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CLINICAL TRIAL

Disseminated tumour cells in the bone marrow in early breast cancer: morphological categories of immunocytochemically positive cells have different impact on clinical outcome

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Abstract Detection of disseminated tumour cells (DTCs) in bone marrow by immunocytochemistry (ICC) includes morphological evaluation of cytokeratin immunopositive cells. The aim of this study was to disclose the prognostic significance of different morphological categories of ICC-positive cells according to treatment status and tumour subtype. Bone marrow samples (at surgery) were analysed for the presence of cytokeratin-positive DTCs by a standard immunocytochemical method. The immunopositive cells were classified into the following categories, prior to any analysis of the association between DTCs and clinical outcome: tumour cells (TC), uninterpretable cells (UIC), hematopoietic cells (HC), and questionable HC (QHC). The analysis included 747 early breast cancer patients. Median follow-up was 84 months for relapse, and 99 months for death. The categorisation of the ICC positive cells revealed TC in 13.3 % of the patients, whereas 13.1, 17.8, and 21.4 % of the cases were positive for UIC, QHC, and HC, respectively. Analysing all patients, only TC and UIC predicted systemic relapse. Separate analysis of all patients not receiving adjuvant systemic treatment (No-Adj; $n = 389$) showed that only QHC were associated with reduced survival (DDFS: $p = 0.008$; BCSS: $p = 0.004$, log rank) and the presence of QHC also remained significant in multivariate analysis. Primary tumour subgroup analysis (of all patients) by hormone receptors (HR) and HER2, demonstrated that only TC/UIC had prognostic impact in the HR+/HER2− patients, whereas presence of QHC was associated with unfavourable outcome only in triple negative patients (DDFS: $p = 0.004$; BCSS: $p = 0.024$). Patients with ≥3HC

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had improved outcome compared to those with fewer/no HC (DDFS: $p = 0.005$; BCSS: $p = 0.009$). Hence, morphological DTC subgroups may differ in clinical significance according to primary tumour subtype and treatment status. This emphasises the importance of DTC characterisation, and separate analyses of DTC categories according to tumour subtype. Hematopoietic ("false positive") cells might predict an immune-related favorable clinical outcome.

Keywords Breast cancer · Disseminated tumour cells · Bone marrow · Morphology · False positive cells · Classification

Abbrevations

DTCs	Disseminated tumour cells
BM	Bone marrow
FU	Follow-up
ICC	Immunocytochemical analysis
MNC	Mononuclear cells
APAAP	Alkaline phosphatase/monoclonal mouse anti-alkaline phosphatase
HC	Hematopoietic cells
TC	Tumour cells
UIC	Uninterpretable cells
pT	Histopathological primary tumor size status
pN	Histopathological lymph node status
G1, 2, 3	Histopathological grade 1–3
IHC	Immunohistochemical staining
ER	Estrogen receptor(s)
PR	Progesteron receptor(s)
HER2	Human epidermal growth factor receptor 2
TMA	Tissue microarray
FISH	Fluorescence in situ hybridization
QHC	Questionable hematopoietic cells
BCSS	Breast cancer-specific survival
DDFS	Distant disease-free survival
HR	Hormone receptor(s)
TN	Triple negative
CTCs	Circulating tumour cells

Introduction

The clinical relevance of disseminated tumour cells (DTCs) in the bone marrow (BM) at diagnosis and during follow-up (FU) has been demonstrated in several early breast cancer studies, including large pooled analyses [1, 2]. So far, the most standardised and validated detection method for DTCs is standard immunocytochemical analysis (ICC) of BM mononuclear cells (MNC) centrifuged onto glass slides (cytospins). The slides are immunostained by anti-cytokeratin monoclonal antibodies and visualised by alkaline phosphatase/monoclonal mouse anti-alkaline phosphatase (APAAP) detection system [3, 4]. In parallel, it has been recommended to stain additional cytospins with a negative control antibody of the same immunoglobulin subtype substituting the specific anti-cytokeratin antibody(ies). Cytospins are screened for immunostained cells by light microscopy, and the presence of DTCs concluded if certain morphological criteria are fulfilled [3, 4]. These criteria for distinction between DTC and occasional false positive hematopoietic cells (HC) have to some extent been clinically validated [5, 6]. However, tumour cells may display different morphological phenotypes, from small, anonymous or HC-like cells to unambigous tumour cell morphology [3].

In a previous study ("Oslo 1 Micrometastasis study") we classified, prior to any knowledge of clinical outcome, ICC-positive cells in BM into four categories: Tumour cells (TC), HC, probable HC, in addition to cells/elements not possible to evaluate because of disruption/degeneration/poor conservation (uninterpretable cells, UIC) [6–9]. The categories TC and UIC were found to harbour cells significantly associated with reduced survival, whereas neither HC nor probable HC affected patient outcome [6]. However, survival analysis according to adjuvant systemic treatment status was not explored. As breast cancer can be classified into distinct subtypes (and morphological appearances) with different prognosis and treatment options [10, 11], the clinical significance of DTCs with different morphologies, should also be studied in relation to primary tumour subtype. In the present study, the immunomorphological cell categories of "DTCs" were revisited, analysing separately their prognostic impact according to adjuvant systemic treatment status and primary tumour subtype.

Materials and methods

Patients

Totally 920 non-metastatic breast cancer patients were enrolled (Oslo Micrometastasis Project; 1995–1998). Studies of prognostic significance of DTCs have been reported previously [1, 2, 6, 8]. In this study, only the cases with both specific test and negative control analysis of comparable and appropriate technical quality were included. In addition, information about systemic adjuvant treatment and clinical outcome (relapse and/or breast cancer death) was required. Totally 747 patients fulfilled these criteria. For 11 patients no relapse status was available. Systemic treatment followed the Norwegian guidelines for 1995–1998 and was administered to patients with pT2pN0G2–3 or pN+ status, as described in detail previously [8]. Clinico-pathologic data were extracted from the

Oslo Micrometastasis Project database. Median FU time was 84 months (range 1–125 months) for relapse. From the Norwegian Death Cause Registry, additional information regarding extended FU time for death was available. Median FU for death was 99 months (range 2–128). The study was approved by the Regional Ethical Committee, and written consent obtained from all patients.

Primary tumour analyses

Primary tumour analyses were performed as described [7]. Grading was centrally performed on whole sections according to Elston/Ellis [12]. In cases of doubt another pathologist was consulted. In general, one paraffin block was examined per case.

Estrogen-, progesterone- and HER2 analysis

Immunohistochemical staining (IHC) for estrogen (ER) and progesterone (PR) receptors and HER2 was performed on whole paraffin sections as previously described [7]. In addition, tissue micro array (TMA) blocks were analysed for HER2 amplification by FISH (HER2 DNA probe kit; Vysis Inc, USA) [13]. The primary tumour was regarded ER or PR-positive if $\geq$10 % of TC nuclei were immunostained with the respective antibodies (according to prevailing recommendations when the study was conducted). Tumours were scored as HER2-positive if $\geq$10 % of the TC showed membranous immunostaining and/or HER2 FISH was positive (HER2/centromere 17 ratio $\geq$2.2) [13]. FISH was performed on all tumours with tissue available for this purpose.

Bone marrow analyses

Mononuclear cells from BM collected at surgery were analysed for DTCs by the standard ICC method, as described [7]. For each sample 4 cytospins (2×10^6 BM MNC) were immunostained for epithelial cells by anti-cytokeratin monoclonal antibodies AE1/AE3 (Sanbio), visualised by APAAP detection [14], and New Fuchsin. In parallel, negative control slides of 2×10^6 BM MNC were prepared from 605 of the 747 patients and incubated with a control antibody of the same immunoglobulin subtype substituting the specific anticytokeratin antibodies. For 139 patients, only 0.5×10^6 MNC were available for negative control analysis, for three patients $1–1.5 \times 10^6$ MNC. The slides were screened in light microscopy (manually or by Ariol SL50 automated screener), and immunostained elements classified according to predefined morphological criteria [3], as TC, UIC, questionable HC (QHC; named probable HC in Naume et al. [6]) or HC. Questionable HC were defined as cells intermediate between TC and HC, and exhibiting some HC characteristics.

A sample was scored as positive for a cell category if no similar positive cells were detected in the corresponding negative control, or if a higher number of cells of the respective cell type were present in AE1AE3-stained slides than in the corresponding negative control slides. The overall analysis of the ICC positive categories included all cases with appropriate negative control quality irrespective of the number of cells included in the control analysis (i.e. $0.5–2.0 \times 10^6$ MNC). This was decided in order not to exclude clinically relevant information from the morphological categories, being aware of the possibility for an increase in false positive cases. Still, patients with the same (or lower) number of ICC positive cells in the specific test than in the negative control analysis of only 0.5×10^6 MNC would be concluded as negative for the studied cell category, thereby reducing the rate of false positivity among these 139 patients. A separate analysis including only cases with equal cell number in specific test and negative control was also performed. Where relevant, the manuscript includes the essential elements of the REMARK criteria [15].

Statistical analysis

The SPSS software (version 17/18) was used. Survival time was measured from time of surgery to time of death or first evidence of recurrence. Breast cancer-specific survival (BCSS) was measured from date of surgery to breast cancer-related death, or otherwise censored at time of last follow-up, or at time of non-cancer-related death. Likewise, distant disease free survival (time to systemic relapse) (DDFS) was measured. Metastases to skeleton, liver, lungs, or central nervous system were recorded as systemic relapse. Kaplan–Meier survival curves for DDFS and BCSS were constructed. P values were computed by log-rank test. Cox proportional hazards regression was used for univariate and multivariate (stepwise backward elimination) analyses of prognostic impact of relevant variables. All p values are two-tailed, and $p < 0.05$ considered significant.

Results

Patients/tumour characteristics and ICC cell category status

Differences in the morphological appearance of ICC positive cells detected in the BM samples are illustrated in Fig. 1. Patient characteristics are presented in Table 1, and the associations between these and the presence of ICC positive cells of TC or UIC morphology and of QHC morphology, are presented in Table 2. Presence of $\geq$1 positive cells of the respective

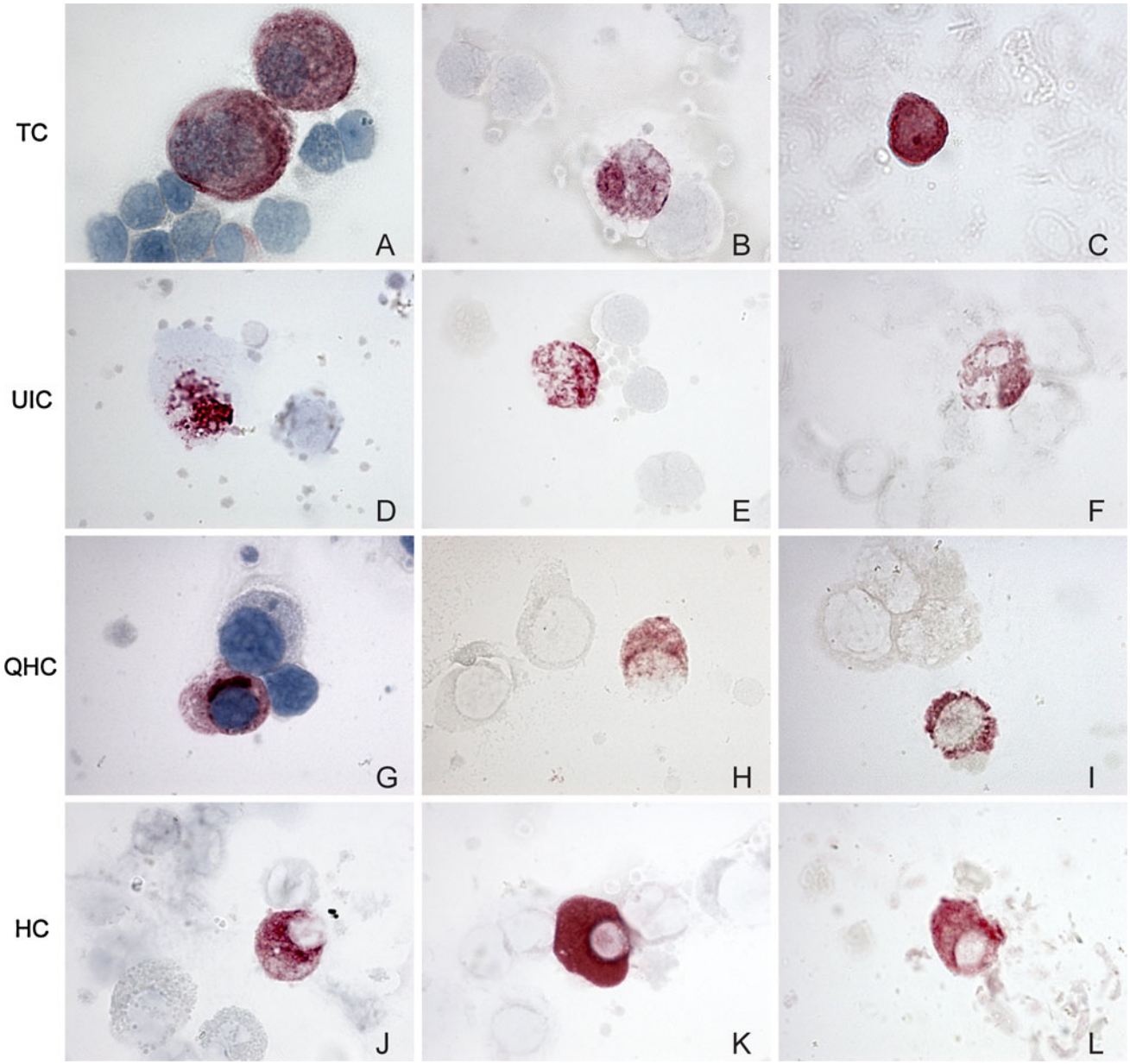

Fig. 1 Examples of morphological features of cells categorised into the tumor cell (TC), uninterpretable cell (UIC), questionable hematopoietic (QHC) and hematopoietic cell (HC) group. The classification was performed prospectively according to published guidelines [3]. TC **a**–**c**: The appearance in clusters, a nucleus clearly enlarged beyond the size of neighbouring bone marrow nuclei and irregular distribution of the cytoplasmic staining, are typical characteristics of TC (**a**). But the nucleus is not necessarily enlarged (**b**, **c**). Strong immunostaining for cytokeratin, partly covering the nucleus (**b**, **c**), with an irregular distribution, sometimes even suggesting a reticular/cytoskeletal network (**b**), is often seen in TC. Strong staining, partly covering the nucleus, and absence of HC characteristica placed cell C into the TC category. UIC (**d**–**f**): These cells/elements are all destroyed/degenerated and with a nucleus not possible to identify, therefore classified in the UIC group. QHC (**g**–**i**): Obvious HC characteristics are lacking, the cytoplasmic staining intensity is somewhat variable, and in cell G irregularly distributed, which could indicate TC. But the nuclei are small, resembling neighbouring cells, and the quite regularly dispersed cytoplasm in H resemble HC cytoplasm, and nuclear covering is lacking/limited. HC (**j**, **k**): Typical HC features with low nuclear/cytoplasmic ratio, regularly dispersed cytoplasm suggesting microvacuolisation (**l**), with pin-point vacuoles (**j**, **k**), and plasma cell-like morphology with a small, eccentric nucleus. Cytoplasmic staining not covering the nucleus

categories was considered positive. Analysing all patients, 13.3 % had the presence of TC, and 13.1, 17.8, and 21.4 % were positive for UIC, QHC, and HC, respectively. Presence of detected TC/UIC was significantly associated with lymph node status, pT-status, and HER2-status, while no significant association between QHC and clinico-pathological variables was found (Table 2). Considering cases with presence of TC, UIC and/or QHC, 72.5 % had cells from only one category.

Table 1 Clinico-pathological characteristics

	Number of pts (%)[a]	No-Adj (%)[b]	Adj (%)[b]	*p* value
All pts	747	389 (52.1)	358 (47.9)	
Menopausal status				
Prem. (<55 years)	295 (40.4)	133 (45.1)	162 (54.9)	0.001
Postm. (≥55 years)	435 (59.6)	249 (57.2)	186 (42.8)	
Missing	17			
Lymph node status[c]				
pN0	483 (64.7)	362 (74.9)	121 (25.1)	<0.001[d]
pN+	243 (32.5)	18 (7.4)	225 (92.6)	
pNX	21 (2.8)			
Tumour size[e]				
pT1	459 (63.2)	312 (69.6)	136 (30.4)	<0.001[f]
pT2–4	267 (36.8)	61 (23.6)	198 (76.4)	
pTX	20 (2.7)			
Missing	1			
Histological tumour subtype				
Ductal	572 (76.6)	295 (51.6)	277 (48.4)	0.524 [g]
Lobular	138 (18.5)	67 (48.6)	71 (51.4)	
Other	37 (5.0)			
Histologic grade[h]				
Grade 1–2	543 (74.3)	299 (55.1)	244 (44.9)	0.005[i]
Grade 3	188 (25.7)	81 (43.1)	107 (56.9)	
Missing	16			
Hormone receptor (HR)				
HR+	565 (78.4)	290 (51.3)	275 (48.7)	0.895
HR−	156 (21.6)	81 (51.9)	75 (48.1)	
Missing	26			
HER2 status				
HER2+	79 (11.1)	38 (48.1)	41 (51.9)	0.595
HER2−	630 (88.9)	323 (51.3)	307 (48.7)	
Missing	38			

[a] Valid percent

[b] The percentages in relation to the clinico-pathological variables

[c] pN1 = 152 (20.3 % of all pts), pN2 = 60 (8.0)

[d] Comparison of pN0 versus pN+

[e] pT2 = 227 (30.4 % of all pts), pT3 = 36 (4.8), pT4 = 4 (0.5)

[f] Comparison of pT1 versus pT2–4

[g] Comparison of infiltrating ductal carcinoma and infiltrating lobular carcinoma

[h] Grade 1 = 186 (25.4 % of all pts), Grade 2 = 357 (48.8), Grade 3 = 188 (25.7)

[i] Comparison of Grade 1–2 versus Grade 3

ICC-positive cell category and clinical outcome

During the median observation time of 84 months (range 1–125 months) for relapse and 99 months (range 2–128) for death, 139 (18.9 %) patients experienced systemic relapse and 111 (14.9 %) died of breast cancer. The association between ICC cell category status and outcome was analysed. When all patients were included in the analysis, only presence of TC and UIC predicted systemic relapse ($p = 0.005$ and $p = 0.030$, respectively, Fig. 2). Separate analysis of patients that had not received adjuvant systemic treatment (No-Adj; $n = 389$) and those that had (Adj; $n = 358$), showed that among No-Adj patients, only those harbouring QHC experienced reduced survival. In contrast, only TC and UIC predicted DDFS and BCSS in Adj patients (Figs. 2, 3; Table 3).

For No-Adj patients, QHC status, and primary tumour factors found to be prognostic in univariate analysis (Table 3) were included in a multivariate analysis. The presence of QHC retained independent prognostic significance both for DDFS and BCSS. Additionally, HER2 status was significant for DDFS, while grade and lymph node status were significant for both DDFS and BCSS (Table 4). Multivariate analysis of Adj patients is presented in Supplementary Table 1 (Online Resource 1).

The patients were subgrouped into three categories according to hormone receptor (HR) and HER2 status of the primary tumour. A prognostic impact of TC and UIC were observed only in HR+/HER− subgroup ($n = 526$) ($p \leq 0.003$), but not in the HER2+ ($n = 79$) or triple negative (TN) subgroup ($n = 116$). In contrast, presence of QHC was associated with worse outcome in TN patients ($p = 0.004$ for DDFS, $p = 0.024$ for BCSS) with no significant prognostic impact in the other two subgroups (Fig. 4; Table 5). In multivariate analyses, the prognostic significance of QHC in TN patients was retained for both DDFS and BCSS (DDFS: Hazard ratio 2.5 (CI 1.2–5.3), $p = 0.017$; BCSS: Hazard ratio 2.4 (CI 1.1–5.4), $p = 0.029$), and BCSS was influenced by the TC/UIC status in HR+/HER2− patients (DDFS: Hazard ratio

Table 2 Association between categories of DTCs and clinico-pathological characteristics

	Number of pts	TC/UIC+ (%)[a]	*p* value	QHC+ (%)[a]	*p* value
All pts	747	168 (22.5)[b]		133 (17.8)[b]	
Menopausal status					
Prem. (<55 years)	295	72 (24.4)	0.236	49 (16.6)	0.431
Postm. (≥55 years)	435	90 (20.7)		82 (18.9)	
Lymph node status					
pN0	483	95 (19.7)	0.001	84 (17.4)	0.792
pN+	243	72 (29.6)		44 (18.2)	
Tumour size					
pT1	459	92 (20.0)	0.031	80 (17.4)	0.644
pT2–4	267	72 (27.0)		50 (18.8)	
Histological tumour subtype					
Ductal	572	127 (22.2)	0.249[c]	100 (17.5)	0.447[c]
Lobular	138	37 (26.8)		28 (20.3)	
Other	37				
Histologic grade					
Grade 1–2	543	116 (21.4)	0.184	98 (18.0)	0.649
Grade 3	188	49 (26.1)		31 (16.6)	
Hormone receptor (HR)					
HR+	565	119 (21.1)	0.165	97 (17.2)	0.655
HR−	156	41 (26.3)		29 (18.7)	
HER2 status					
HER2+	79	26 (32.9)	0.018	112 (17.8)	0.985
HER2−	630	133 (21.1)		14 (17.7)	

[a] The percentages in relation to the clinico-pathological variables

[b] Valid percent

[c] Comparison of infiltrating ductal carcinoma and infiltrating lobular carcinoma

1.6 (CI 1.0–2.7), $p = 0.076$; BCSS: Hazard ratio 2.1 (CI 1.2–3.8), $p = 0.016$) (Supplementary Table 2; Online Resource 2).

Additional survival analyses were also performed after exclusion of patients with presence of DTCs with TC morphology. As presented in Fig. 5, QHC status had the same impact on clinical outcome. Furthermore, the exclusion of the 140 patient samples from which the negative controls included less than 1.5×10^6 MNC, did not alter the results (Supplementary Fig. 1; Online Resource 3).

A separate analysis of the tumour subgroups according to the morphological categories of DTCs (i.e., TC, UIC, and QHC) in No-Adj patients revealed that TN patients with the presence of QHC had markedly reduced DDFS ($p = 0.004$) and BCSS ($p = 0.002$) (Supplementary Figs. 2 and 3; Online Resource 4 and 5). In the same subgroup of patients (No-adj, TN), the presence of TC was associated with reduced DDFS ($p = 0.027$), but not BCSS ($p = 0.224$). No association was observed for UIC for any No-Adj subgroup.

Analysis of number of false positive HC

The presence of ≥1 HC did not negatively affect clinical outcome, compatible with the unequivocal hematopoietic cell morphology of these cells. Comparison of 2×10^6 MNC stained by AE1AE3 with the same number of cells stained by negative control antibody, showed a similar frequency of HC (26.7 and 23.9 %, respectively). Of all patients, 91.4 % had either presence of none (61.4 %) or 1–2 (30.0 %) ICC-positive cells with definite HC morphology in specific test and negative controls added together. In the remaining cases 3–7 cells were detected. As these cells were defined as unspecific, we performed an analysis based on the total number of HC present in all slides analysed (both AE1AE3 and negative control slides). Patients with ≥3 HC had significantly improved DDFS ($p = 0.005$, log-rank) and BCSS ($p = 0.009$, log-rank) compared to those with fewer or no HC (Fig. 6). Only 4.8 % of the patients with a total of ≥3 HC experienced metastasis, versus 20.2 % of those with <3 HC. Overlapping survival curves were observed for patients with the presence of 1 HC, 2 HC, or no HC (data not shown).

Discussion

We have previously reported morphological cell classification of immunopositive elements to increase the clinical significance of DTCs in BM [6]. Morphological interpretation of detected elements is also part of the standardised method for detection of circulating tumour cells in blood (CTCs) by the Cellsearch system [16]. There is, however, a morphological

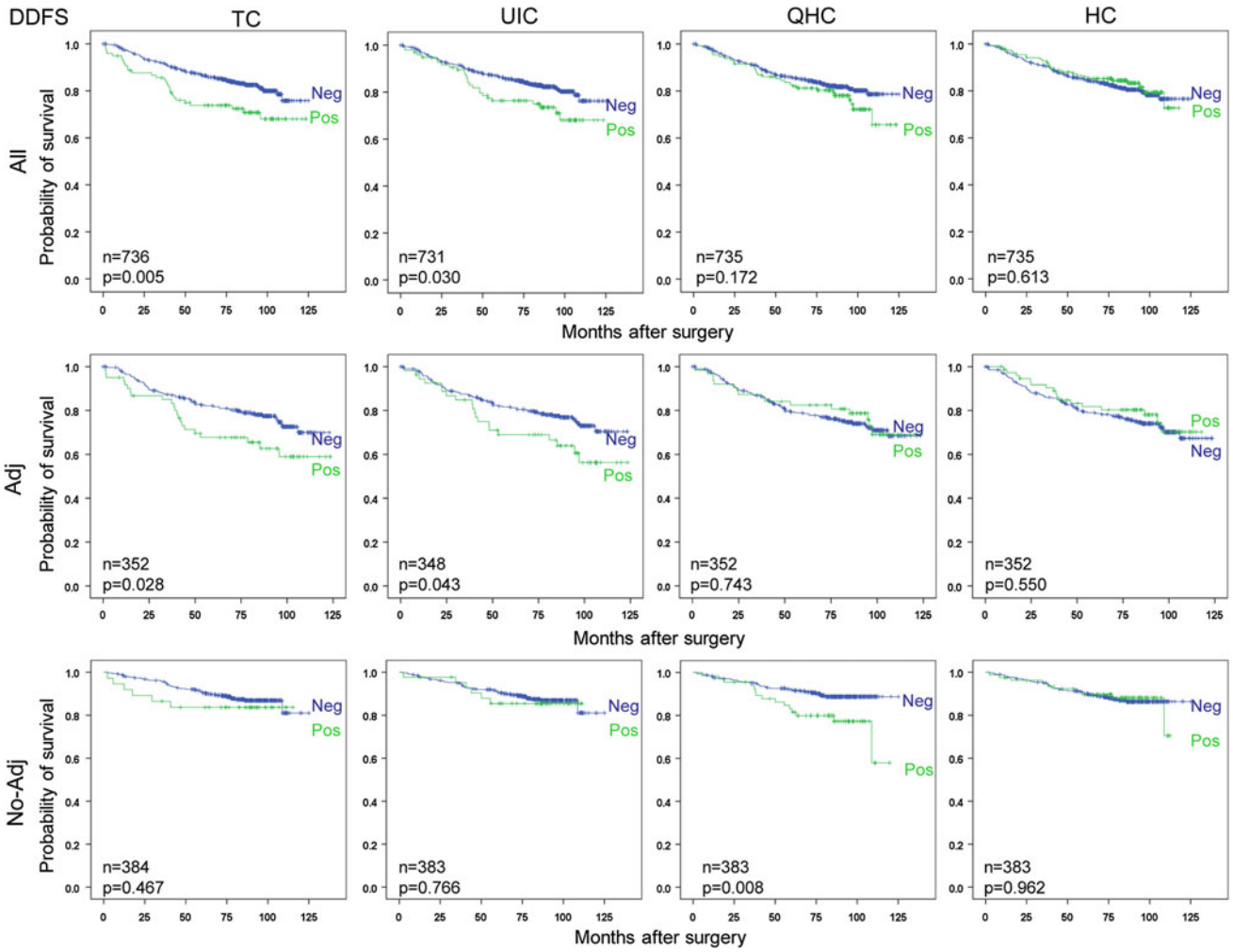

Fig. 2 Distant disease-free survival among patients with (Pos) or without (Neg) ICC-positive cells in the bone marrow within the indicated morphological cell categories; for all, for Adj, and for No-Adj patients. *P* values were computed by log-rank test. Due to missing data in the database for a few patients, the number of patients included in the various survival analyses differ

overlap between real tumour cells and false positive HC, as illustrated by the occasional presence of cells with morphology compatible with tumour cells in negative control specimens and in BM samples from healthy donors [3, 17, 18].

This study shows a clinically significant biological heterogeneity within the DTC population. The results demonstrate that QHC positivity is associated with reduced survival among patients that did not receive systemic adjuvant treatment, indicating presence of DTCs within this cell category. In addition, TN patients (irrespective of treatment status) with presence of QHC had reduced survival, whereas in the HER2+ or HR+/HER2− subgroups, QHC positivity did not affect survival. It cannot be excluded that misclassification of some HC as QHC might have influenced the results, but the contrasting finding of TC/UIC presence associated with reduced survival only for the systemically treated patients, and predominantly for patients with HR+/HER2− status, indicates biological differences. These results show that a definition of DTC positivity based on a combination of different cell morphologies, would have reduced the clinical significance of DTCs. Furthermore, the inclusion of the entire patient population (Adj + No-Adj) in the analyses diminishes the clinical information obtained from DTC analysis. The data indicate that the DTC population is morphologically heterogenous, including cells with clear epithelial tumour cell morphology as well as anonymous cells with some resemblance to HC. Differences in cell biology and possibly in treatment sensitivity between these cell categories, seem to be reflected in their morphological appearance. The interpretation of these results is hampered by the overlap in morphology between ICC false positive cells, as they appear in healthy controls, and tumour cells [3, 17, 18]. Furthermore, the possibility to perform quantitative analysis is restricted because of the low number of these cells in the samples. Analysis of a higher number of BM

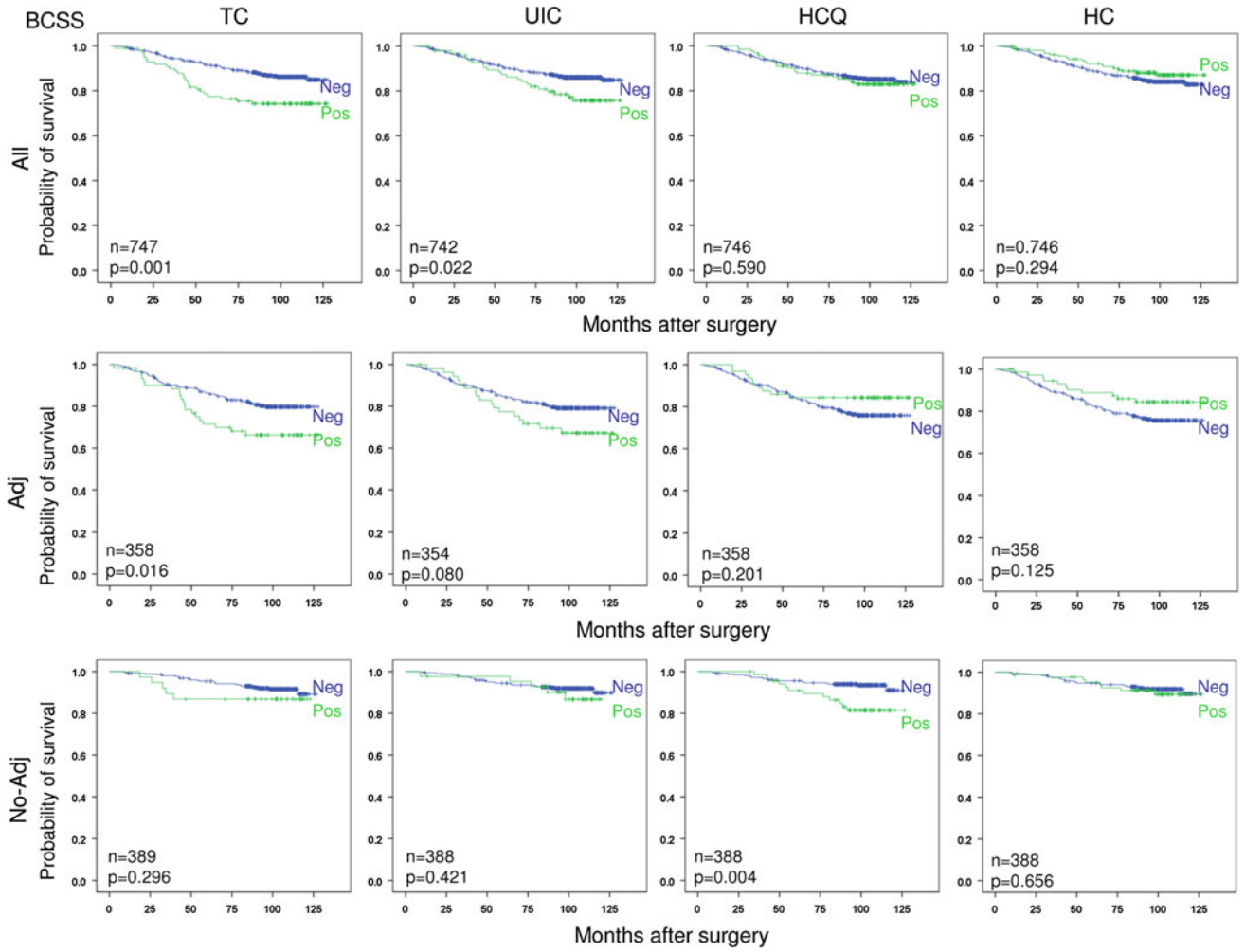

Fig. 3 Breast cancer-specific survival among patients with (Pos) or without (Neg) ICC-positive cells in the BM within the indicated morphological cell categories; for all, for Adj, and for No-Adj patients. Due to missing data in the database for a few patients, the number of patients included in the various survival analyses differ

cells would probably give additional information, if combined with additional characterisation of the cells. Unfortunately, additional cytospins are not available for this purpose. Uninterpretable cells were defined as cells/elements not possible to evaluate because of disruption/degeneration/poor conservation. The finding of prognostic significance for this equivocal ICC positive category is not unique. Analysis for CTCs by the Cellsearch system has also shown that cytokeratin positive cell fragments have prognostic significance [19].

The patients in this study were selected for adjuvant therapy on the basis of standard risk markers, as tumour size, lymph node metastasis and tumour grade, according to Norwegian guidelines at the time of inclusion. The *Adj* patients therefore represented higher risk individuals compared to the No-Adj. It might be speculated if the TCs, with their quite recognisable tumour cell morphology, represent dissemination/desquamation from the bulk of tumour, in patients with generally more advanced disease. Indeed the frequency of TC/UIC was clearly higher in lymph node positive patients than in lymph node negative, which was not the case for QHC (Table 2). As only the presence of cells in the TC/UIC group predicted reduced survival among the HR+/HER2− patients (enriched for luminal A patients), the tumour-initiating cells of this tumour subtype might predominate in the TC/UIC subpopulation. It has been proposed that tumor cells all along the differentiation/hierarchical tree in luminal-like breast cancers may have tumour-initiating properties [20]. As the Luminal A subgroup also has the best prognosis, patients at very early stages of this disease (with node negative status, not receiving systemic treatment) would have a reduced chance of developing metastasis, despite presence of a low number of DTCs. The lack of metastasis formation might be explained by unsupportive microenvironment, elimination of tumour cells by the immune system and/or

Table 3 Cox univariate analysis. Prognostic significance of DTC categories, primary tumour factors and axillary lymph node metastasis, according to adjuvant treatment status

	DDFS			BCSS		
	Hazard ratio	95 % CI	*p*	Hazard ratio	95 % CI	*p*
ICC- subcategories						
No-Adj						
TC/UIC	1.3	0.7–2.6	0.410	1.9	0.9–4.0	0.085
QHC	2.2	1.2–4.1	0.010	2.7	1.3–5.5	0.006
Adj						
TC/UIC	2.0	1.3–3.0	0.002	2.0	1.2–3.1	0.004
QHC	0.9	0.5–1.6	0.743	0.7	0.3–1.3	0.205
Primary tumour factors						
No-Adj						
HER2 (Pos vs. neg)	3.7	2.0–7.0	<0.001	4.5	2.2–9.2	<0.001
HR (Neg vs. pos)	3.7	2.1–6.5	<0.001	5.0	2.6–9.9	<0.001
Grade (3 vs. 1, 2)	5.0	2.8–8.7	<0.001	7.9	3.9–15.9	<0.001
pN (pN+ vs. pN0)	6.8	3.3–14.0	<0.001	10.4	4.9–22.4	<0.001
pT (pT2–4 vs. pT1)	2.6	1.4–4.8	0.002	2.7	1.3–5.6	0.006
Adj						
HER2 (Pos vs. neg)	2.3	1.3–3.9	0.002	2.5	1.5–4.4	0.001
HR (Neg vs. Pos)	2.2	1.4–3.5	0.001	2.6	1.6–4.2	<0.001
Grade (3 vs. 1,2)	2.1	1.4–3.2	0.001	2.2	1.4–3.5	<0.001
pN (pN+ vs. pN0)	3.0	1.7–5.2	<0.001	4.5	2.2–9.0	<0.001
pT (pT2–4 vs. pT1)	2.0	1.2–3.2	0.004	2.6	1.5–4.5	0.001

Table 4 Cox multivariate analysis in No-Adj patients

	DDFS			BCSS		
	Hazard ratio	95 % CI	*p*	Hazard ratio	95 % CI	*p*
QHC (Pos vs. neg)	2.1	1.1–4.1	0.019	2.6	1.3–5.4	0.010
HER2 (Pos vs. neg)	2.0	1.0–4.0	0.043	2.0	1.0–4.4	0.066
HR (Neg vs. pos)	1.9	1.0–3.7	0.057	2.1	1.0–4.4	0.060
Grade (3 vs. 1–2)	2.8	1.4–5.5	0.004	3.6	1.6–8.2	0.002
pN (pN+ vs. pN0)	3.2	1.4–6.9	0.004	4.6	2.0–10.5	<0.001
pT (pT2–4 vs. pT1)	1.2	0.6–2.4	0.636	0.9	0.4–2.2	0.809

dormancy state of the scarce DTCs [21, 22]. Among the patients with TN tumours, our results indicate that DTCs within the QHC group include tumour-initiating cells. The anonymous, undifferentiated appearance typical of QHC, often with a modest cytokeratin staining compatible with a low/downregulated cytokeratin expression, might signify epithelial-mesenchymal transformation/stemness [23]. This does not exclude presence of tumour-initiating cells within the TC/UIC categories. A separate analysis of the TC group (excluding UIC) showed that TN No-Adj patients with presence of TC actually had reduced DDFS, but BCSS was not significantly affected. Analysis of the No-Adj patients according to QHC status in the TN patients, showed that presence of QHC had the strongest prognostic impact in the TN patients (Supplementary Figs. 2 and 3; Online Resource 4 and 5). The lack of significance of QHC in the entire Adj group (Fig. 2) may in part be explained by the fact that only a smaller fraction of the Adj patients, 53 out of 358, were TN. Furthermore, a better elimination of tumour-initiating, disseminated cells by the adjuvant treatment in TN patients than in the large group of HR+/HER2− patients is also a possible explanation [24, 25]. Outcome comparison of Adj versus No-Adj patients revealed a hazard ratio for systemic relapse of 2.3 for the HR+/HER2− patients ($p = 0.001$) and 1.9 for the TN patients ($p = 0.086$).

We have previously identified plasma cells/pre-plasma cells as responsible for false positive reactions in DTC diagnostics [26]. The beneficial outcome for those with ≥3 HC in our study might support an association between the B cell lineage response and inhibition of tumour progression, as part of an immunological response [27]. Cancer progression, as well as the response to anti-cancer therapy, are influenced and modulated by the immune system.

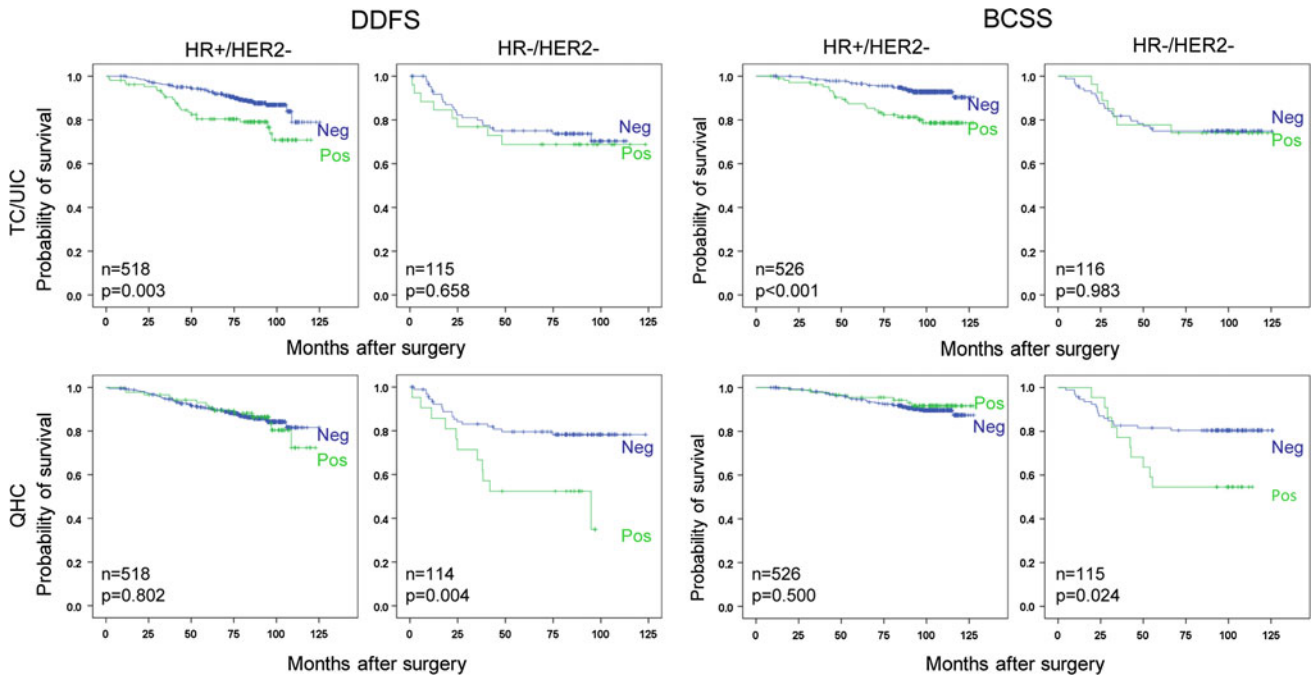

Fig. 4 Survival analyses (DDFS and BCSS) for patients with (Pos) or without (Neg) the indicated DTC subcategory (TC/UIC versus QHC) detected in the BM; for HR+/HER2− and for HR−/HER2− patients. Due to missing data in the database for a few patients, the number of patients included in the various survival analyses differ

Table 5 Cox univariate analysis. Prognostic significance of DTC categories according to primary tumour subgroups

	DDFS			BCSS		
	Hazard ratio	95 % CI	*p*	Hazard ratio	95 % CI	*p*
HR+/HER−						
TC/UIC	2.1	1.3–3.4	0.004	3.1	1.8–5.4	<0.001
QHC	1.1	0.6–1.9	0.803	0.8	0.3–1.7	0.502
Any HER2+						
TC/UIC	1.4	0.7–2.9	0.387	1.2	0.6–2.6	0.633
QHC	1.0	0.4–2.5	0.973	0.9	0.4–2.4	0.873
HR−/HER2−						
TC/UIC	1.2	0.5–2.7	0.658	1.0	0.4–2.4	0.983
QHC	2.9	1.4–6.0	0.006	2.4	1.1–5.2	0.028

Different molecular signatures, signaling patterns and susceptibility loci of importance have been identified [28–30]. It has been reported that cytotoxic T-lymphocytes and natural killer cells exhibit antitumour activity [31]. In a recent study by Kristensen et al. [29], primary tumour immune signatures were shown to predict survival. Although they demonstrated that patients with a Th1-expression profile signature had the most favourable outcome, it has been shown that B-lymphocyte responses may both inhibit and potentiate tumour progression [27, 29, 31].

In addition to morphological classification, further characterisation of single DTCs at the molecular level is desirable. Several methods have been presented for this purpose, although not yet as high-throughput routine analyses. These include array CGH, multiplex immunological and FISH analyses, and analyses of gene expression [32–38]. These techniques are resource demanding and still lack appropriate standardisation and thorough testing on BM samples. Therefore, morphological subclassification of detected elements combined with an optimised interpretation, taking aspects as therapy and primary tumour molecular subtype into account, might serve as an important basic/screening analysis for selection of clinical relevant samples for such additional DTC analyses.

Studies comparing CTCs and DTCs have shown differences in their clinical significance [39, 40], and different

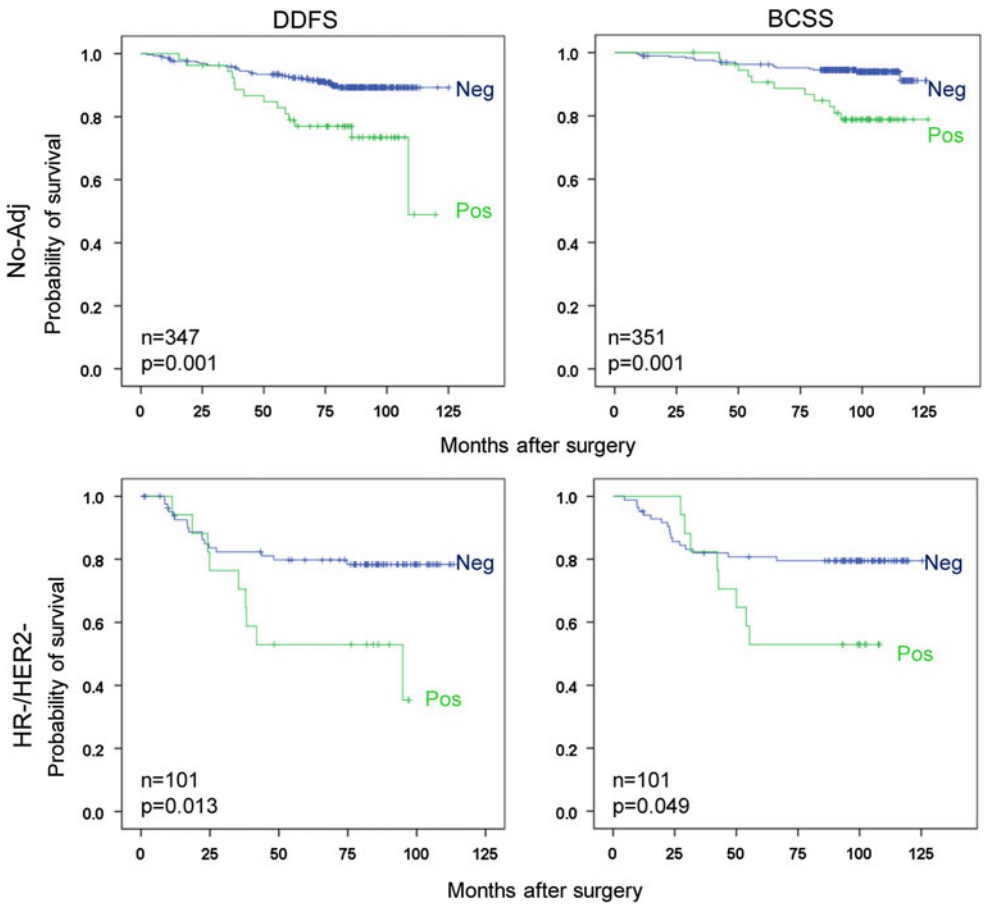

Fig. 5 Survival analyses (DDFS and BCSS) for patients with (Pos) or without (Neg) QHC detected in the BM, patients harbouring TC excluded; for all No-Adj patients, and for all triple negative (HR−/HER2−) patients

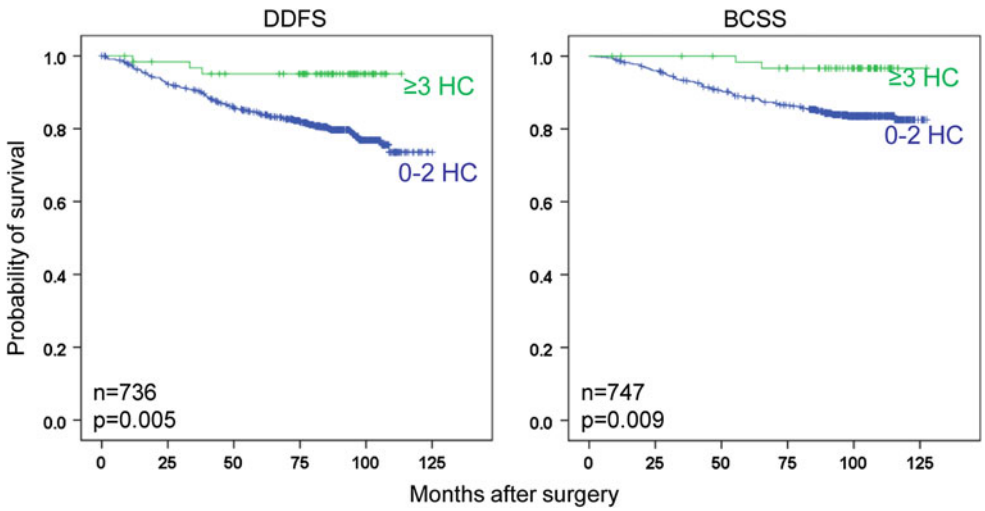

Fig. 6 Survival analyses (DDFS and BCSS) among patients harbouring a total of ≥3 HC versus patients harbouring 0–2 HC, in the bone marrow. Patients with ≥3 HC had significantly improved DDFS (Hazard ratio 0.2, 95 %CI 0.1–0.7, $p = 0.010$) and BCSS (Hazard ratio 0.2, 95 %CI 0.05–0.8, $p = 0.019$) compared to those with fewer or no HC

prognostic relevance of CTCs versus DTCs according to primary tumour characteristics [41, 42]. Although it cannot be excluded that such differences may partially have methodological reasons, these observations have similarities with our results. It has also been reported that certain primary tumour signatures are associated with presence of DTCs or CTCs [43–45]. Therefore, optimal monitoring of DTCs and CTCs should incorporate information about primary tumour expression profiles (if available) and other clinico-pathological parameters.

In conclusion, this study reveals a biologically and clinically relevant heterogeneity within the DTC population, and highlights the importance of further characterisation of DTC at the single cell level. Studies of DTCs

should also take into account the molecular subtype of the primary tumour.

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Conflict of interest The authors declare no conflicts of interests.

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