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Primary breast cancer patients with high risk clinicopathologic features have high percentages of bone marrow epithelial cells with ALDH activity and $CD44^+CD24^{lo}$ cancer stem cell phenotype

James M. Reuben [a,h,*], Bang-Ning Lee [a,h], Hui Gao [a,h], Evan N. Cohen [a,h], Michel Mego [a,h,i], Antonio Giordano [a,h], Xuemei Wang [c,h], Ashutosh Lodhi [b,h], Savitri Krishnamurthy [d,h], Gabriel N. Hortobagyi [e,h], Massimo Cristofanilli [f,h], Anthony Lucci [b,h], Wendy A. Woodward [g,h]

[a] Department of Hematopathology, Unit 54, The University of Texas M.D. Anderson Cancer Center, Houston, Texas 77030, USA
[b] Department of Surgical Oncology, The University of Texas M.D. Anderson Cancer Center, Houston, Texas 77030, USA
[c] Department of Biostatistics, The University of Texas M.D. Anderson Cancer Center, Houston, Texas 77030, USA
[d] Department of Pathology, The University of Texas M.D. Anderson Cancer Center, Houston, Texas 77030, USA
[e] Department of Breast Medical Oncology, The University of Texas M.D. Anderson Cancer Center, Houston, Texas 77030, USA
[f] Fox Chase Cancer Center, Philadelphia, PA, USA
[g] Department of Radiation Oncology, The University of Texas M.D. Anderson Cancer Center, Houston, Texas 77030, USA
[h] Morgan Welch Inflammatory Breast Cancer Research Program, The University of Texas M.D. Anderson Cancer Center, Houston, Texas 77030, USA

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ABSTRACT

Background: Cancer stem cells (CSCs) are purported to be epithelial tumour cells expressing $CD44^+CD24^{lo}$ that exhibit aldehyde dehydrogenase activity ($Aldefluor^+$). We hypothesised that if CSCs are responsible for tumour dissemination, disseminated cells in the bone marrow (BM) would be positive for putative breast CSC markers. Therefore, we assessed the presence of $Aldefluor^+$ epithelial ($CD326^+CD45^{dim}$) cells for the presence of the $CD44^+CD24^{lo}$ phenotype in BM of patients with primary breast cancer (PBC).

Methods: BM aspirates were collected at the time of surgery from 66 patients with PBC. Thirty patients received neoadjuvant chemotherapy (NACT) prior to aspiration. BM was analysed for $Aldefluor^+$ epithelial cells with or without $CD44^+CD24^{lo}$ expression by flow cytometry. BM aspirates from three healthy donors (HD) were subjected to identical processing and analyses and served as controls.

Results: Patients with triple-receptor-negative (TN) tumours had a significantly higher median percentage of $CD44^+CD24^{lo}$ CSC within $Aldefluor^+$ epithelial cell population than patients with other immunohistochemical subtypes ($P = 0.018$). Patients with TN tumours or with pN2 or higher pathologic nodal status were more likely to have a proportion of $CD44^+CD24^{lo}$ CSC within $Aldefluor^+$ epithelial cell population above the highest level of

* Corresponding author at. University of Texas M.D. Anderson Cancer Center, Department of Hematopathology, 1515 Holcombe Blvd, Unit 54, Houston, TX 77030, United States. Tel.: +1 713 7923559, fax: +1 713 7924296.
E-mail address: jreuben@mdanderson.org (J.M. Reuben).
[i] Present address: National Cancer Institute, Klenova 1, 833 10 Bratislava, Slovak Republic.
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HD. Furthermore, patients who received NACT were more likely to have percentages of Aldefluor$^+$ epithelial cells than the highest level of HD ($P = 0.004$).

Conclusion: The percentage of CD44$^+$CD24^{lo} CSC in the BM is higher in PBC patients with high risk tumour features. The selection or enrichment of Aldefluor$^+$ epithelial cells by NACT may represent an opportunity to target these cells with novel therapies.

1. Introduction

Approximately 5% of patients with breast cancer have clinically detectable metastases at the time of initial diagnosis and 30–40% of patients who appear clinically free of metastases harbour occult metastases.[1–4] It is presumed that tumour cells that shed from the primary lesions are released into the peripheral circulation as circulating tumour cells (CTC) that express epithelial-lineage markers, such as CD326 (i.e. EpCAM, epithelial cell adhesion molecules). CTC migrate to the bone marrow microenvironment where there is a selection to maintain a non-proliferative stem-cell like phenotype or to be induced to become cancer-initiating stem cells (CSC) that initiate metastases.[5,6]

Evidence for the existence of CSC, a limited population of tumour cells responsible for giving rise to heterogeneous tumour, was first demonstrated in patients with acute myeloid leukaemia.[7,8] Later, Weissman et al. provided proof of principle that inhibiting the tumour stem cell can prevent the recurrence of leukaemia.[9] Al-Hajj and colleagues used cell-surface markers to isolate a subpopulation of highly tumourigenic breast cancer cells from the bulk of human breast tumour.[10] They observed that CD44$^+$CD24^{lo} human breast tumour cells have an increased ability to form tumours when injected into the cleared mammary fat pad of NOD/SCID mice than cells without this phenotype. While as few as 10^2 CD44$^+$CD24^{lo} human breast tumour cells re-capitulated the human tumours from which they were derived, injection of 10^4 cells of other phenotypes failed to form tumours.[10]

It has been demonstrated that the majority of cytokeratin positive (CK$^+$) tumour cells in the bone marrow, also known as disseminated tumour cells (DTC), are CD44$^+$CD24^{lo} even though these cells were observed in only a small proportion of bone marrow aspirates.[11] Furthermore, the tumour outgrowth potential of CD44$^+$CD24^{lo} cells resides within a subpopulation of epithelial cells with ALDH activity measured by the Aldefluor® method (STEMCELL Technologies, Vancouver, BC).[8] As bone marrow serves as a reservoir for occult disease and CD44$^+$CD24^{lo} CSC within the Aldefluor$^+$ epithelial (CD326$^+$CD45^{dim}) cells may serve as prognostic factors for breast cancer, we measured these cells in bone marrow (BM) of patients with primary breast cancer (PBC) and correlated the findings with their clinicopathological characteristics.

2. Materials and methods

2.1. Study population

We conducted a prospective laboratory-based study (Lab04-0657, Principal Investigator: Anthony Lucci, M.D.) that was approved by the institutional review board of The University of Texas M.D. Anderson Cancer Center. Enrolment eligibility criteria included patients with a diagnosis of PBC, stages I–III without metastatic disease, and elected to undergo definitive surgery for primary tumour and lymph nodes dissection. All patients provided informed consent according to institutional guidelines. From September 2006 to October 2008, 66 PBC patients were enrolled and provided a BM specimen either at the time of placement of a central venous catheter for delivering neoadjuvant chemotherapy (NACT) ($N = 6$) or at the time of surgery ($N = 60$). Thirty patients received NACT prior to bone marrow aspiration. We noted that NACT group had a significantly higher proportion of patients with N2 or higher nodal status ($P = 0.000$) or with HER-2+ primary tumour ($P = 0.001$). As a control, BM aspirates from three healthy donors (HD) were purchased from STEMCELL Technologies (Vancouver, BC) and analysed in the same manner as patient BM samples.

2.2. Primary breast tumour characterisation

The primary breast tumours were graded histologically on a scale from 1 to 3 based on a modified, combined Nottingham histologic grading system. Immunostaining for ER, PR, and HER2 was performed using primary antibodies against ER (clone 6FII, dilution 1:35; Novocastra Laboratories Ltd., Newcastle upon Tyne, United Kingdom), PR (clone PgR 1294, dilution 1:200; Dako, Carpenteria, CA) and HER2/neu (Lab Vision, clone neu Ab8, dilution 1:300; Lab Vision, Fremont, CA). Primary breast tumours that expressed nuclear staining in 1% of tumour cells were regarded as positive for ER and PR. Immunostaining results for HER2 were scored as 1þ when <10% of the tumour cells had complete membranous staining; as 2þ when weak-to-moderate, complete membranous staining was present in >10% of tumour cells; and as 3þ when strong, complete membranous staining was present in >30% of tumour cells. All 2þ and 3þ cases were evaluated by fluorescence in situ hybridisation (FISH) for HER2 gene amplification using the HER2 DNA probe kit (Abbott Laboratories, Abbott Park, Ill). A HER2/CEP17 ratio >2.2 was regarded as positive for HER2 gene amplification.

2.3. Detection of epithelial cells in BM

To assess for the presence of epithelial cells in BM, 5–8 mL of BM were aspirated from bilateral iliac crests of 66 PBC patients, the specimen dispensed into EDTA-coated tubes and transported to the laboratory immediately for processing within 4 h. To maximise epithelial cell recovery, the bilateral BM samples were pooled prior to the isolation of mononuclear cells (MNC) by ficoll-hypaque density gradient centrifugation at 400*g* for 30 min. The MNC were harvested and washed twice with ice-cold phosphate-buffered saline (PBS).

MNC samples were interrogated for ALDH activity using the Aldefluor® assay and manufacturer's protocol (STEMCELL Technologies). Briefly, 4×10^6 MNC from patients and HD were suspended in Aldefluor buffer which contains a proprietary ATP-binding cassette transport inhibitor. One third of the cells were reacted with 5 μL of the ALDH inhibitor, diethylamino-benzaldehyde (DEAB), as a negative control. Both the test reaction and the negative control were incubated for 35 min at 37 °C in a 5% CO_2 atmosphere. Purified anti-CD44 monoclonal antibody (BD Pharmingen, San Diego, CA) was conjugated with Alexa700 using the Zenon® antibody labelling kit (Invitrogen, Carlsbad, CA) prior to reaction with the Aldefluor-labelled cells. Additionally, pre-conjugated antibodies to CD24 (PE) and CD45 (PE-Cy7) both from BD Pharmingen (San Diego, CA), and CD326 (APC, Miltenyi Biotec, Auburn, CA) were used to label cells at room temperature protected from light for 30 min. An additional tube of Aldefluor-labelled cells was stained with the appropriate isotype-matched controls. The stained cells were washed twice with PBS and the cell pellet suspended in 200 μL of PBS prior to analysis on a LSR-II flow cytometer capable of discriminating 6-colour fluorescence (BD Biosciences, San Jose, CA). Cellular debris was excluded from the analysis based on low forward light scatter. For analysis and throughout the manuscript, epithelial cells in BM were defined as cells exhibiting the phenotype $CD326^+$ and $CD45^{dim}$. Within the $Aldefluor^+$ epithelial cell population, a subset of cancer-initiating stem cells (CSC) was defined as cells with a $CD44^+CD24^{lo}$ phenotype.

Table 1 – Characteristics of patients.

Characteristics	Number	%
Median age (min, max)	52 (27, 77)	
Ethnicity		
Caucasian	52	78.8
Hispanic	7	10.6
Black	5	7.6
Asian	2	3.0
Tumour markers		
ER	49	74.2
PR	36	54.5
HER2 (IHC 3+ or FISH)	11	16.7
Triple receptor negative	10	15.2
Tumour grade		
Grade 1	8	12.1
Grade 2	28	42.4
Grade 3	30	45.5
Tumour size		
T1	32	48.5
T2	20	30.3
T3	6	9.1
T4	8	12.1
Lymph node status		
N0	36	54.5
N1	15	22.7
N2	3	4.5
N3	12	18.2
Neoadjuvant chemotherapy (NACT)	30	45.5
Pathological complete responses (pCR) after NACT	10	15.2

2.4. Statistical analysis

In this prospective study descriptive statistics were computed for $Aldefluor^+$ epithelial cells and $CD44^+CD24^{lo}$ CSC in BM specimens of PBC patients and HD. Frequency and percentages of epithelial cells and CSC subsets were summarised for categorical variables and means, medians, standard deviations and standard errors of mean were used to summarise the continuous variables. The Wilcoxon rank sum tests[12] and Mann-Whitney *U* tests were used to compare the difference of continuous variables among and between clinicopathological groups, respectively.[13] The Fisher's exact test was used to compare the categorical variables between groups. Statistically significant differences were set at $P < 0.05$. All analyses were conducted in SPSS 16 (SPSS Inc., Chicago, IL).

3. Results

3.1. Patient population

A total of 66 PBC patients, stages I–III, and undergoing surgery with ($N = 30$) or without ($N = 36$) prior NACT were studied (Table 1). The median age of the patient population was

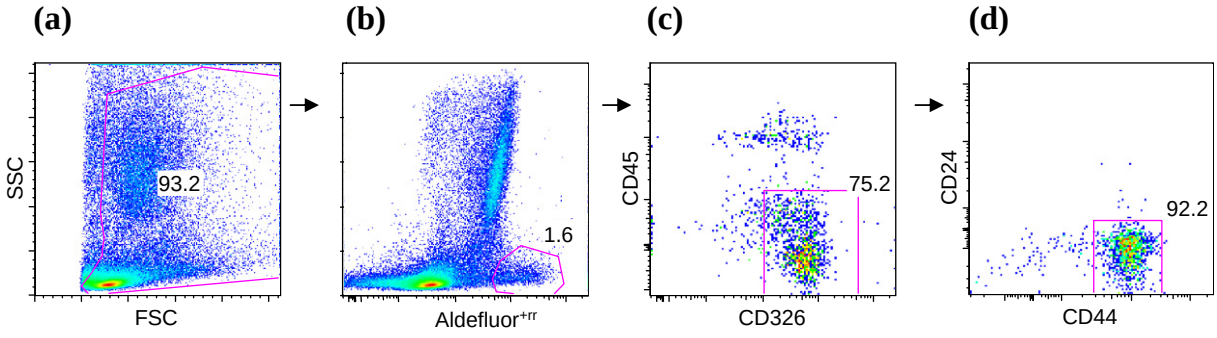


Fig. 1 – Enumeration of $CD44^+CD24^{lo}$ CSC within the $Aldefluor^+$ epithelial ($CD326^+CD45^{dim}$) cell population. Bone marrow (BM) mononuclear cells were analysed for the ALDH activity using the Aldefluor® assay kit (b). The percentage of epithelial ($CD326^+CD45^{dim}$) cells was calculated within the $Aldefluor^+$ population (c) and the percentage of $CD44^+CD24^{lo}$ CSC was calculated within the $Aldefluor^+$ epithelial cell population (d).

52 years (range: 27–77 years). There were 45 patients with Hormonal Receptor (HR)+/HER2–, 4 patients with HR+/HER2-amplified (HER2+), 7 patients with HR–/HER2+ and 10 patients with triple-receptor-negative (TN) tumour subtypes according to the immunohistochemical (IHC) staining of the primary tumour using ER/PR and HER2-neu status. Tumour grade consisted of eight grade 1, 28 grade 2 and 30 grade 3. Tumour sizes consisted of 32 T1, 20 T2, 6 T3 and 8 T4 (Table 1). Lymph node status of patients consisted of 36 N0, 15 N1, 3 N2 and 12 N3. Staging was based on the revision of the American Joint Committee on Cancer staging system for breast cancer.[14]

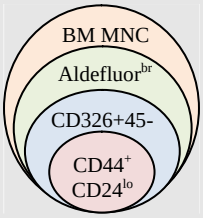
3.2. Assessment of epithelial cells and CSC for ALDH activity

Patient BM samples were analysed for the presence of ALDH activity using the Aldefluor® assay kit (Fig. 1). Cells were initially analysed for their ALDH activity (Aldefluor$^+$) and then assessed for the percentage of epithelial (CD326$^+$CD45^{dim}) cells within the Aldefluor$^+$ population. The mean percentage (±SEM) of Aldefluor$^+$ cells in MNC, irrespective of their epithelial or haematopoietic origin, was 1.40% (±0.11%) (Table 2). The mean percentage of Aldefluor$^+$ epithelial cells was 48.6% (±2.21%; alternatively expressed as 0.68% of total MNC counted) and the mean percentage of CD44$^+$CD24^{lo} CSC within the Aldefluor$^+$ epithelial cell population was 43.0 ± 3.59% (Table 2; alternatively expressed as 0.30% of total MNC counted).

3.3. Relationship between immunohistological subtypes and percentage of CSC within the Aldefluor$^+$ epithelial cells

Comparing the entire patient cohort to the HD samples, there was a statistically significantly higher median percentage of Aldefluor$^+$ epithelial cells (independent of CD44$^+$CD24^{lo} expression) in BM of PBC patients when compared with that of HD (50.6% versus 37.7% $P = 0.045$). Overall, the proportion of the CD44$^+$CD24^{lo} CSC subset within Aldefluor$^+$ epithelial cells was not statistically different between patients and HD (Fig. 2b). However, the subgroup of patients with TN tumours had a significantly higher percentage of CD44$^+$CD24^{lo} CSC when compared with patients with other tumour IHC subtypes (66.8% versus 35.6%, $P = 0.02$) (Fig. 2d). In addition, these patients were more likely to have the proportion of CSC greater than the highest level of the HD (35.1%) ($P = 0.019$, Table 4).

Table 2 – The percentage of Aldefluor$^+$ CD326$^+$ epithelial cells and CSC of patients with different immunohistologic tumour subtypes.



N = 66	Median (%)	Mean (%)	SEM
Aldefluor$^+$	1.18	1.40	0.11
CD326$^+$CD45^{dim}	47.0	48.6	2.21
CD44$^+$CD24^{lo}	39.2	43.0	3.59

Tumour histological subtypes		% Aldefluor$^+$	% CD326$^+$CD45^{dim}	% CD44$^+$CD24^{lo}
HR$^+$ HER2$^-$	N	45	45	45
	Median	1.25	46.90	36.10
	Mean	1.37	46.61	40.39
	Standard error of mean	0.13	2.71	4.36
HR$^+$ HER$^+$	N	4	4	4
	Median	1.07	61.15	21.49
	Mean	1.48	61.65	33.64
	Standard error of mean	0.47	7.51	18.74
HR$^-$ HER2$^+$	N	7	7	7
	Median	0.95	55.40	30.80
	Mean	1.24	52.27	43.61
	Standard error of mean	0.30	7.66	14.41
Triple negative	N	10	10	10
	Median	1.09	54.55	66.85
	Mean	1.46	55.21	64.26
	Standard error of mean	0.42	5.34	6.03
Healthy donors (HD)	N	3	3	3
	Median	2.39	37.30	19.60
	Mean	1.98	30.47	22.73
	Standard error of mean	0.58	7.85	6.43

HR$^+$ = hormone receptor$^+$ (ER$^+$ and/or PR$^+$); HER2$^+$ = HER2/neu amplified.

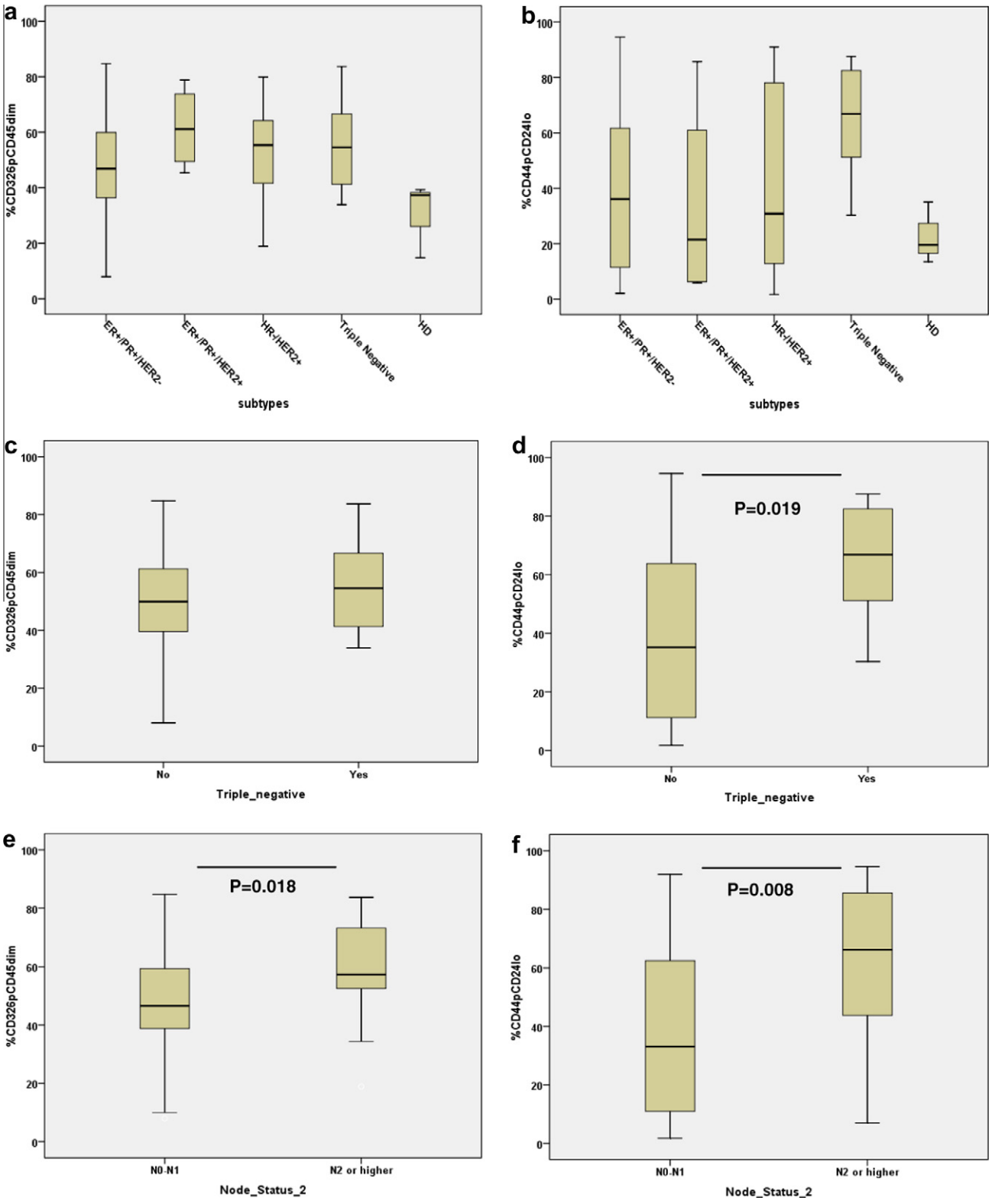


Fig. 2 – Percentages of Aldefluor$^+$ epithelial cells (a, c, e) and CD44$^+$CD24^{lo} CSC within Aldefluor$^+$ epithelial cells (b, d, f) in subsets of patients. While patients with different clinicopathological characteristics have similar percentages of Aldefluor$^+$ epithelial cells (a), patients with triple-receptor-negative (TN) tumours have a significantly higher median percentage of CD44$^+$CD24^{lo} CSC within Aldefluor$^+$ epithelial cells (d) than patients with other tumour subtypes. In addition, patients with pN2 or higher nodal status have a significantly higher median percentage of Aldefluor$^+$ epithelial cells (e) and CD44$^+$CD24^{lo} CSC within Aldefluor$^+$ epithelial cells (f) than patients with pN1 or pN0 status.

Table 3 – Relationship between clinicopathological characteristics and percentage of Aldefluor$^+$ epithelial cell population.

Variable	Aldefluor$^+$ epithelial cells > 39.3%[a]		P-values[b]
	No N (%)	Yes N (%)	
Lymph node status			
pN0	10 (27.8%)	26 (72.2%)	0.219
pN1–3	5 (16.7%)	25 (83.3%)	
Lymph node status			
pN0–1	13 (25.5.0%)	38 (74.5%)	0.270
pN2–3	2 (13.3%)	13 (86.7%)	
Tumour size			
pT1	9 (28.1%)	23 (71.9%)	0.236
pT2–4	6 (17.6%)	28 (82.4%)	
High tumour grade			
3	3 (10.0%)	27 (90.0%)	0.023
1–2	12 (33.3%)	24 (66.7%)	
Oestrogen receptor			
Negative	3 (17.6%)	14 (82.4%)	0.416
Positive	12 (24.5%)	37 (75.5%)	
Progesterone receptor			
Normal	6 (20.0%)	24 (80.0%)	0.428
Amplified	9 (25.0%)	27 (75.0%)	
HER2 status			
Normal	13 (23.6%)	42 (76.4%)	0.262
Amplified	1 (9.1%)	10 (90.9%)	
Triple receptor negative			
No	13 (23.2%)	43 (76.8%)	0.319
Yes	1 (10.0%)	9 (90.0%)	
Immunohistochemical (IHC) subtypes			
HR+/HER2–	12 (26.7%)	33 (73.3%)	0.352
HR+/HER2+	0 (0.0%)	4 (100%)	
HR–/HER2+	1 (14.3%)	6 (85.7%)	
Triple receptor negative	1 (10.0%)	9 (90.0%)	
Neoadjuvant therapy			
No	11 (30.6%)	25 (69.4%)	0.039
Yes	3 (10.0%)	27 (90.0%)	
pCR			
No	2 (10.0%)	18 (90.0%)	0.719
Yes	1 (10.0%)	9 (90.0%)	

[a] The highest value of the HD.
[b] Fisher exact test.

3.4. *Relationship between the tumour size and percentage of CSC in bone marrow*

There were 34 patients with T2 or greater tumours. There were no differences in the percentage of Aldefluor$^+$ epithelial cells or proportion of CD44$^+$CD24^{lo} CSC within the Aldefluor$^+$ epithelial cell population between patients with T1 tumours and patients with T2 or greater tumours. However, compared with patients with grade 1–2 tumours (24/36 or 66.7%), patients with grade 3 breast cancer (27/30 or 90%) were more likely to have a proportion of Aldefluor$^+$ epithelial cells above 39.3%, the highest level of HD bone marrow aspirates ($P = 0.023$, Table 3). Nevertheless, patients with grade 1–2 and grade 3 tumours had similar proportion of CD44$^+$CD24^{lo} CSC within the Aldefluor$^+$ epithelial cell population.

3.5. *Relationship between lymph node status and CD44$^+$CD24^{lo} CSC in bone marrow*

Compared with patients with pN1 or negative nodes, the percentage of Aldefluor$^+$ epithelial cells (46.6% versus 57.3%; $P = 0.018$) and the proportion of CD44$^+$CD24^{lo} CSC within Aldefluor$^+$ epithelial cell cluster (33.1% versus 66.2%; $P = 0.008$) were significantly higher for patients with pN2 or higher nodal status (Fig. 2f). Furthermore, patients with pN2 or higher nodal status were more likely to have the proportion of

Table 4 – Relationship between clinicopathological characteristics and percentage of $CD44^+CD24^{lo}$ CSC within $Aldefluor^+$ epithelial cell population.

Variable	$CD44^+CD24^{lo}$ CSC > 35.1%[a]		P-values[b]
	No N (%)	Yes N (%)	
Lymph node status			
pN0	16 (44.4%)	20 (55.6%)	0.563
pN1–3	13 (43.3%)	17 (56.7%)	
Lymph node status			
pN0–1	26 (51.0%)	25 (49.0%)	0.031
pN2–3	3 (20.0%)	12 (80.0%)	
Tumour size			
pT1	13 (40.6%)	19 (59.4%)	0.391
pT2–4	16 (47.1%)	18 (52.9%)	
High tumour grade			
1–2	17 (47.2%)	19 (52.7%)	0.368
3	12 (40.0%)	18 (60.0%)	
Oestrogen receptor			
Negative	5 (29.4%)	12 (70.6%)	0.132
Positive	24 (49.0%)	25 (51.0%)	
Progesterone receptor			
Negative	11 (36.7%)	19 (63.3%)	0.201
Positive	18 (50.0%)	18 (50.0%)	
HER-2 status			
Normal	22 (40.7%)	32 (59.3%)	0.215
Amplified	7 (58.3%)	5 (41.7%)	
Triple receptor negative			
No	28 (50.0%)	28 (50.0%)	0.019
Yes	1 (10.0%)	9 (90.0%)	
IHC subtypes			
HR+/HER2–	22 (48.9%)	23 (51.1%)	0.114
HR+/HER2+	3 (60.0%)	2 (40.0%)	
HR–/HER2+	4 (57.1%)	3 (42.9%)	
Triple receptor negative	1 (10.0%)	9 (90.0%)	
NACT			
No	16 (44.4%)	20 (55.6%)	0.563
Yes	13 (43.3%)	17 (56.7%)	
pCR			
No	9 (45.0%)	11 (55.0%)	0.554
Yes	4 (40.0%)	6 (60.0%)	

[a] The highest value of the HD.
[b] Fisher exact test.

$CD44^+CD24^{lo}$ CSC higher than 35.1%, the highest level of HD's bone marrow aspirates ($P = 0.031$, Table 4).

3.6. *Effect of neoadjuvant chemotherapy on CSC in bone marrow*

Patients who received NACT had a significantly higher median percentage of $Aldefluor^+$ epithelial cells (55.5% versus 43.2%; $P = 0.002$), but not $CD44^+CD24^{lo}$ CSC, when compared with those of untreated patients. In addition, compared with patients who did not receive NACT (25/36 or 69.4%), NACT-treated patients (27/30 or 90%) were more likely to have a proportion of $Aldefluor^+$ epithelial cells greater than 39.3%, the highest level of HD bone marrow aspirates ($P = 0.039$, Table 3).

Among the 30 patients who received NACT, 10 patients achieved pathological complete responses (pCR); however, the responses were not associated with lower percentage of $Aldefluor^+$ epithelial cells (Table 3) or lower proportion of $CD44^+CD24^{lo}$ CSC (Table 4).

4. Discussion

As part of an on-going prospective trial to study the prognostic value of disseminated epithelial cells in patients with operable breast cancer, we report for the first time the distribution of putative breast cancer stem cell markers measured by flow cytometry in the bone marrow of patients with PBC. This cohort represents an unselected group with stages I–III breast

cancer and the expected distribution of clinical tumour markers. In this study, we demonstrated the presence of epithelial cells ($CD326^+CD45^{dim}$) with ALDH activity ($Aldefluor^+$) in the bone marrow of PBC patients is significantly greater than that seen in HD. In particular, the percentage of epithelial cells expressing Aldefluor and putative CSC markers was highest in patients with TN tumour and advanced nodal disease. This is consistent with the hypothesis that CSCs are associated with early micrometastatic disease in patients who eventually relapse. These are preliminary but important translational data confirming the presence of these phenotypes in clinical samples and supporting the need for further studies to examine the mechanisms of chemotherapy resistance of these cells.

In our study, we used multi-parameter flow cytometry to detect $CD326^+CD45^{dim}$ epithelial cells and to enumerate the $CD44^+CD24^{lo}$ cells within the $Aldefluor^+$ epithelial cells population in BM of PBC patients. We detected epithelial cells in the BM of all patients examined. While others used immunocytochemistry (ICC) and PCR-based technology for the detection of CSCs in the BM,[3] we employed multi-parameter FACS analysis and found it to be sensitive and reliable for detecting of putative CSC in BM of PBC patients. A major disadvantage of employing the current putative markers (CD326, CD44, CD24, and Aldefluor) to identify CSC is that these characteristics are not lineage specific. Indeed, CD326 can be expressed by $CD45^+$ haematopoietic cells and by $CD34^+$ early progenitor cells in BM of HD[15] and we find them at lower levels in HD samples. Nevertheless, these markers represent the current state of the art for immunophenotypic characterisation of CSC and have been reported by others as well.[15]

CD44, a cell surface receptor for hyaluronic acid, is involved in cell adhesion, migration and metastasis of cancer cells,[16] and the lineage negative $CD44^+CD24^{lo}$ phenotype prospectively and selectively identified cells which re-generated breast tumours when transplanted into immunocompromised mice.[10] In further studies of primary tumours, the $CD44^+CD24^{lo}$ phenotype was more common in basal-like tumours[17] and associated with BRCA1 hereditary breast cancer.[17] Others have also reported that patients with the $CD44^+CD24^{lo}$ phenotype had TN tumours.[18] Thus, it is consistent that the proportion of $CD44^+CD24^{lo}$ CSC within the $Aldefluor^+$ epithelial cells in the bone marrow was indeed highest in patients with TN tumours. In addition, patients with pN2 or higher nodal status, regardless of immunohistological subtypes, tend to have a higher percentage of $CD44^+CD24^{lo}$ CSC within the $Aldefluor^+$ epithelial cell population when compared to patients with pN0 or pN1 nodal status (Table 4). This finding is unprecedented and provocative given the strong correlation between nodal status and outcome. In this study, patients receiving NACT had a significantly higher median percentage of $Aldefluor^+$ epithelial cells than untreated patients (55.5% versus 43.2%, $P = 0.008$), but no significant difference in $CD44^+CD24^{lo}$ CSCs. We highlight that these were not paired samples and as such comparisons are subject to biases between any two unrelated cohorts. In this case, the NACT group had a significantly higher proportion of patients with N2 or higher nodal status or with HER-2+ primary tumours. It is, therefore, possible that the higher % of Aldefluor+ epithelial cells seen in treated patients in these data reflects worse prognostic features or that while in some patients NACT is effective in reducing tumour burden, one may expect a concomitant enrichment of tumour cells with the stem cell phenotype in other patients (ALDH activity). Achieving pCR did not correlate with the percentages of $Aldefluor^+$ epithelial cells or with the proportion of $CD44^+CD24^{lo}$ CSC in patients who received NACT, however, these are based on small numbers of patients. No prior studies have correlated putative CSC markers in the bone marrow to NACT, however, several studies on primary tumour tissue suggest a relationship between CSCs and NACT. Tanei et al. observed similar results with an increase in ALDH1 expressing cells in the post chemotherapy primary samples, but no increase in $CD44^+CD24^{lo}$ cell population,[19] while Li et al. reported NACT increased the percentage of $CD44^+CD24^{lo}$ cells in PBC patients.[20] In contrast to these studies, a recent comparative analysis of ALDH1 expression in tumour cells by Resetkova et al. did not show a significant increase in the ALDH1 expressing population in the post chemotherapy samples.[21] Nevertheless, in the bone marrow, the persistence of epithelial cells with ALDH activity following NACT implies resilience of these cells to chemotherapy which is concerning given the relevance of this phenotype to cancer-initiating properties. While the presence of $Aldefluor^+$ epithelial cells in PBC patients who received NACT may select appropriate cohorts for future stem cell targeted therapy trials, the small sample size and lack of paired samples in the current study prohibit definitive conclusions regarding NACT.

It remains to be determined if putative CSCs in the bone marrow predict for distant metastases, a critical correlation, particularly among patients for whom standard clinical risk factors appear favourable. The small number of events (three patients have had relapse of disease) precludes this analysis here. While the correlation of putative CSCs in bone marrow with high risk features is important and consistent with the CSC hypothesis, patients with advanced disease and poor prognostic features can already be selected for additional adjuvant therapies to eradicate occult disease. Finding a biomarker to select the small percentage of patients in the low risk cohort who will eventually relapse without further treatment is a major challenge. Rigorously testing a stem cell biomarker is particularly difficult as functional stem cell assays are needed to definitively prove the biomarker is a meaningful stem cell marker, and obtaining sufficient live cells from patients for functional studies is often not feasible. This is indeed a significant limitation of the work described here. Attempts to characterise functional end-points from the material gained from the bone marrow were unsuccessful due to the limited material obtained. Herein, we report that the proportion of $CD44^+CD24^{lo}$ CSC in a subset of patients considered low risk is higher than that of HD controls. Consistent with these results, in the only previous study of cancer stem cell markers in the bone marrow of patients with early stage disease, Balic et al. employed spectral imaging in conjunction with double marker immunohistochemistry to examine the simultaneous expression of CD44 and CD24 on cytokeratin-positive DTCs in the bone marrow of 50 patients with early stage breast cancer and detected $CD44^+CD24^{lo}$ cells in all cytokeratin positive BM samples with a median prevalence of 66%.[11] It is tempting to speculate, but indeed remains unproven, that low risk patients that harbour putative CSCs in the bone marrow may be at higher risk for relapse.

A significant concern related to studies looking at DTCs or stem cell markers in the bone marrow is that, thus far, these studies appear to select a larger group of patients than are expected to relapse based on clinical data. In the Balic study,[11] DTC selection with cytokeratin followed by stem cell marker assessment selected a gratifyingly small subset of patients, consistent with the clinical expectations that the majority of low risk patients will not relapse. However, using a differentiation marker such as cytokeratin may increase the false negative rate by not detecting cancer stem cells lacking differentiation markers. Omitting cytokeratin as done herein leads to detection of putative CSCs in a larger population of patients even if a threshold for positivity is set well above that observed in HD controls. While this seems to refute the hypothesis or suggests this approach is not sufficiently specific, it is analogous to the accepted correlation between axillary metastases and clinical outcomes. Two randomised trials have clearly demonstrated a significant proportion of patients with pathologically demonstrable metastases in the axilla not subjected to local therapy will not experience an axillary recurrence.[22,23] These data underscore how much is unknown about cancer cell biology and quiescence and demonstrate the danger of rejecting data that are inconsistent with our beliefs instead of challenging our beliefs in the face of new data.

In conclusion, we demonstrated that the $CD326^{+}CD45^{dim}$ epithelial cell population of PBC patients contained a subset of $CD44^{+}CD24^{lo}$ CSC. Clinicopathological characteristics, such as TN tumour and higher nodal status (pN2 or higher) are factors that favored the presence of a higher percentage of CSC than found in healthy donors consistent with the hypothesis that these cells mediate recurrence. The markers examined are clearly present in the bone marrow of healthy donors at low levels and we were unable to determine if this is a true positive or false positive finding given the limitations of the current study. Further work is clearly needed to expand the study of healthy donors. Without feasible functional stem cells assays the correlation of biomarker studies to long-term clinical follow up is critical, and without them these findings are appropriately considered preliminary and hypothesis-generating. Nevertheless, they are consistent with the cancer stem cell hypothesis and lend credence to the hypothesis that current NACT regimens may not be effective in eliminating epithelial cells with stem cell phenotype. With further study we may well find that novel targeted therapy is required to eliminate CSC in some breast cancer patients.

Conflict of interest statement

None declared.

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