PI (red) and have an intact morphology. (b) Dead cells are either positive for EpCAM and PI or only positive for EpCAM and negative for PI because of the destroyed cell membrane. Scale bar: 10μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Cambridge, USA) at a final concentration of 2μg/ml (Fig. 2b). Finally, cells were visually inspected for green and red surface staining, and additionally well-preserved nucleus and the percentage of double positive CETCs for the respective staining was determined. The isotype controls for ER and PR (rabbit IgG PE, abcam, Cambridge, USA) were used at the same concentrations.

Fluorescence-in-situ-hybridization (FISH) of human epidermal growth factor receptor 2 (HER2)

For the investigation of HER2 gene amplification on chromosome 17 in 20 patients prior to RT, a fluorescence-*in-situ-hybridization* assay was performed with the PathVysion HER2 DNA Probe Kit. This kit includes a control probe specifically against the alpha satellite DNA sequence in the centromeric region of chromosome 17 (*chromosome enumeration probe* 17, 17q11.1-q11.1 CEP 17, green fluorescence). In addition, this kit includes the probe, which is specific for the HER2 gene locus (*locus specific identifier*, 17q11.2-q12 LSI, orange fluorescence). Patient cells, which were previously labeled with the anti-EpCAM-FITC antibody (see above) were transferred onto Poly-L-Lysin coated slides. Slides were fixed with 4% paraformaldehyde before hybridization for 10 min and treated for further 10 min with proteinase K at room temperature. Afterwards, the cells were denatured for 5 min at 72°C in 70% formamide/2x standard saline citrate solution, air dried and dehydrated in 70%, 85% and 96% ethanol. Slides were hybridized overnight at 37°C in a humidified chamber, washed, air dried and counterstained with 0.2 μM DAPI in an anti-fade solution. Twenty nuclei per sample were analyzed. CETCs were positive for HER2 amplification when more than 3 HER2 signals in one cell were counted (Fig. 2c). Finally, the results for HER2 amplification were calculated as percentage of 20–30 visually inspected EpCAM positive cells.

Analysis of stem cell properties of cCSCs

For the cell culture of cCSCs, we used the stemtrac approach as described previously [31]. For the identification of the population of circulating cancer stem cells capable to grow clonally into tumorspheres *in vitro*, we cultured CETCs together with leukocytes at a density of 2×10^5 cells/ml for up to 3 weeks in RPMI-1640 supplemented with L-glutamine, HEPES, penicillin/streptomycin and growth factors such as EGF, insulin and hydrocortisone. Cells were maintained at 37°C and 5% CO_2 without movement of the culture flasks. Depending on the morphology of the tumorspheres observed in the culture flask under an inverted light microscope (Primo Vert, Zeiss, Germany) at 40x magnification, tumorspheres were collected and prepared for immunostaining analogous to the maintrac® staining. Tumorspheres were stained with an

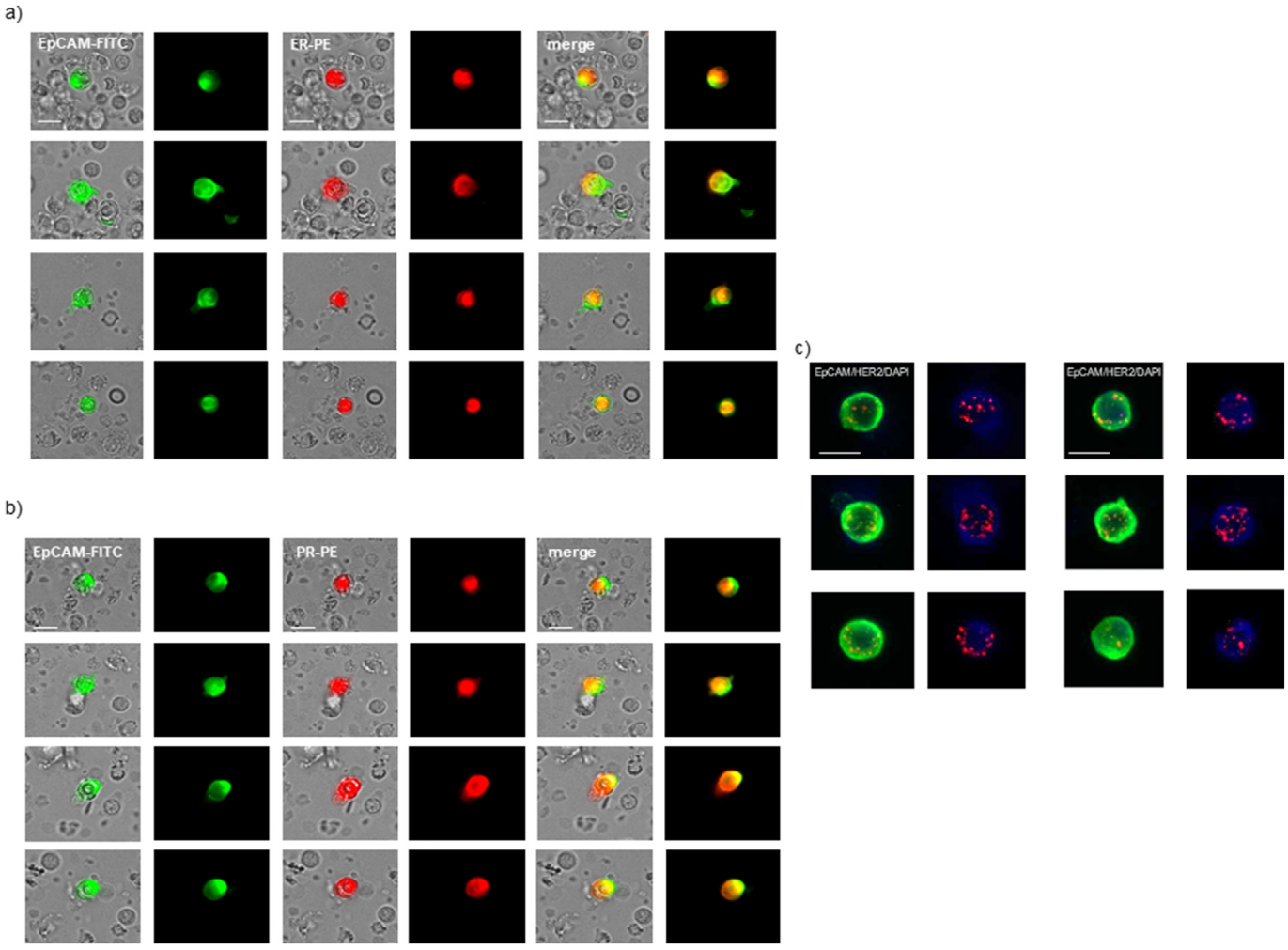

Fig. 2. Typical CETC images of double antibody staining for (a) EpCAM (green) and ER (red) and (b) EpCAM (green) and PR (red) from one patient. (c) Typical cell gallery of HER2 amplified CETCs from one patient. Cells are positive for EpCAM (green), and DAPI (blue). Cells are HER2 amplified (red signals). Scale bar 10μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

anti-EpCAM antibody at the same concentration as for the CETCs and afterwards measured with the same fluorescence microscope as used for the CETCs. Tumorspheres cultured from cCSCs could easily be distinguished from single or aggregated cells because they develop a solid spherical formation. This assay can be used to estimate the percentage of cancer stem cells present in a population of tumor cells.

For the characterization of cultured tumorspheres from cCSCs we used the typical combination of markers for breast cancer stem cells. EpCAM positive tumorspheres were additionally stained for CD24 (clone ML5, mouse anti-human, BD Bioscience, USA) and CD44 (clone 515, mouse anti-human, BD Bioscience, USA) PE-conjugated antibody [31].

Aldehyde dehydrogenase isoform 1 (ALDH1) activity is a universal marker for the identification and isolation of cancer stem cells. To confirm tumor stem cell properties of isolated cells, we used an ALDH1 enzymatic assay [31], which quantifies the ALDH1 activity of tumorspheres. We used an ALDEFLUOR assay kit (Stem Cell Technologies™, Canada) according to the manufacturer´s protocol. Tumorspheres expressing high levels of ALDH1 become brightly fluorescent. The number of such tumorspheres was counted.

Statistical methods

Statistical analysis was performed using the software program SigmaPlot version 13.0 (Systat Software Inc., Chicago, USA) for windows. Based on the antidromic behavior of CETC numbers during RT patients were dichotomized into 2 groups: patients with increasing (n=25) and decreasing (n=27) CETC numbers during RT. Comparisons between the variables were performed with the independent sample t test (dichotomous variables) or ANOVA (variables with more than two categories), taking into account the possibility of using nonparametric tests. The screening criterion used was a p-value of <0.05.

Results

CETCs and clinicopathological features

Peripheral blood samples from healthy volunteers (n=20) were used as a negative control and processed analogous to the samples from breast cancer patients. In these samples no CETCs were found and no tumorsphere formation *in vitro* was observed during cultivation.

Patient characteristics of all patients with primary breast cancer are shown in Table 1. CETCs were compared with the primary tumor tissue with regard to the hormone receptor expression and HER2 status. ER and PR status in CETCs were evaluable in all patients prior to and at the end of RT. The HER2 status in CETCs was determined in a subset of 20 patients prior to RT. Most patients with positive ER, PR or HER2 receptors expressed in the primary tumor tissue (immunohistochemical > 1%), had also ER (89% of patients) (Fig. 3a), PR (70% of patients) (Fig. 3b) and HER2 (100% of patients) (Fig. 3c) positive CETCs. Most

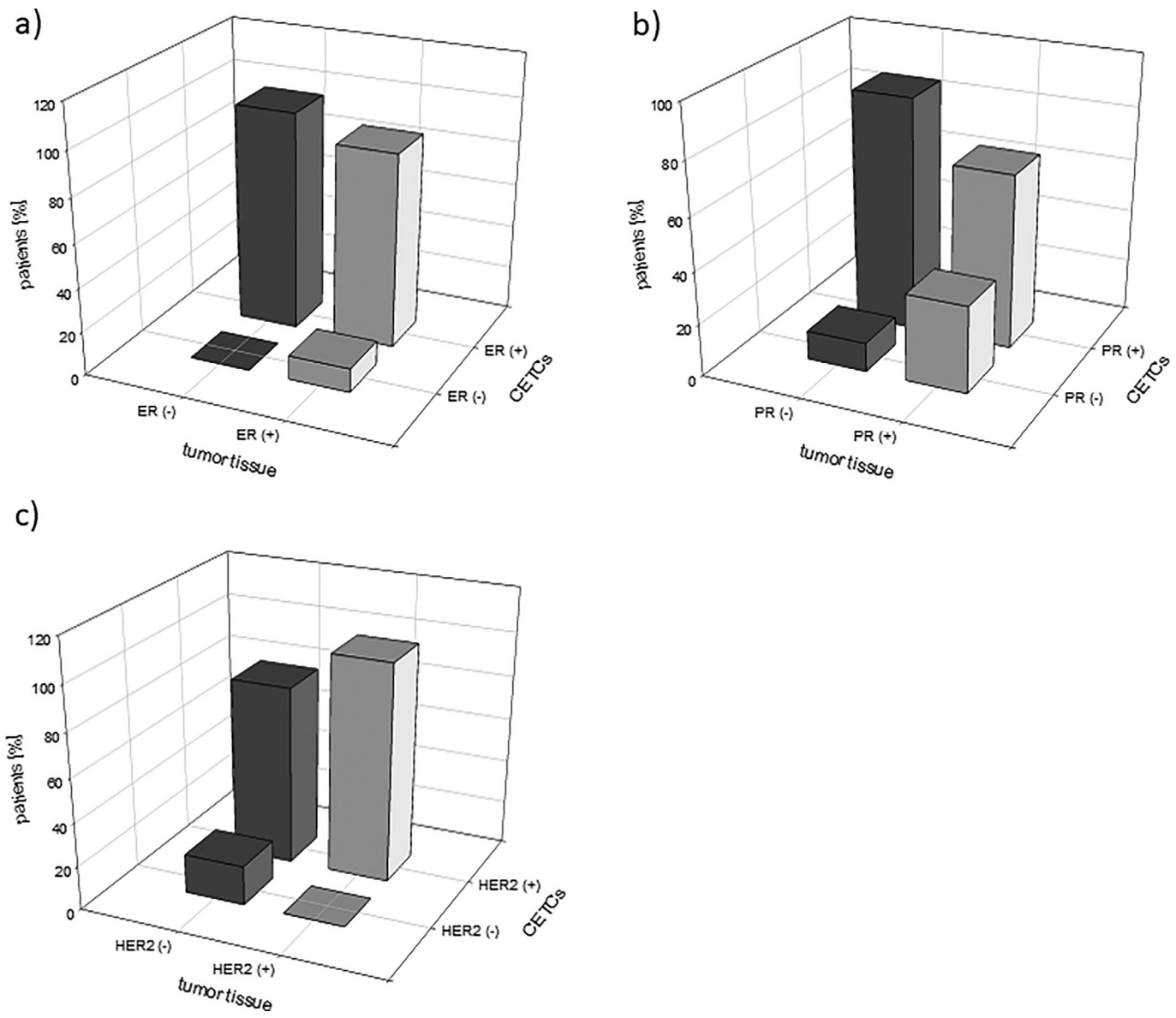

Fig. 3. Percentage of patients comparing (a) ER, (b) PR and (c) HER2 status in CETCs and tumor tissue.

patients with negative hormone receptor status and/or negative HER2 status in the primary tumor tissue had at least 1% of CETCs with expression of these receptors (Fig. 3).

We compared the ER and PR status in CETCs prior to RT with the receptor status at the end of RT. There was no influence of the RT on the hormone receptor status in CETCs when comparing the values prior to and at the end of RT, which means that patients who were positive or negative for ER or PR prior to RT remain positive or negative for the respective marker at the end of RT.

In addition, we examined the number of CETCs with regard to the different molecular subtypes of breast cancer before initiation of RT. According to international definition, Luminal A cancers were defined as ER and/or PR positive, HER2-negative, low level Ki67 (n=23; 44%), Luminal B ER and/or PR positive, HER2-negative, high level Ki67 (n=11; 21%), basal-like tumors were defined as ER, PR and HER2 negative (triple negative) (n=7; 14%), and HER2-like tumors were defined as HER2-positive (n=11; 21%). By comparing the molecular subtypes of the primary tumors with respect to the number of CETCs detected prior to RT, triple negative breast cancer patients had significantly more CETCs as compared to patients with luminal A or luminal B subtypes (median 16 vs. 5 CETCs/100µl of cell suspension, $p<0.05$) (Fig. 4a). Patients with HER2 positive/hormone receptor negative tumors had the highest median CETC numbers but due to the low number of patients with this subtype the difference was not significant.

In addition, lymph node involvement had an influence on the number of CETCs prior to RT. Patients with positive lymph nodes, who had not yet received chemotherapy, had statistically significantly more CETCs before start of RT as compared to patients without lymph node involvement (median 10 vs. 5 CETCs/100µl of cell suspension; $p<0.05$) (Fig. 4b). Patients with lymph node involvement, who previously had received chemotherapy (neoadjuvant or adjuvant) did not have higher CETC numbers as compared to patients without lymph node involvement prior to RT.

Follow-up of CETC counts during RT

During our study and the analysis we were surprised that RT does not have the same impact on CETCs in all patients. We identified 2 patient groups with antidromic behavior of CETC numbers during RT. Both groups were equally distributed with respect to T- and N-status, molec-

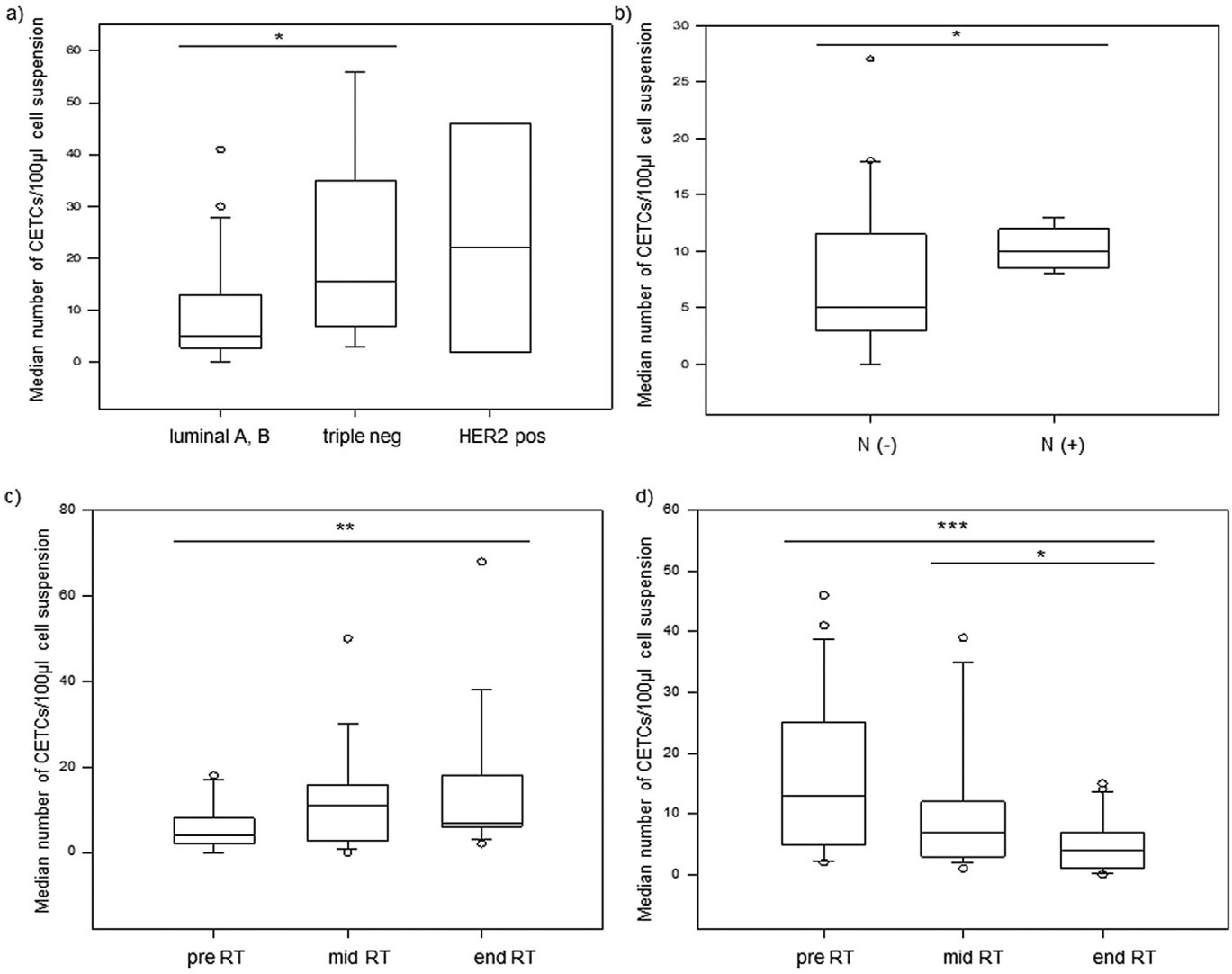

Fig. 4. Box plots of the median numbers of CETCs depending on (a) different molecular subtypes of breast cancer and (b) lymph node metastases. (c) box plots for the median number of CETCs in the patient group with increasing CETC numbers during RT and (d) in the patient group with decreasing CETC numbers.

ular subtype, histology, grading and administration of hormone therapy (Table 1). The only difference between the two groups was the observation that more patients with increasing cell numbers have had adjuvant chemotherapy whereas in the patient group with decreasing CETC numbers more patients had had neoadjuvant chemotherapy. 25 patients had increasing CETC numbers during RT whereas in 27 patients we observed decreasing CETC numbers. Pre RT CETC numbers differed significantly between the two groups (median 4 vs. 13 CETCs/100µl cell suspension; $p<0.01$), respectively (Fig. 4c and 4d).

In the patient group with increasing CETCs, cell numbers peaked at midterm of RT in 53% of patients. At the end of RT, the CETC numbers decreased but the median CETC numbers of all patients were still significantly higher as compared to the median value prior to RT (4 vs. 7 CETCs/100µl cell suspension; $p<0.01$) (Fig. 4c). The mean increase in CETC numbers from pre RT to end of RT was 2.91 ± 2.09 fold.

In the patient group with decreasing CETC numbers the mean decrease was 4.46 ± 3.68 fold. A continuous decrease in the CETC numbers was observed in this patient group. The difference in the CETC numbers was statistically significant between start of RT and end of RT (median 13 vs. 4 CETCs/100µl of cell suspension; $p<0.001$), as well as between midterm of RT and end of RT (median 7 vs. 4 CETCs/100µl of cell suspension, $p<0.05$) (Fig. 4d).

Number of CETCs in the context of prior chemotherapy

As cytostatic chemotherapy might have an influence on the number of CETCs, we investigated the CETC count dependent on chemotherapy before initiation of RT. 25 patients were treated with chemotherapy, 13 of which received neoadjuvant chemotherapy. 27 patients had not received any cytostatic chemotherapy. In the patient group treated with neoadjuvant chemotherapy, treatment led to pathological complete response (pCR) in 3 patients (23%). Patients who had received neoadjuvant chemotherapy had statistically significantly higher CETC numbers prior to RT as compared to patients who had received adjuvant chemotherapy or who had not received any chemotherapy (median 21, 4 and 6 CETCs/100µl cell suspension, respectively, $p<0.05$) (Fig. 5a). Patients who received adjuvant chemotherapy had slightly lower CETC numbers prior to RT as compared to patients who did not receive any cytotoxic chemotherapy before initiation of radiotherapy. In patients who received neoadjuvant chemotherapy, the number of CETCs decreased significantly at the end of RT from 21 (prior to RT) to 5 (at the end of RT) CETCs/100µl of cell suspension (Fig. 5b). Patients who received adjuvant chemotherapy had a trend to increasing median CETC numbers during RT (median 4, 11 and 11 CETCs/100µl cell suspension, prior to RT, midterm and end of RT, respectively) (Fig. 5c). In patients who did not receive chemotherapy there was only a marginal, nonsignificant

Table 1
Patient characteristics and CETC examination results.

Clinicopathological characteristics	Number of patients	Median number of CETCs	Number of patients with decreasing CETCs during RT	Number of patients with increasing CETCs during RT
Age				
≤50 years	13	**18**	**9**	**3**
>50 years	39	**6**	**13**	**18**
Tumor size				
pT1	30	**7**	**15**	**15**
pT2	19	**8**	**9**	**9**
pT3	2	**17**	**1**	**1**
pT4	1	**2**	**1**	
Lymph nodes				
pN0	31	**6**	**15**	**16**
pN1	17	**9**	**9**	**7**
pN2	3	**11**	**1**	**2**
pN+	1	**0**	**0**	**1**
pN3				
Stage				
I	24	**6**	**12**	**12**
II	20	**9**	**10**	**9**
III	8	**8**	**4**	**4**
ER status				
Positive (>1%)	42	**6**	**19**	**22**
Negative (≤1%)	10	**22**	**7**	**3**
PR status				
Positive (>1%)	34	**5**	**15**	**18**
Negative (≤1%)	18	**16**	**11**	**7**
HER2 status				
Positive (3+)	11	**10**	**5**	**6**
Negative (<3+)	41	**7**	**21**	**19**
Grading				
G1	10	**6**	**5**	**4**
G2	29	**10**	**15**	**14**
G3	13	**6**	**6**	**7**
Ki-67 index				
< 13%	30	**7**	**13**	**15**
> 13%	22	**10**	**12**	**9**
Molecular subtype				
Luminal A,B	34	**5**	**15**	**12**
Basal like	7	**21**	**5**	**2**
HER2 enriched	11	**10**	**5**	**6**
Histology				
invasive ductal	44	**9**	**23**	**21**
invasive lobular	6	**16**	**2**	**4**
tubular	1	**0**	**0**	**1**
mucinous	1	**2**	**1**	**0**
Chemotherapy				
No	27	**6**	**13**	**13**
adjuvant	12	**4**	**3**	**9**
neoadjuvant	13	**21**	**10**	**3**
Endocrine therapy				
Yes	23	**6**	**9**	**13**
No	29	**8**	**17**	**11**

change in the CETC numbers during RT. In contrast to chemotherapy, endocrine therapy had no influence on the median numbers of CETCs (data not shown).

Characterization of circulating cancer stem cells (cCSCs)

Cell culture for the identification of cCSCs was performed for each patient at each time of measurement. The formation of tumorspheres from cCSCs *in vitro* was controlled every 7 days under the light microscope. After 18-21 days of culture tumorspheres were collected, processed as described above and counted. As shown in Fig. 6a tumorspheres cultured from cCSCs were very compact. They were positive for EpCAM and reached a diameter of 50-90 µm. The EpCAM staining was very heterogeneous in the individual cells within individual tumorspheres 6b.

Tumorspheres had typical stem cell characteristics being positive for ALDH1 as shown in Fig. 6c. ALDH1 positivity varied strongly between individual tumorspheres from one patient and ranged between 60%-90%. In addition, they were positive for EpCAM, positive for CD44 (Fig. 6d) and negative or marginally positive for CD24 (Fig. 6e). CD44 staining varied strongly between individual cells in one individual tumorsphere.

Follow-up of cCSCs counts during RT

Irrespective of the molecular tumor subtype, tumorsphere formation *in vitro* was observed in 29/52 patients at least at one point of measurement. Prior to RT tumorspheres were detected in 31% of patients, midterm of RT in 25% of patients and at the end of RT in 41% of patients. In 44% of patients no tumorsphere formation was observed at any measurement point.

There were 15 patients with increasing and 14 patients with decreasing numbers of tumorspheres during RT. The mean increase in the patient group with increasing tumorsphere numbers was 6 ± 5 fold. There were significant differences between the number of tumorspheres prior to and at the end of RT (mean 1 vs. 7 tumorspheres/100µl of cell suspension, $p<0.001$) as well as between midterm of RT and end of RT (mean 4 vs. 7 tumorspheres/100µl of cell suspension, $p<0.05$) (Fig. 7a). The mean decrease in the patient group with decreasing tumorsphere numbers was 5 ± 7 fold. Differences in tumorsphere numbers between start of RT and midterm of RT (mean 7 vs. 4 tumorspheres/100µl of cell suspension, $p<0.05$) as well as between start of RT and end of RT (mean 7 vs. 1 tumorspheres/100µl of cell suspension, $p<0.01$) were statistically significant (Fig. 7b).

Chemotherapy had an effect on the number of tumorspheres grown *in vitro* prior to RT, yet this was not statistically significant. Comparable to the number of CETCs prior to RT, the number of tumorspheres *in vitro* was higher in the patient group after neoadjuvant chemotherapy as compared to patients who received adjuvant chemotherapy (mean 7 vs 4 tumorspheres/100µl of cell suspension (Fig. 7c). The highest tumorsphere number was observed in patients who did not receive chemotherapy prior to RT (mean 10 tumorspheres/100µl of cell suspension).

Tumorsphere formation *in vitro* was observed more frequently in patients after neoadjuvant chemotherapy (61%) as compared to patients with adjuvant chemotherapy (42%) or without chemotherapy (48%).

In 22 patients out of the 29 patients (76%), CETC and tumorsphere numbers developed in parallel: in 14 patients, both the number of CETC and the number of tumorspheres decreased during the course of RT, whereas in 8 patients, the number of tumor cells and the number of tumorspheres increased. However, in the patient group after neoadjuvant chemotherapy as well as in the patient group who did not receive chemotherapy an inverse development in tumorsphere and CETC numbers was observed: most patients in this patient group had increasing tumorsphere numbers but decreasing CETC numbers during RT.

Discussion

Although nowadays breast cancer is often diagnosed at an early stage it is still a leading cause of cancer mortality [3]. Due to intra- and intertumoral heterogeneity but especially due to potential changes in tumor cell properties during the course of disease, which can be caused by treatment effects, longitudinal analysis of circulating tumor cells promises a more complete understanding of the complex processes underlying treatment response or relapse[3]. Biomarkers from the primary tumor may help to identify low- and high risk patients but the analysis of the actually disseminated tumor cells would be preferable [3]. Especially in early stage where treatment is expected to be curative, monitoring circulating tumor cells could predict early disease recurrence and survival [20,36].

It has been shown that the characteristics of the cells released from the primary tumor may change during disease progression leading to

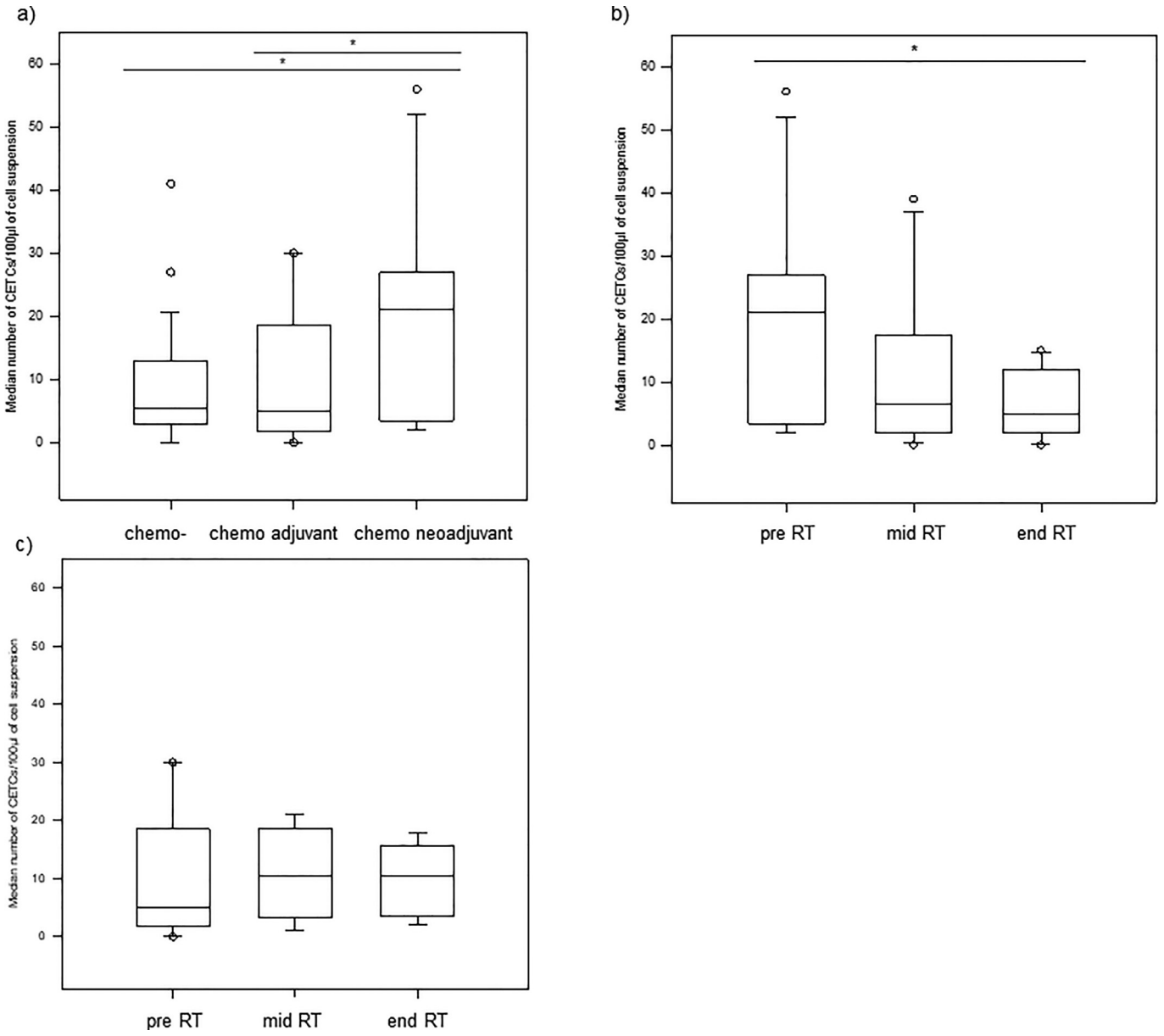

Fig. 5. (a) Median number of CETCs prior to RT with respect to chemotherapy. (b) median number of CETCs during RT in patients who received neoadjuvant chemotherapy and (c) median number of CETCs during RT in patients who received adjuvant chemotherapy.

ineffective treatment in the absence of the respective marker as indicated by the discrepancy in hormone receptor and HER2 status between primary tumor and metastatic lesions in one-third of metastatic breast cancer patients [37]. Our study shows that the expression of hormone receptors and amplification of HER2 in CETCs initially reflect hormone receptor and HER2 status of the primary tumors and was concordant in 70% for ER, 46% for PR and 55% for HER2 expression indicating that these cells are highly probably tumor cells disseminated from the primary tumor. These results are in agreement with the study of Kalinsky et al, who observed a concordance of 68% in ER/PR status between primary tumor and circulating tumor cells in early stage breast cancer [38] and Zhang et al reporting a concordance between HER2 status in primary tumor and circulating tumor cells in 38% of patients [39]. During subsequent RT almost no changes in hormone receptor status were observed suggesting that RT does not have any selective effect on marker expression and RT does not selectively eliminate cell populations.

In agreement with Xu et al. we observed higher CETC numbers in triple negative and HER2 positive patients as compared to luminal A/B subtypes before initiation of RT, indicating that patients with highly aggressive disease have increased CETC numbers and higher risk for metastasis formation [40]. Currently, the treatment of patients with triple negative breast cancer is the biggest challenge in breast cancer. In early stages, the use of neoadjuvant chemotherapy is the standard of care and achieving a pCR after neoadjuvant chemotherapy correlates with improved survival outcomes [41]. In our short follow up of 7 months we observed one relapse in a triple negative breast cancer patient. The patient suffered from a stage II (T2N1M0) breast cancer and had been treated with neoadjuvant chemotherapy. As she did not achieve a pCR she was additionally treated with adjuvant chemotherapy (Fig. 7d). Her numbers in CETCs and tumorspheres, grown *in vitro* from cCSCs, increased during RT followed by a relapse 7 months after end of RT. If this observation is confirmed in a higher number of patients monitoring CETCs during the course of RT could help in decision making for fur-

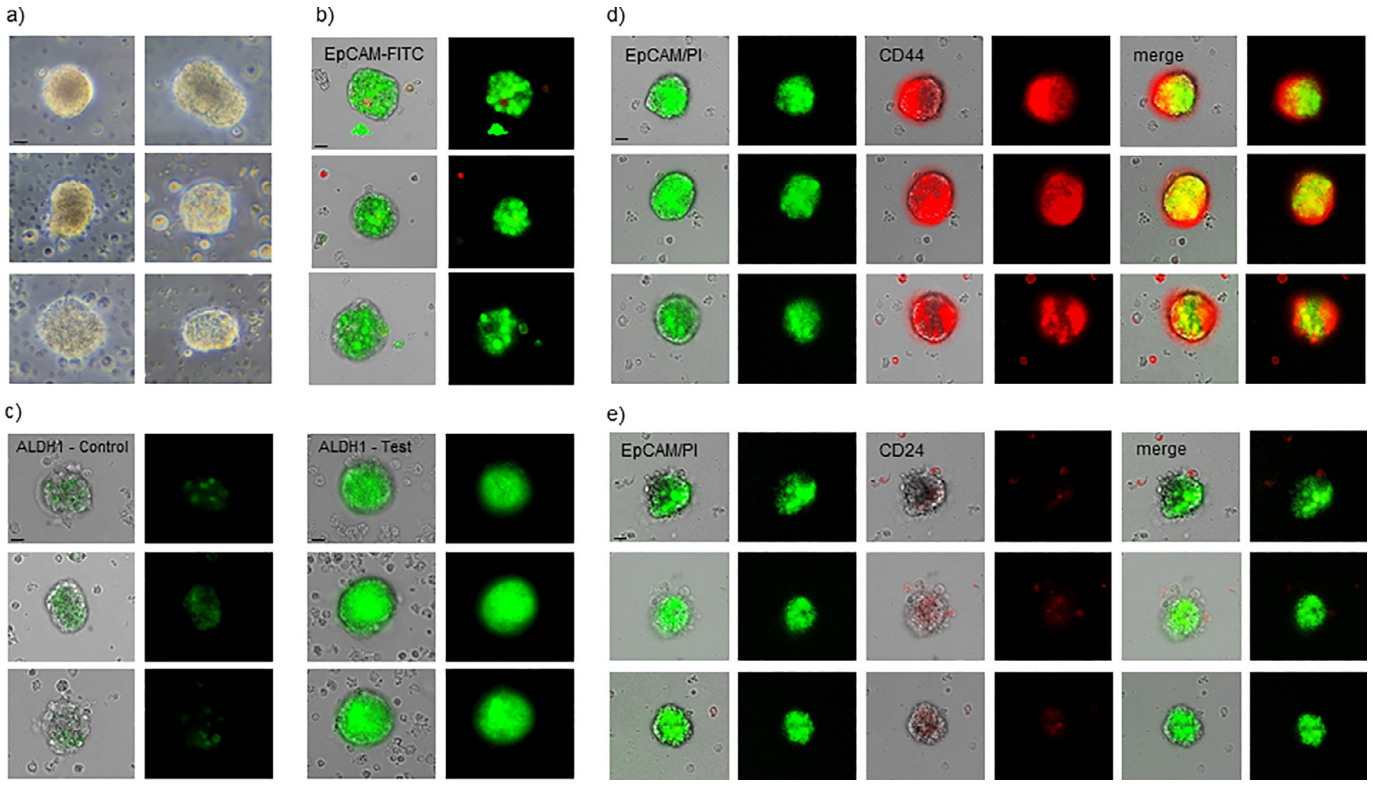

Fig. 6. Typical images of tumorspheres grown *in vitro* from cCSCs from one breast cancer patient (a) from culture flask and (b) fluorescence scanning microscope. (c) ALDH1 activity of tumorspheres. (d) Images of tumorspheres being positive for EpCAM and CD44 and (e) Images of tumorspheres being negative or marginally positive for CD24. Scale bar: 10μm.

ther diagnostic and therapeutic interventions to early detect recurrence, especially in this aggressive subgroup of breast cancer.

Furthermore, in accordance with other studies [12,36] the number of CETCs was associated with patients' nodal stage. Positive axillary lymph nodes are a predictor of increased risk for local and distant recurrence and directly correlated with overall survival [42].

Goodman et al showed that patients in whom circulating tumor cells can be detected (24% of all patients with at least 1 cell/20 ml of blood) fare better with RT after BCS than without RT. RT seems to reduce the risk of recurrence and death from breast cancer suggesting elimination of microscopic tumor foci [33] which might seed cells into the circulation. In the present study population, breast conserving procedure was performed in 90% of patients. Increasing CETC numbers were observed in 40% of patients and decreasing CETC numbers in 44% of patients from the start of RT to end of RT. Regarding clinicopathological features, age and endocrine therapy there was no difference in the groups with increasing and decreasing cell numbers. The only difference is that in the patient group with decreasing cell numbers almost all patients (80%) were treated with neoadjuvant chemotherapy and that these patients after neoadjuvant chemotherapy had statistically significantly more CETCs before RT than patients with adjuvant chemotherapy or without chemotherapy. While neoadjuvant therapy enables tumor downsizing for BCS by disintegration of tumor tissue, assessing *in vivo* response to therapy and provide prognostic information based on pathological response [2] the reduction in tumor size and the destruction of tumor tissue may lead to the mobilization and release of tumor cells into the bloodstream. Monitoring CETCs during neoadjuvant chemotherapy in early breast cancer patients [22] showed that the initial decrease in the number of CETCs correlated with the reduction in tumor size. Comparable observations during RT have been made in NSCLC patients where increased numbers of circulating tumor cells during the course of neoadjuvant RT were detected due to mobilization of viable tumor cells from intact tumors into the circulation [43].

The group of patients with increasing CETC numbers during RT mainly comprised patients who had not received chemotherapy or who had received adjuvant chemotherapy. In this subpopulation we observed in half of the patients a peak in CETC numbers at midterm of RT followed by a decrease.

It is not easy to distinguish dormant circulating tumor cells from those with proliferating capacity initiating new tumors at distant sites [3]. It is assumed that only a small subpopulation of circulating tumor cells, the so called circulating cancer stem cells, can grow into metastases. They have properties of stemness and represent the most aggressive fraction of cells [44]. Different approaches for the identification of cancer stem cells from the primary tumor are available, one of them a functional assay, the sphere-formation assay [45] is commonly used to study stem cell properties of CSCs. In the present study we used a tumorsphere-formation assay developed by us to grow circulating cancer stem cells from the peripheral blood. To our knowledge, this is the first study to monitor the number of circulating cancer stem cells during RT in breast cancer patients. In about one half of patients tumorsphere formation *in vitro* could be observed at least at one point of measurement expressing typical stem cell markers for breast cancer. Again, there were two patient groups, one with increasing and one with decreasing tumorsphere numbers *in vitro* during RT. The group with increasing tumorsphere numbers *in vitro* comprised mainly patients with an unfavorable hormone receptor status and higher Ki-67 index. In addition, in this patient group, many patients had not received any chemotherapy. Thus, as reported previously [31], the number of tumorspheres correlated with unfavorable characteristics of the primary tumor. In patients who had received neoadjuvant chemotherapy, tumorsphere formation *in vitro* was more frequently observed as compared to patients who received adjuvant chemotherapy or no chemotherapy. After neoadjuvant chemotherapy an increase in tumorsphere numbers during RT was found in 63% of patients and in 69% of patients who had not received any chemotherapy. Like CETCs cells with cancer stem cell properties may be-

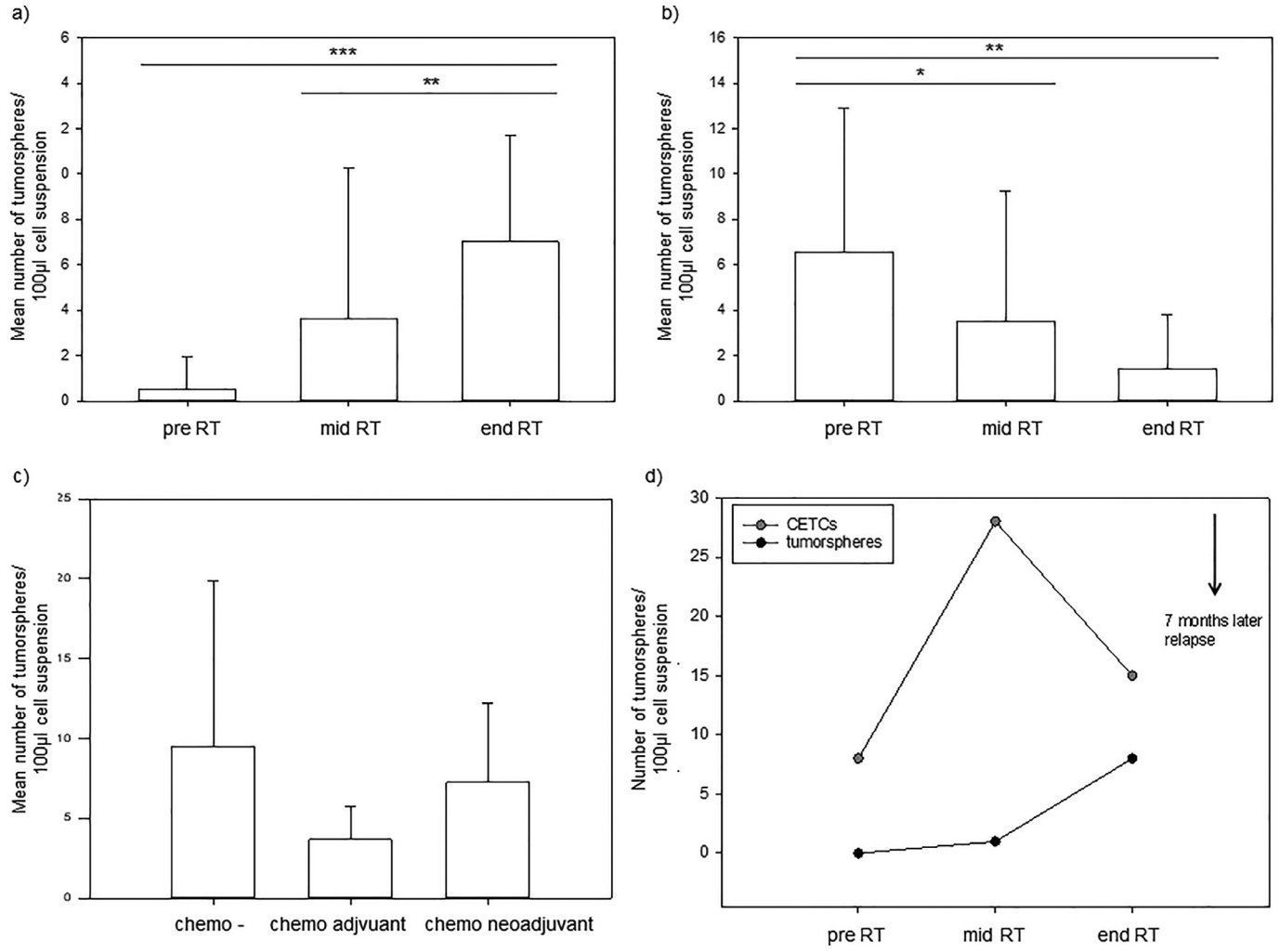

Fig. 7. Mean variation in the number of tumorspheres during RT in the patient group with (a) increasing tumorsphere numbers and (b) decreasing tumorspheres numbers. (c) Mean numbers of tumorspheres prior to RT with respect to chemotherapy. (d) Course of CETC and tumorsphere numbers during RT in one breast cancer patient after neoadjuvant and adjuvant chemotherapy.

come released from the primary tumor during neoadjuvant chemotherapy. In the patient group after neoadjuvant chemotherapy we observed more frequently a decrease in the number of CETCs antidromic to an increase in the number of tumorspheres grown from cCSCs *in vitro* than in the other patients, indicating that cancer stem cells might be radioresistant.

Our results of decreasing single CETC numbers during RT but increasing numbers of tumorspheres after neoadjuvant treatment might also support the hypothesis, that under conditions of radiation-induced stress, cancer cells may undergo dedifferentiation into stem cell-like cells. These cells might subsequently obtain phenotypes and functions of CSCs, including radioresistance, which indicates that radiation may directly result in the generation of novel CSCs from non-stem cancer cells [46]. Changes in the number of CETCs, but especially changes in the number of tumorspheres cultured from cCSCs *in vitro*, could be relevant for recurrence of cancer disease.

Conclusion

We could show that CETCs before RT have the same molecular properties as the cells in the primary tumor, which in turn indicates that the detected cells are tumor cells. Furthermore, we were able to identify a subpopulation of CETCs which were able to grow clonally *in vitro* into so called tumorspheres and possess stem cell properties. Our preliminary results shows that changes in the number of CETCs, but especially changes in the number of tumorspheres cultured *in vitro* from cCSCs during adjuvant radiotherapy could reflect the activity of residual tumor burden after breast conserving surgery. This could help to identify patients with increased risk of local recurrence.

Declaration of Competing Interest

All authors declare that they have no competing interests.

CRediT authorship contribution statement

Dorothea Sonja Schott: Conceptualization, Methodology, Validation, Investigation, Writing - original draft, Visualization, Supervision, Project administration. **Monika Pizon:** Methodology, Validation, Writing - original draft, Visualization. **Ulrich Pachmann:** Writing - review & editing. **Katharina Pachmann:** Conceptualization, Methodology, Writing - review & editing, Project administration. **Rainer Schobert:** Writing - review & editing, Project administration. **Andrea Wittig:** Writing - review & editing, Project administration. **Matthias Mäurer:** Conceptualization, Writing - original draft, Supervision, Project administration.

Ethics approval and consent to participate

All subjects have provided written informed consent before participation in the study which has been approved by the Ethics Committee of Jena.

Consent for publication

All authors have agreed to the submission of the manuscript.

Availability of data and material

All data are fully available without restriction.

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