Cytological, Histological Uni- and Multi-Immunohistochemical Marrow Examinations in Detecting Early Disseminated Tumor Cells in De Novo Breast Cancer Patients.

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Abstract: Many studies have demonstrated the independent prognostic value of detecting bone marrow (BM) disseminated tumor cells (DTCs) at initial diagnosis of 1^{ry} breast cancer (BC) patients. Therefore, an accurate detection of these DTCs in the BM is very critical and must be obtained by using the most reliable and sensitive detection methodologies. In this respect, our study aimed to evaluate the detection capacities of the cytological, histological, uni- and multi-immunohistochemical (IHC) marrow examinations for early DTCs in the BM of newly diagnosed patients with non-stage IV 1^{ry} BC. This study included 80 of these patients that were subjected to CBC, BM aspiration/biopsy and IHC staining by a panel of monoclonal antibodies (McAbs) including Cytokeratin (CK), Mammaglobin and cancer antigen 15-3 (CA15-3). The detection rate of the histological BM examinations (11.3%) was significantly higher than that of the cytological one (2.5%), *p*-value =0.04. Our individual interpretation of the uni-IHC marrow examinations, using the above mentioned 3 McAbs, revealed that their detection rates (21.3%, 26.3% and 35%) were considerably variable but were significantly higher than that of the routine histological ones, *p*-values= 0.049, 0.035 and 0.02, for CK, Mammaglobin and CA15-3, respectively. The results obtained from the uni-IHC marrow examinations, using the same 3 McAbs, also showed variable degrees of agreement between each other. Therefore, a total interpretation of multi-IHC marrow examinations for these 3 McAbs was established. From the quantitative point of view, our multi-IHC total interpretation revealed a detection rate (47.5%) significantly higher than that of our histological interpretation (11.3%), *p-value* = 0.01; also, from the qualitative point of view, our results of both histological and multi-IHC total interpretations showed a highly significant statistical difference, *p*-value = 0.001. We concluded that for optimal increase in the detection capacity for early DTCs in the BM of de novo patients with non-stage IV 1^{ry} breast cancer, a total interpretation for combined histological/multi-IHC marrow examinations must be performed.

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1. Introduction

Breast cancer (BC) is considered a systemic disease in which a hematogeneous dissemination of tumor cells, essentially to bone marrow (BM), may occur at very early stages of primary tumor development and form an occult isolated tumor cells, called disseminated tumor cells (DTCs) or micro-metastases that subsequently lead to an overt metastases **[1]**. Over the last two decades many studies have demonstrated the independent prognostic value of detecting BM micro-metastases or DTCs at initial diagnosis of 1^{ry} BC patients **[2 - 7]**. Other studies described a significant correlation between the presence of DTCs in BM and an unfavorable clinical outcome **[8-10]**.Therefore, accurate detection of BC micro-metastases in BM is very critical and must be based on standardized detecting methodologies. Nevertheless, among the currently used procedures, the reported incidence of BM micro-metastatic cell detection fluctuates considerably. This might be due to variations in patient series, stage distribution, expression of targeted antigen, sensitivity and/or specificity of the used monoclonal antibody (McAb) and in sensitivity of the procedure itself **[11]**. The detecting procedure for DTCs in BM is still investigational according to the American Society of Clinical Oncology, 2007 update of recommendations for the use of tumor markers in breast cancer **[12]**. At initial diagnosis of any 1^{ry} BC, especially in non-stage IV patients, the continuous real challenge for hematopathologists is the uppermost increase of detection capacity for any DTCs hidden in the BM or peripheral blood. In this respect, many different procedures have been used **[11, 13 - 15]**, but some limiting factors were always experienced with some of these detection methods. Therefore a combination of techniques and markers might help to overcome limitations experienced with these detection procedures **[16]**. In this study, we aimed to evaluate the detection capacities of cytological, histological, uni- and multi-immunohistochemical (IHC) marrow examinations for early DTCs in the BM of de novo patients diagnosed with non-stage IV 1^{ry} BC.

2. Material and Methods

This study included 80 newly diagnosed female patients with apparently non-metastatic 1ry BC staged [I-III] and received neither chemotherapy nor radiotherapy. These patients were selected from the surgical out-patient clinics, in National Cancer Institute – Cairo University, between October 2008 and July 2011. All patients' medical records were reviewed for data concerned with clinical examinations, pathological reports and radiological findings that confirm their clinical staging. Patients were subjected to CBC, BMA, BMB and IHC staining of BM sections using a panel of monoclonal antibodies (McAbs) including CK with (AE1/AE3) clone, Mammaglobin and CA15-3.

Bone marrow sampling, preparation, routine staining and interpretation:

Bone marrow biopsy cores (≥ 2cm long) and aspirates were consequently obtained from each patient under local infiltration anesthesia. BM smears were stained with routine leishman stain. BMB cores were fixed, decalcified, processed, paraffin embedded, sectioned and H&E-stained according to the well known routine techniques **[17].** Their morphological interpretation was accomplished according to the following precise criteria **[18, 19]**:-

Morphologically positive BM:

The BM was considered morphologically positive based on: **(1)** BMA smears showed large pleomorphic neoplastic cells with hyperchromatic coarsely reticular nuclear chromatin and moderately abundant variably basophilic cytoplasm with or without vaculation. These cells were arranged in tight small clusters either in syncytial formation or in cell columns pattern named "Indian Files". Solitary individually dispersed cells were also taken in consideration. **(2)** BMB sections showed these neoplastic cells occurred in randomly scattered small aggregates [2-4 cells] forming "micro-metastases" and associated with stromal reactions like [a-fibrous reaction among the involved areas b-interstitial increase in marrow eosinophils, histiocytes, plasma cells and/or fibroblasts c- active angiogenesis and/or d- increased osteoblastic/ osteoclastic activities with occurrence of trabecular bone erosion and/or widening].

Morphologically suspicious BM:

The BM was considered morphologically suspicious based on: **(1)** Absence of frank non-haemopoietic cells in BM smears and rare or occasional presence of their single or clustered "bare" nuclei associated with increased marrow osteoblasts and/or osteoclasts, **(2)** Absence of frank micro-metastatic colonies in BM sections and presence of one or more of the above-mentioned marrow stromal reactions and **(3)** The morphologic expectation of hidden metastatic cells that might be entangled among a prominent fibrotic reaction.

Morphologically negative BM:

The BM was considered morphologically negative based on: **(1)** Morphological absence of frank or suspicious individual cells, micro-metastatic colonies or sheets in all examined BM smears and sections **(2)** Absence of any suspicious marrow stromal reactions in marrow sections.

Immunohistochemical staining and interpretation for BM sections:

The BM sections were subjected to IHC staining using (DAKO Envision™ + System, peroxidase (HRP)/DAB, Mouse, K4006) as universal visualization system. BM sections were first de-paraffinized, in 2 changes of xylene, and re-hydrated in descending ethyl alcohol concentrations till distilled water. The concentrated primary antibodies [Mammaglobin, DAKO, code M3625, Cytokeratin (AE1/AE3), BioGenex, code MU071-UC and CA15.3, BioGenex, code MU323-UC] were diluted by antibody diluent in ratios of 1:100, 1:50-100 and 1:15-30, respectively. Sections were then pretreated by heat-induced epitope retrieval (DAKO 10x citrate buffer solution, Ph 6.1, S1699) for 15 minutes in microwave. After blocking the endogenous peroxidase activity by incubating the sections 10 min in a blocking solution, the diluted primary Abs were applied for 1 h and after washing in 3 changes of phosphate buffer saline (PBS) the HRP-labeled Polymer was applied for 30 min. After washing, the staining was completed by applying freshly prepared DAB + substrate-chromogen solution (20 µl DAB+Chromogen to 1 ml buffered substrate) to the sections and leaving them in dark for 8 minutes to produce a brown-colored precipitate at the antigen-sites. After washing, the sections were counter stained by light green stain 5% for 20 minutes, washed by distilled water, dehydrated in ascending grades of ethyl alcohol, cleared by xylene and finally mounted by DPX to be ready for microscopic examination. Unless test sections showed specific surface and/or cytoplasmic brown coloration in non-haemopoietic cells as positive control sections, they were considered negative. Also the positive staining intensity in test sections was assessed within the context of any non-specific background staining appeared in the negative control sections.

Individual interpretation of uni-IHC-staining [using a single McAb]:

To avoid false positive staining and to increase specificity of each individual McAb, its interpretation was accomplished in view of the standardized objective morphological criteria established by **Borgen *et al.* [20]** for the evaluation of immuno-stained DTCs in the BM. These criteria include: the occurrence of DTCs in clusters (e.g. in doubles, triples or more) and/or the presence of large-sized cells showing high N/C ratio, strong cytoplasmic staining with or without

large nucleoli. Sections showed cells with these criteria were considered positive. Sections showed absence of any immuno-stained cells or showed immuno-stained cells but without these criteria were considered negative.

Total interpretation of multi-IHC-staining [using a panel of McAbs]:

The total interpretation of multi-IHC staining (using 3 McAbs) was obtained depending on (1) the individual interpretation of each McAb as mentioned above (2) the detection rate of each McAb in the used panel and (3) the degrees of agreement between the used McAbs i.e. Firstly, out of the 3 used McAbs, the two with highest detection rates and substantial degree of agreement were selected for the total interpretation. Secondly, among these selected two McAbs, all cases that showed at least one McAb positive were totally interpreted as positive, while, all cases that showed the two selected McAbs negative were totally interpreted as negative.

Statistical Analysis

Data was analyzed using [SPSSwin] statistical package version 15.0.1 (Echo Soft Corporation, Chicago, IL). Quantitative (numerical) data, for non-parametric results, were expressed as median (50^{th} percentile) and interquartile range (IQR: 25^{th} – 75^{th} percentile). Qualitative data were expressed as frequency and percentage. Chi-square test was used to examine the relation between qualitative variables. Sign test was used to examine the relation between 2 related qualitative variables. Kappa (interrater reliability) was used to examine the agreement between two tests on the assignment of categories of a categorical variable. The probability of being by chance (p-value) was calculated for all parameters and was evaluated as follows: p-value ≥ 0.05 was considered non-significant and p-value < 0.05 was considered significant.

3. Results

This study included 80 females with de novo apparently non-metastatic 1ry BC; staged I to III and received neither chemotherapy nor radiotherapy. Their ages ranged from 27 to 77 years with a median of 53 years. Table (1) shows the relation between the detection rates of cytological versus histological marrow examinations in detecting early DTCs in these 80 patients. We noted that the histological detection rate (11.3%) was significantly higher than that of the cytological one (2.5%), (*p*-value = 0.04, Figs. 1, 2 and 3).

Cytokeratin (AE1/AE3), Mammaglobin and CA15-3 McAbs were used to highlight early DTCs in the IHC-stained marrow sections of the 80 patients included in this study. Individual interpretation of the uni-IHC marrow examinations revealed different detection rates of these McAbs. Cytokeratin (AE1&AE3) McAb showed the least detection rate (17/80, 21.3%), Mammaglobin showed a higher detection rate (21/80, 26.3%) and CA15-3 McAb showed the highest detection rate (28/80, 35%). Table (2) shows the relation between the detection rates of the histological marrow examination and that of each uni-IHC marrow examinations; using the above mentioned McAbs. The obtained results revealed that the detection rates of all uni-IHC marrow examinations, using CK (AE1/AE3), Mammaglobin and CA15-3 McAbs were significantly higher than that of the histological marrow examination, (*p*-values= 0.049, 0.035 and 0.02, respectively, Figs. 4, 5 and 6).

In table (3) the degrees of agreement between results of the marrow uni-IHC staining of the three McAbs were highlighted. The results of CA15-3 McAb showed a substantial agreement with that of Mammaglobin McAb (Kappa = 0.644) and a moderate agreement with that of Cytokeratin (AE1&AE3) McAb (Kappa = 0.515). Mean-while, the results of Mammaglobin McAb showed a weak agreement with that of Cytokeratin (AE1&AE3) McAb (Kappa = 0.275).

Our individual interpretation of the uni-IHC marrow examinations for CK (AE1/AE3), Mammaglobin and CA15-3 McAbs showed a considerable variation in their detection rates as well as their degrees of agreement; therefore a total interpretation of multi-IHC marrow examinations for these three McAbs was established based on recruiting only two of them (CA 15-3 and Mammaglobin McAbs) that showed the highest detection rates as well as a substantial degree of agreement. In this respect, all cases that showed positive staining of at least one of these two McAbs were multi-IHC totally interpreted as positive, while, cases that showed negative staining of these two McAbs were multi-IHC totally interpreted as negative. Accordingly, from the quantitative point of view, our total interpretation of the multi-IHC marrow examinations revealed a detection rate of (47.5%) which is higher than those of the individually interpreted uni-IHC marrow examinations (mentioned above) and, as shown in table (4), is significantly higher than that of the histological marrow examination (11.3%), *p*-value = 0.01. However, from the qualitative point of view, the relation between results of the histological interpretation of BM sections and that of the total interpretation of their multi-IHC staining for the 80 patients is studied and revealed, as shown in table (5), that all the 9 cases that histologically interpreted as positive were also multi-IHC totally interpreted as positive. Mean-while, among the 45 cases that were histologically interpreted as negative, 14 were multi-IHC totally interpreted as positive.

Also, among the 26 cases that were histologically interpreted as suspicious, only 15 were multi-IHC totally interpreted as positive and 11 cases were interpreted as negative. Thus, the results of both histological and multi-IHC total interpretations showed a highly significant statistical difference (*p*- value = 0.001).

Table (1): The relation between the detection rates of cytological and histological marrow examinations for detecting early DTCs in patients with de novo 1ry breast cancer

		BM examinations for 80 patients		*p*-value
		Cytological (BMA)	**Histological (BMB)**	
Detection rates	Positive marrow	2 (2.5%)	9 (11.3%)	**0.04**
	Negative marrow	73 (91.2%)	45 (56.2%)	
	Suspicious marrow	5 (6.3%)	26 (32.5%)	

BM= bone marrow, **DTCs=** disseminated tumor cells, **BMA=** BM aspiration, **BMB=** BM biopsy.

Table (2): The relation between the detection rates of the histological and each of the uni-IHC marrow examinations for detecting early DTCs in patients with de novo 1ry breast cancer; using CK, Mammaglobin and CA 15-3 McAbs.

		BM examinations for 80 patients		*p*-value
		Histological (BMB)	**Uni-IHC by CK (AE1/AE3) McAb**	
Detection rates	Positive marrow	9 (11.3%)	17 (21.3%)	**0.049**
	Negative marrow	45 (56.2%)	63 (78.7%)	
	Suspicious marrow	26 (32.5%)	-	

		Histological (BMB)	**Uni-IHC by Mammaglobin McAb**	
Detection rates	Positive marrow	9 (11.3%)	21 (26.3%)	**0.035**
	Negative marrow	45 (56.2%)	59 (73.7%)	
	Suspicious marrow	26 (32.5%)	-	

		Histological (BMB)	**Uni-IHC by CA 15-3McAb**	
Detection rates	Positive marrow	9 (11.3%)	28 (35%)	**0.02**
	Negative marrow	45 (56.2%)	52 (65%)	
	Suspicious marrow	26 (32.5%)	-	

IHC= immunohistochemical, **BM=** bone marrow, **DTCs=** disseminated tumor cells, **BMA=** BM aspiration, **BMB=** BM biopsy, **CK**= Cytokeratin, **McAb**= monoclonal antibody.

Table (3): Degrees of agreement between different McAbs used in the IHC-staining for detecting early DTCs in BM of patients with de novo 1ry breast cancer **[a]** agreement between CA15-3 and Mammaglobin McAbs, **[b]** agreement between CA15-3 and CK (AE1/AE3) McAbs and **[c]** agreement between Mammaglobin and CK (AE1/AE3) McAbs.

	Patients number=80		**CA15-3 McAb**		**Kappa**
			28 + ve	**52 - ve**	
[a]	**Mammaglobin McAb**	**21 +ve**	20	1	**0.644**
		59 - ve	8	51	

	Patients number=80		**CA15-3 McAb**		**Kappa**
			28 + ve	**52 - ve**	
[b]	**CK (AE1/AE3) McAb**	**17 +ve**	15	2	**0.515**
		63 - ve	13	50	

	Patients number=80		**Mammaglobin McAb**		**Kappa**
			21 +ve	**59 - ve**	
[c]	**CK (AE1/AE3) McAb**	**17 +ve**	5	12	**0.275**
		63 - ve	16	47	

McAbs= monoclonal antibodies, **IHC=** immunohistochemical, **DTCs=** disseminated tumor cells, **BM=** bone marrow, **CA15-3=** cancer antigen 15-3, **CK=** cytokeratin

Table (4): The relation between the detection rates of histological and multi-IHC marrow examinations for detecting early DTCs in patients with de novo 1ry breast cancer

		BM examinations for 80 patients		*p*-value
		Histological (BMB)	Multi-IHC staining	
Detection rates	Positive marrow	9 (11.3%)	38 (47.5%)	**0.01**
	Negative marrow	45 (56.2%)	42 (52.5%)	
	Suspicious marrow	26 (32.5%)	-	

BM= bone marrow, **BMB=** BM biopsy, **IHC=** Immunohistochemical.

Table (5): The relation between the outcome of histological interpretation of BMB sections and that of the total interpretation of their multi-IHC staining for patients with de novo 1ry breast cancer

Patients number=80		Histological interpretation of BMB sections			*p*-value
		Positive BM (n=9)	Negative BM (n=45)	Suspicious BM (n=26)	
Total interpretation of multi-IHC staining	Positive BM (n=38)	9	14	15	**0.001**
	Negative BM (n=42)	0	31	11	

BM= bone marrow, **BMB=** BM biopsy, **IHC=** Immunohistochemical.

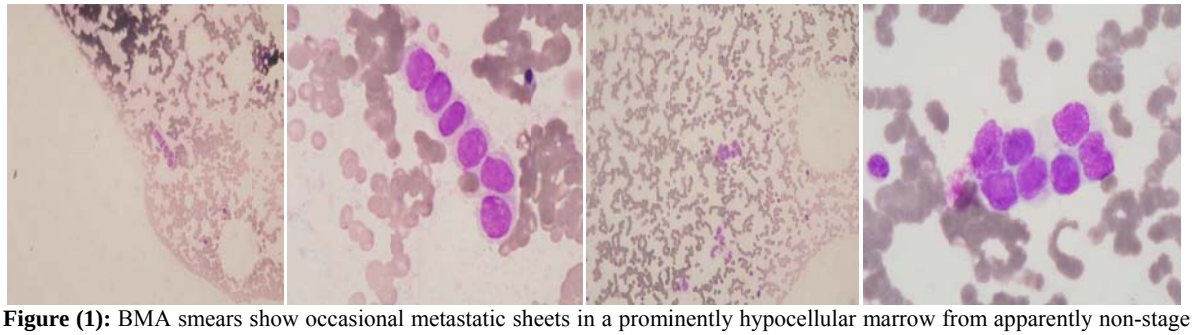

Figure (1): BMA smears show occasional metastatic sheets in a prominently hypocellular marrow from apparently non-stage IV 1^{ry} breast cancer patients, Leishman's stain, 20x and 100x.

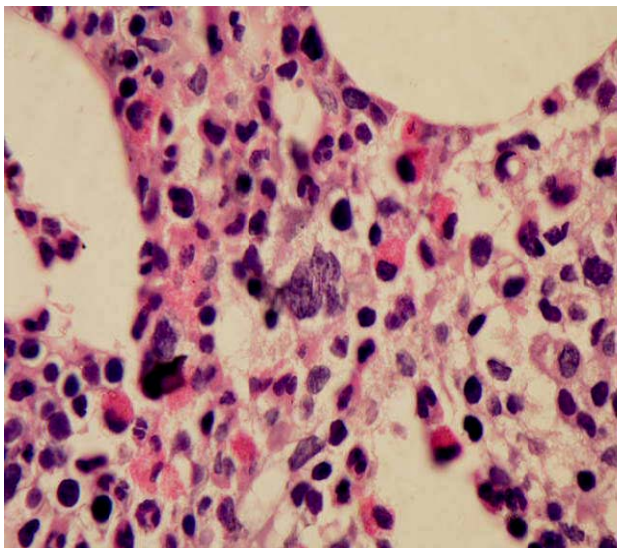

Figure (2): BMB section, from apparently non-stage IV 1ry cancer breast patient, shows a collection of 2 to 3 disseminated tumor cells (in center); associated with reactive interstitial increase in marrow eosinophils, H&E, 100x.

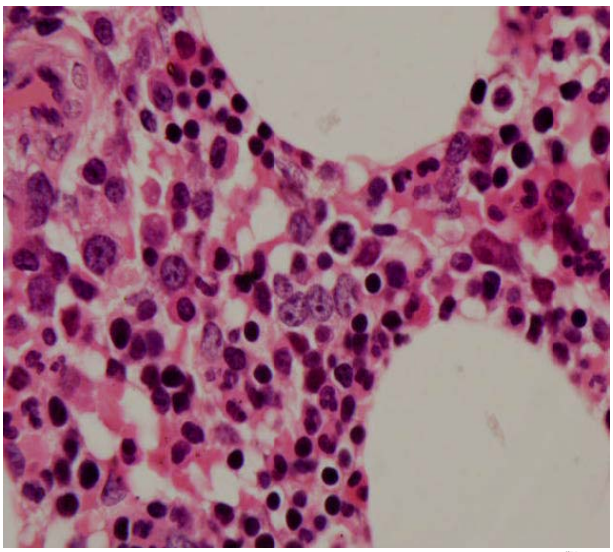

Figure (3): BMB section, from apparently non-stage IV 1^{ry} breast cancer patient, shows 3 to 4 morphologically suspicious (malignant-looking) cells (in center) arranged in an Indian file pattern, H&E, 100x.

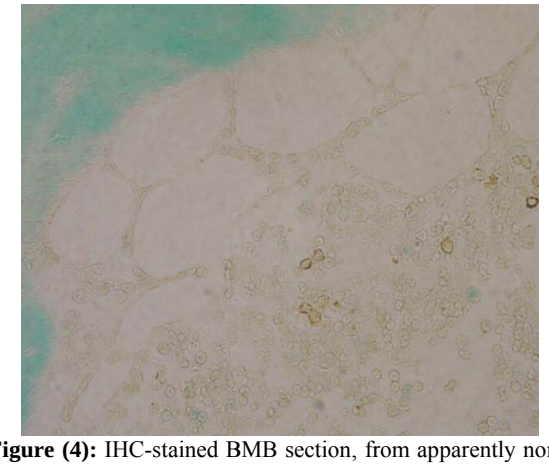

Figure (4): IHC-stained BMB section, from apparently non-stage IV 1ry breast cancer patient, shows few DTCs positive for Cytokeratin (AE1/AE3) McAb and arranged in singles, doubles and triples, 40x

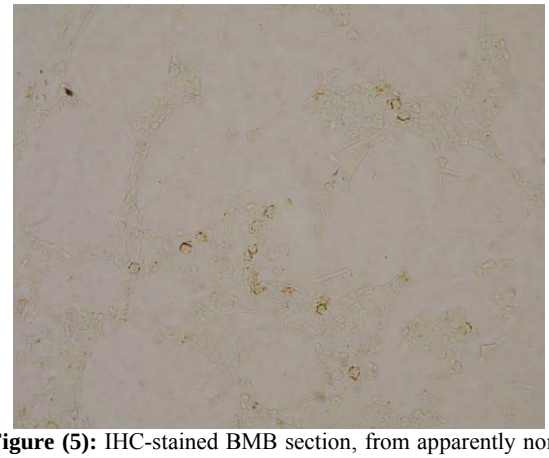

Figure (5): IHC-stained BMB section, from apparently non-stage IV 1ry breast cancer patient, shows some DTCs positive for Mammaglobin McAb and arranged in singles, and doubles, 40x

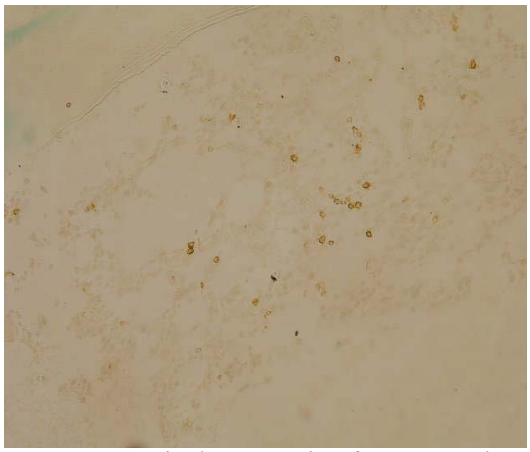

Figure (6): IHC-stained BMB section, from apparently non-stage IV 1ry breast cancer patient, shows multiple scattered DTCs positive for CA 15-3 McAb and arranged either individually, in doubles or in a collection showed an Indian file pattern, 20x

4. Discussion

In the haematopathology practice the cytological, histological and immuno-histochemical marrow examinations are known to be frequently used methodologies to detect metastatic lesions in BM of BC patients. In this respect, our study aimed firstly to evaluate the detection capacities of these different marrow examinations in detecting early DTCs in de novo cases of 1ry BC patients that clinically appear healthy and considered non-stage IV. Secondly, in view of the priority of cost/benefit relationship in our low socioeconomic communities, we tried to verify the impact of the step-wise combining of cytological, histological and immuno-histochemical marrow examinations on their detection capacities for early DTCs in the BM and eventually whether an individual interpretation of a uni-IHC staining will be satisfactory and reliable or a total interpretation of a multi-IHC staining should be performed.

In our results, the cytological BM examinations for early DTCs showed the weakest detection capacity with the lowest detection rate (2.5%). When the histological BM examinations are combined to the cytological one, the detection capacity became stronger and the detection rate was significantly increased to (11.3%), *p*-value =0.04. However, because of our histological interpretation of BM sections was restricted to precise morphological criteria, 32.5% of the histologically examined cases were still morphologically considered as suspicious and 56.2% were considered as negative for DTCs. Therefore, further BM examinations for early DTCs were required by an immunohistochemical staining.

As cancer breast is an epithelial cell tumor and the BM is normally free of any epithelial cells, the detection of DTCs in BM by immuno-staining procedures was based on using different McAbs raised against epithelial markers on the breast carcinoma cells **[11, 16]**. The specificity of some antibodies, used to characterize epithelial cells, remains controversial as they have been shown to cross-react with some hematopoietic cells **[21]**. In this study the IHC staining were performed using three properly selected McAbs; including CK [AE1/AE3], Mammaglobin and CA15-3. Our morphological interpretation to their immunohistochemical staining was preliminary accomplished on individual basis i.e. we performed an individual interpretation of the uni-IHC marrow examination for each McAb alone. These individual interpretations were done in view of the standardized objective morphological criteria established by Borgen et al. for the evaluation of immuno-stained DTCs **[20]**.

Cytokeratins are proteins that constitute a part of the cytoskeleton of epithelial cells, hence are regularly expressed by these epithelial cells and their malignant descendents **[22]**. They belong to a large multi-gene family thus are expressed at various levels and

compositions in all epithelial tumors **[23]**. We used CK McAb with the clone [AE1/AE3] that reacts with basic and acidic keratins covering a large spectrum of cytokeratins (e.g. CK1-8, CK10, CK14-16 and CK19). Among our 80 cases, its uni-IHC marrow examination for early DTCs showed a detection rate of 21.3% which is the lowest rate among the 3 selected McAbs. However, this detection rate is still significantly higher than that of the histological BM examinations (11.3%), *p-value*= 0.049. In comparison to previous studies, **Landys *et al.* [24]** used CK McAb with the same AE1/AE3 clone in uni-IHC BM examinations for 128 BC patients. They obtained a 19% detection rate which is a bit close to ours. **Salvadori *et al.* [25]** also performed uni-IHC marrow examinations on biopsies from 121 BC patients by using MBr1 McAb. They reported a detection rate of 17% which is obviously lower than ours. This is may be due to their use of a different McAb. Also their larger number of cases may partially explain their lower detection rate.

Up to our knowledge, we considered Mammaglobin and CA15-3 McAbs as novel markers; being used as McAbs in IHC marrow examinations for detecting early DTCs in patients with non-stage IV 1ry breast cancer. Mammaglobin is a member of the Uteroglobin/Clara cell protein [secretoglobin] superfamily **[26]**. It has been discussed as a promising diagnostic marker in breast cancer for almost ten years **[27 - 29]**.It is almost exclusively expressed in breast epithelial cells and is also over expressed in 61–93% of 1^{ry} and metastatic breast cancer tissues **[30, 31]**. Furthermore, **Ferrucci *et al.* [32]** included Mammaglobin among a new comprehensive gene expression panel to study tumor micro-metastases in patients with high-risk breast cancer. **Li *et al.* [33]** specified the detection of Mammaglobin m-RNA, by reverse transcriptase PCR, and considered it a superior biomarker for circulating tumor cells in BC patients. We could not find any previous studies that used Mammaglobin as McAb in immunohistochemical detection of early DTCs in the BM. Our individual interpretation of uni-IHC marrow examinations, for early DTCs, using Mammaglobin McAb revealed a detection rate of 26.3% which is higher than that of CK [AE1/AE3]McAb and is significantly higher than that of the histological BM examinations (11.3%), *p-value*= 0.035. Recently, **Liu *et al.* [34]** studied the expression of human Mammaglobin m-RNA, in the BM of 102 patients with stage I-III breast cancer, by RT-PCR. They reported a positive expression rate of 38.2% which is much higher than ours. This may be attributed to the higher detection capacity (sensitivity) of RT-PCR technique for detecting Mammaglobin m-RNA than that of uni-IHC staining using single anti-Mammaglobin McAb.

CA15-3 is a common well-known breast tumor marker. It is considered one of the markers that showed evidence of clinical utility and was recommended for use in practice **[12]**. **Velaiutham *et al.* [35]** reported that CA15-3 has an independent prognostic impact in both uni- and multi-variate analysis. In our study, CA15-3 is the third McAb selected in our immunohistochemical staining. Its clone [BGX323A] is considered very specific to react with CA15-3 antigen in mammary cancer cells and, as stated by its manufacturer, it has no cross reactivity with human CEA or CA125 and has no binding with non-specific tissues or cells. We could not find any previous studies that used CA15-3 as McAb in an immunohistochemical detection of early DTCs in the BM. In our individual interpretation of uni-IHC marrow examinations for early DTCs, CA15-3 McAb showed a detection rate of 35% which is higher than that of CK [AE1/AE3] and Mammaglobin McAbs (21.3% and 26.3%, respectively) and is significantly higher than that of the histological BM examinations (11.3%), *p-value*=0.02. Thus, our individual interpretation of the uni-IHC marrow examinations, using the above mentioned 3 McAbs, revealed a considerable variation in their detection capacities for early DTCs in the BM. This variation could be attributed to the heterogeneity of expression of these markers in breast carcinoma cells. Also, the results obtained from the uni-IHC marrow examinations, using the same 3 McAbs showed variable degrees of agreement between each other (Table 3). Therefore, a total interpretation of multi-IHC marrow examinations for these 3 McAbs was established on the basis of recruiting only two of them (CA15-3 and Mammaglobin) that showed the highest detection rates as well as a substantial degree of agreement. Accordingly, from the quantitative point of view, our total interpretation of the multi-IHC marrow examinations revealed an obvious increase in the detection rate (47.5%) which is higher than those of the individually interpreted uni-IHC marrow examinations (Table 2) and, also, is significantly higher than that of the histological BM examinations (11.3%), *p-value* = 0.01 (Table 4). On the contrary of our results, **Mathieu *et al.* [36]** performed first multi-IHC staining on 12 histologically positive BM biopsies; using a panel of McAbs including anti-CK with different clones (KL1, AE1/AE3 and CAM-5) and anti-EMA. Secondly, they selected out of these McAbs the most sensitive one to be used in the IHC detection of any occult metastases among series of 93 BM biopsies negative by conventional histological examinations. They found only one case stained positive with CK (KL1) demonstrating isolated tumor cells. Therefore, they stated that "Single BM biopsy techniques whether stained by conventional or IHC methods do not appear to be useful tests to detect occult BM metastases, especially at initial diagnosis of clinically M0 breast carcinoma patients".

Also, in comparison to our study, **Vannucchi *et al.* [37]** evaluated the presence of BM micrometastases in bilateral BM biopsies obtained at diagnosis of 33 patients with stage II/IIIA breast cancer using RT-PCR assay for CK19 m-RNA, histology and multi-IHC marrow examinations with a panel of three McAbs. They detected CK19 transcripts in one or both BM samples in 48% of patients, with an overall 85% concordance with the results of their multi-IHC analysis. On the other hand, 56% of PCR- and IHC-positive BM samples were diagnosed as 'normal' on histological analysis. Previously, **Lyda *et al.* [38]** used a combination of different CK clones; including AE1/AE3, CAM5-2 and 35ßH11, in performing multi-IHC marrow examinations for 65 BM biopsies from 54 patients with lobular breast carcinoma. They obtained a detection rate (30.8%) higher than that of their routine histological marrow examinations. The detection rate of their multi-IHC marrow examinations is lower than ours (47.5%) because in our multi-IHC marrow examinations we used 3 totally different McAbs (CK, Mammaglobin and CA15-3) rather than 3 different clones of the same McAb.

From the quantitative point of view, our multi-IHC total interpretation revealed a detection rate (47.5%) significantly higher than that of our histological interpretation (11.3%), *p-value* = 0.01; also, from the qualitative point of view, our results of both histological and multi-IHC total interpretations showed a highly significant statistical difference, *p*-value = 0.001. Therefore both interpretations are complementary to each other and neither of them can substitute the other.

In this study, we confirmed the popular finding that ensured the superiority of the histological detection capacity over the cytological one for DTCs in the BM. We found that the combined histological/uni-IHC interpretations significantly increase the detection capacity but to a different rates according to the individually used McAb and with variable degrees of agreement between the used McAbs. Eventually, we concluded that for optimal increase in the detection capacity for early DTCs in the BM of de novo patients diagnosed clinically with non-stage IV 1ry breast cancer, a total interpretation for combined histological/multi-IHC marrow examinations must be performed.

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