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RESEARCH ARTICLE

ROLE OF CIRCULATING ENDOTHELIAL PROGENITOR CELLS IN PATIENTS WITH BREAST CANCER

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ABSTRACT

Objectives: To detect the relation between the circulating endothelial progenitor cells (CEPCs) and stages of breast cancer patients.

Background: Breast cancer is a major cause of death. Immunocytochemistry and RT-PCR have been widely used for the detection of circulating endothelial progenitor cells (CEPCs) in patients with breast cancer but their specificity is limited. Our purpose was to utilize a convenient and specific technology to detect CEPCs in breast cancer patients. Given a potential, predictive and therapeutic value of EPCs in breast cancer, level of CEPCs in breast cancer would correlate with extent of the disease.

Material and Methods: This study included 75 newly diagnosed patients with breast cancer females, their ages ranged between 19 and 65 years old. Twenty age and sex matched healthy females were included as controls. All patients were subjected to: full history taking, clinical examination, radiological investigations, histopathological examination of the breast mass for surgically removed breast cancer tissue: Estrogen Receptor (ER), Progesterone Receptor (PR), human epidermal growth factor receptor 2 (HER2), routine laboratory investigations including liver and renal function tests, complete blood picture, alkaline Phosphatase, prothrombin time (PT) and routine breast tumor markers Carcinoembryonic antigen (CEA), Cancer Antigen 15-3 (CA15.3), and flow cytometry detection of CD14, CD133, and VEGFR2 (vascular endothelial growth factor receptor 2) positive CEPCs in the peripheral blood.

Results: In this study, there was statistically significant increase CEPCs numbers in breast cancer patients when compared with control (2.05 ± 0.6x103 CEPCs/5x106 PBMC). The level of CEPCs was significantly correlated with the stage of breast cancer. The least significant was stage I < II < III < IV (7.71 ± 1.40 <8.67 ± 1.65 <30.01 ± 9.74 <76.19 ± 13.25 x 103 CEPCs/5x106 PBMC) Also, there was highly statistically significant increase of CEPCs mean fluorescence intensity (Mx, My) with disease progression respectively (31.18 ± 3.38<37.80 ± 8.02<65.78 ± 9.51<85.50 ± 9.15) (54.24 ± 8.81<65.0 ± 11.44<101.28 ± 19.83<190.40 ± 45.92) as compared with controls (27.70 ± 5.54) (42.20 ± 8.61).

Conclusion: CEPC elevation in the blood of breast cancer patients established as a useful marker of tumor angiogenesis and progression. From existing research data, it is apparent that CEPCs monitoring is efficient, specific and reproducible as early predictors of metastatic potential in breast cancer patients with sensitivity 100 and specificity 100% at 52850 /5x106 PBMC as a cutoff point.

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INTRODUCTION

Breast cancer (BC) is the leading cause of cancer deaths among women. The prognosis of BC treatment results is a serious problem since a variety of clinical, morphological, molecular, biological and molecular genetic factors influences the behavior of tumor cells and their response to the treatment (Senchukovo *et al*., 2015). Breast cancer remains the most commonly diagnosed cancer among women and the second leading cause of cancer mortality in the United States. Despite major advances in adjuvant therapy for early stage breast cancer, patients still have a 20–50% chance of relapse over10 years. Metastasis, the final step of cancer progression, is responsible for most cancer-related deaths and may occur after an extraordinarily long period of time after initial diagnosis and treatment (Jain *et al*., 2012). In the past few years, a number of studies have shown that adult stem and progenitor cells play a role in tumor progression. Deregulation in the self-renewal programs of adult stem cells leads to cell transformation, contributing to the formation and development of new tumors. Although angiogenesis (the formation of new blood vessels from preexisting vasculature) plays a beneficial role in many physiological processes, such as wound healing it also contributes to the growth and metastasis of tumors. It is showed that bone marrow derived endothelial progenitor cells (EPCs) not only have therapeutic application but also are

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involved in the pathological neo-vascularization of tumors (Marcola and Rodrigues, 2015). Peripheral blood progenitor cells (PBPC) are increasingly used as an alternative to autologous bone marrow (BM) rescue for patients with solid tumors as well as hematological malignancies. Endothelial progenitor cells can be detected and quantified in peripheral blood samples. Given the potential predictive and therapeutic value of EPCs in breast cancer, Brugger and colleagues hypothesized that the level of circulating EPCs in breast cancer patients would correlate to extent of the disease (Brugger *et al*., 2016).

Aim

To measure CEPCs, correlate CEPCs with stages of breast cancer patients and evaluate CEPCs as disease progression marker.

Patients and Method

This study included 75 newly diagnosed patients with breast cancer females, their ages ranged between 19 and 65 years old. Twenty age and sex matched healthy females were included as controls. The present study was carried out in the duration between April 2016 and July 2017. The patients were selected from the outpatient clinics of the Surgical Oncology, Menoufia University Hospital. This study was done at clinical pathology department, faculty of medicine, Menoufia. The study was approved by the ethics committee of our medical faculty and written informed consents were obtained from all subjects before study entry.

Patients were divided into four groups

Group I: includes 17 female patients with stage I breast cancer.
Group II: includes 20 female patients with stage II breast cancer.
Group III: includes 18 female patients with stage III breast cancer.
Group IV: includes 20 female patients with stage IV breast cancer.

Exclusion criteria

Patient excluded from this study were those: patients with other diseases on breast, patient on chemotherapy treatment and there was metastatic secondaries to the breast.

MATERIALS AND METHODS

All patients and controls were subjected to the following:

Demographic data recording, Medical history, Clinical General Examination: Chest, heart and abdomen examination. Local examination: the two breasts were examined. Radiological investigations: including mammography with complementary ultrasound, chest X-ray, abdominal ultrasound, bone scan and MRI.

Histopathological examination of the breast mass: immunohistological examination of ER, PR and HER2

Laboratory Investigations: including liver and renal function tests, complete blood picture and routine breast tumor markers, CEA, CA153, Alkaline Phosphatase and (PT) and Flow cytometric detection of CD14, CD133, and VEGFR2 positive CEPCs in the Peripheral blood.

Sampling:

A venous blood sample (15 ml) was withdrawn from each individual under aseptic condition by a clean venipuncture and then dispensed into four tubes:

1-10 ml of blood was delivered into EDTA containing tube for flow cytometry.

2-5ml of blood for other tests:

a. 1.8 ml of blood was delivered into a tube containing (0.2 ml trisodium citrate), in which plasma was separated by centrifugation at 4000 rpm for 10 minutes, top yellow plasma layer was withdrawn for PT.
b. 3.2 ml of blood was delivered into a plan tube for other tests.

1-Complete blood count (CBC):

CBC was measured by Sysmex1 XN-1000 Automated Hematology Analyzer (Sysmex Corporation, Japan)

2-Blood chemistry & Serology:

Liver and kidney function tests were done on auto analyzer AU 480 from (Beckman coulter, AU chemical analyzer, USA):

- Total and direct bilirubin were done by quantitative colorimetric diazo method.
- Alanine amino transferase (ALT) was done by quantitative kinetic method.
- Aspartate amino transferase (AST) was done by quantitative kinetic method.
- Alkaline phosphatase (ALP): was done by quantitative kinetic method
- Albumin was done by quantitative modified bromocresol green colorimetric method.
- Prothrombin time was measured by automated method using Stago-STA Compact Max Analyzer (Diagnostic Stago, France).
- Estimation of serum creatinine was done by quantitative buffered kinetic Jaffe reaction.

Quantification of CD14, CD 133 and VEGFR2 dual expression on peripheral mononuclear cells (Hristov *et al*., 2009). Reagents: Monoclonal antibodies used are antibody label CD14 FITC (fluorescein isothiocyanate), CD133 PE (phycoerythrin), VEGFR2 PerCP (chloropyll protein-conjugated), Phosphate Buffered Saline (PBS) and Ficoll Hi paque (1077 g/ml). Washed cells were incubated with labeled monoclonal antibody which binds to cells expressing the antibody of interest, unbound antibody was then washed from the cells, and the cells expressing monoclone were fluorescently stained with the intensity of staining directly proportional to the density of expression of the monoclone. Peripheral blood mononuclear cells (PBMCs) were isolated from 10ml of whole blood samples, which had been collected in ethylene di amine tetra-acetic acid coated sterile tubes. Blood was mixed with an equal volume of phosphate buffered saline (PBS, pH, 7.4), layered on Ficoll reagent (1077 g/ml)

and centrifuged at 1800 g for 20 min. The buffy coat containing mononuclear cells was recovered, mixed with 50 ml PBS and centrifuged at 3000 g for 10 min then the supernatant was discarded and the cell pellet was suspended in 1 ml PBS adjusting the count of PBMCs to be $1x10^6$ /ml. For staining, for each sample two tubes were prepared, one for the stained sample and the other for unstained control.100µl cells suspension in PBS was incubated with 10µl fluorescein isothiocyanate (FITC)-conjugated anti-CD14 antibodies [Clone JS-14, anti-human immunoglobulin G (IgG), BD PharMingen, and San Diego, CA], PBMC were co stained with R-phycoerythrin (PE) 10µl conjugated anti-CD133 (Clone RPA-T4, anti-human IgG, BD PharMingen) and also co stained with10µl (PerCP) peridinin chloropyll protein-conjugated anti-VEGFR2 (Clone HIT8a, mouse anti-human IgG, BD PharMingen). The frequency of circulating EPCs was determined by measurement of cells exhibiting CD133 and VEGFR2 co-expression after gating for CD14positive cells. The results were reported as number of EPCs per 5 million ($5x10^6$) MNCs. Incubate for 30 min at 4° C and subsequently wash twice with PBS. The cells were suspended in 300 µl of PBS for final flowcytometric analysis. As a control for analysis, unstained 100 µl of cells and completed to300 µl by PBS in a separate tube are run as auto-control. All samples were analyzed using (FACS Calibur, BD immune cytometry system, San Jose, CA, USA) flow cytometer. All time-points of monocyte preparations relating to one patient were stained and quantified on the same day. A control for analysis, 100 µl of cells in a separate tube were run as auto-control without any stain. Data were acquired on a FACS caliber flow cytometer (BD immune cytometry systems, San Jose, CA). The instrument set up was checked weekly using QC windows beads (flow cytometry standard, San Juan, PR). A representative flow cytometry gating strategy for the identification of EPCs is shown in Figure 1. EPCs were identified as CD14/CD133þ/VEGFR2 cells.

Statistical analysis

Data were fed to the computer and analyzed using IBM SPSS software package version 20.0. (Armonk, NY: IBM Corp) Qualitative data were described using number and percent. The Kolmogorov-Smirnov test was used to verify the normality of distribution. Quantitative data were described using range (minimum and maximum), mean, standard deviation and median. Significance of the obtained results was judged at the 5% level. P value non-significant difference if $P > 0.05$, significant difference if $P < 0.05$ and highly significant difference if $P < 0.001$.

RESULTS

In this study, there was statistically significant increase CEPCs numbers in breast cancer patients when compared with control ($2.05 \pm 0.6x10^3$ CEPCs/$5x10^6$ PBMC). The level of CEPCs was significantly correlated with the stage of breast cancer. The least significant was stage I < II < III < IV (7.71 ± 1.40 <8.67 ± 1.65 <30.01 ± 9.74 <$76.19 \pm 13.256x10^3$ CEPCs/$5x10^6$ PBMC) Also, there was highly statistically significant increase of CEPCs mean fluorescence intensity (Mx, My) with disease progression respectively (31.18 ± 3.38<37.80 ± 8.02<65.78 ± 9.51<85.50 ± 9.15) (54.24 ± 8.81<65.0 ± 11.44<101.28 ± 19.83<190.40 ± 45.92) as compared with controls (27.70 ± 5.54) (42.20 ± 8.61)as shown in Table (1) and Figure 1. In this study, there was statistically significant increase between control and cases and statistically significant increase with disease stage progression as regard metastasis (size and site). Metastasis only found in stage IV (100%), according to the size of metastasis in stage IV, 50% was ≤1.5 cm and 50% was ≥1.5 cm, 80% of them were bone metastasis, 10% were liver and10% were brain.

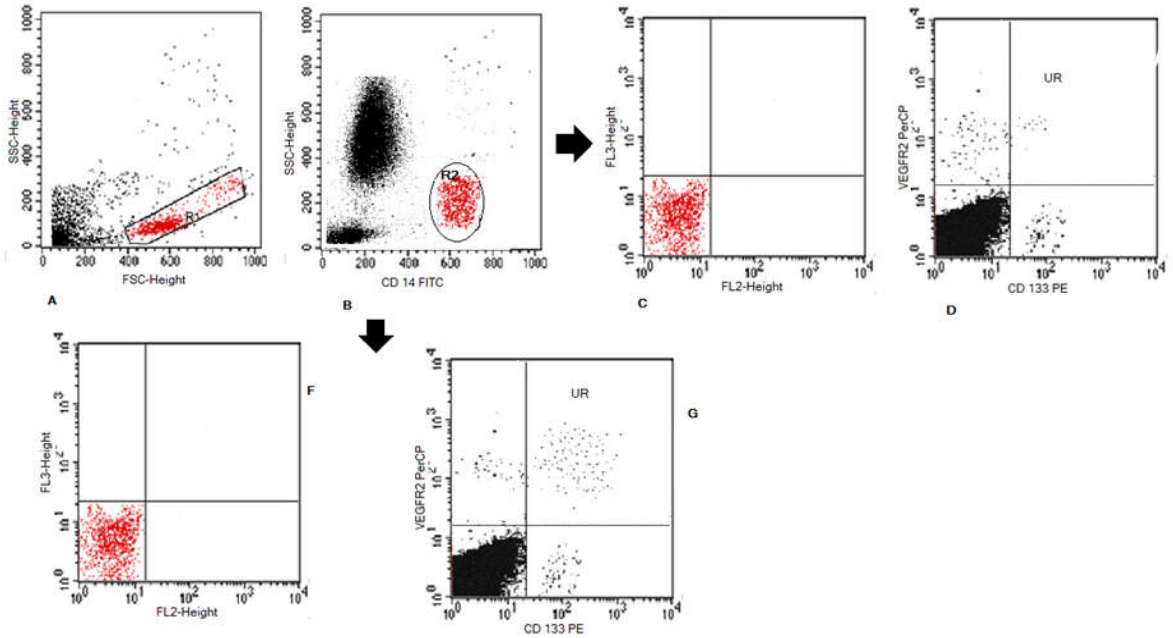


Figure 1. Representative flow cytometric plots indicating identification of circulating endothelial progenitor cells (EPCs). EPCs were defined as CD14 þ / CD133þ/vascular endothelial growth factor receptor 2 (VEGFR2) þ cells.: (A) forward (FSC) and side scatter (SSC) plot of cells and gating region R1 to include all mononuclear cells ; (B) CD14 þ gating to exclude lymphocytes, CD 14 against side scattering; (C) Auto unstained control sample cells; (D) stained control sample the UR region represent CD 14 þ / CD 133 þ / VEGFR2þ circulating endothelial progenitor cells;(E) Auto unstained cancer breast Patient sample;(F) stained cancer breast Patient sample the UR region represent CD 14 þ / CD 133 þ / VEGFR2þ circulating endothelial progenitor cells. Finally, comparing UR region between fig D & fig F confirming increased circulating endothelial progenitor cells in breast cancer patients to controls

Table 1. Comparison between the control and breast cancer patients as regard flowcytometry count and Mean Fluorescence Intensity (MFI)

Flowcytometry CD/14+CD/133+VEGFR2	Breast cancer stage				F	P
	I (n = 17)	II (n = 20)	III (n = 18)	IV (n = 20)		
Count of flow cytometer (x103)						
Range	5.50 – 10.20	5.90 – 11.60	14.20 – 47.0	58.70 – 101.40	329.582*	<0.001*
Mean ± SD	7.71 ± 1.40	8.67 ± 1.65	30.01 ± 9.74	76.19 ± 13.25		
Median	7.30	8.80	29.70	74.75		
Pcontrol	0.157	0.048*	<0.001*	<0.001*		
Sig. bet. grps.	p_1=0.995,p_2<0.001*,p_3<0.001*,p_4<0.001*,p_5<0.001*p_6<0.001*					
Mx						
Range	25.0 – 37.0	25.0 – 51.0	50.0 – 80.0	74.0 – 111.0	220.387	<0.001*
Mean ± SD	31.18 ± 3.38	37.80 ± 8.02	65.78 ± 9.51	85.50 ± 9.15		
Median	31.0	36.50	66.50	86.50		
Pcontrol	0.60	0.001*	<0.001*	<0.001*		
Sig. bet. grps	p_1=0.068,p_2<0.001*,p_3<0.001*,p_4<0.001*,p_5<0.001*p_6<0.001*					
My						
Range	45.0 – 77.0	45.0 – 82.0	72.0 – 135.0	132.0 – 320.0	122.949	<0.001*
Mean ± SD	54.24 ± 8.81	65.0 ± 11.44	101.28 ± 19.83	190.40 ± 45.92		
Median	50.0	66.0	94.50	180.0		
pcontrol	0.553	0.028*	<0.001*	<0.001*		
Sig. bet. grps	p_1=0.655,p_2<0.001*,p_3<0.001*,p_4<0.001*,p_5<0.001*p_6<0.001*					

CD =Cluster Of Differentiation
Mx= Mean Fluorescence Intensity

Table 2. Correlation between Count of flow cytometer (x103) and tumor markers: carcino embryonic antigen (CEA) and cancer antigen 15-3 (CA15.3) and Alkaline hosphatase

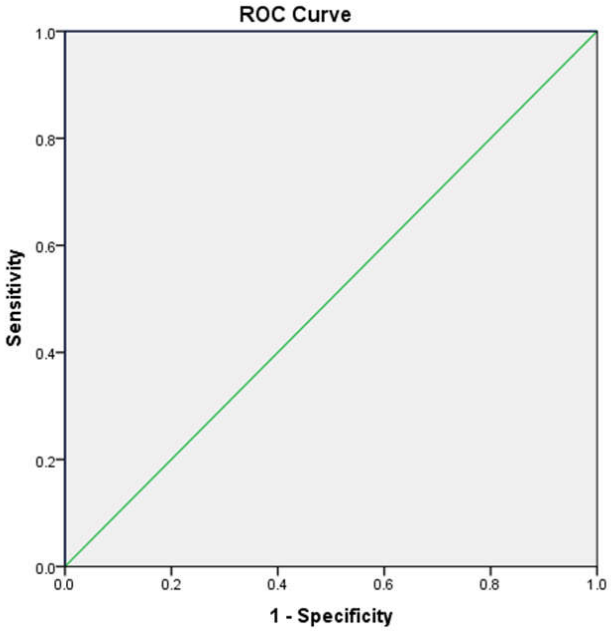
		Count of flow cytometer ($x10^3$)						
		Control	Breast cancer stage					Total Sample
			I	II	III	IV	Total cases	
CEA (mcg/l)	r	-0.21*	-0.297	-0.417	0.511*	0.048	0.910*	0.910*
	p	0.929	0.248	0.067	0.030*	0.841	<0.001*	<0.001*
CA15.3 (u/ml)	r	0.072	0.034	0.434	0.541*	-0.182	0.852*	0.859*
	p	0.764	0.897	0.056	0.020*	0.443	<0.001*	<0.001*
Alkaline phosphatase (Iu/L)	r	0.269	-0.096	0.085	0.018	-0.010	0.773*	0.801*
	p	0.252	0.714	0.723	0.943	0.966	<0.001*	<0.001*

r: Pearson coefficient
*: Statistically significant at $p \leq 0.05$

Table 3. Comparison between the control and breast cancer patients as regard tumor markers: carcino embryonic antigen (CEA) and cancer antigen 15-3(CA15.3)

	Control (n = 20)	Breast cancer stage				F	P
		I (n = 17)	II (n = 20)	III (n = 18)	IV (n = 20)		
CEA (mcg/l)							
Range	0.60 – 2.50	0.80 – 2.0	0.60 – 2.0	1.80 – 7.50	15.0 – 46.0	361.12	<0.001*
Mean ± SD	1.56 ± 0.59	1.35 ± 0.43	1.26 ± 0.43	3.93 ± 1.52	33.20 ± 6.93	2*	
Median	1.50	1.20	1.20	3.55	33.0		
$p_{control}$		1.000	0.998	0.177	<0.001*		
Sig. bet. grps.		p_1=1.000,p_2=0.146,p_3<0.001*,p_4=0.096,p_5<0.001*,p_6<0.001*					
CA15.3 (u/ml)							
Range	10.0 – 30.0	10.0 – 28.0	13.0 – 31.0	25.0 – 60.0	100.0 – 290.0	122.34	<0.001*
Mean ± SD	20.90 ± 6.74	16.88 ± 5.38	22.85 ± 5.31	41.22 ± 10.45	170.85 ± 55.67	7*	
Median	22.0	16.0	22.50	40.0	165.0		
$p_{control}$		0.990	0.999	0.133	<0.001*		
Sig. bet. grps.		p_1=0.959,p_2=0.057,p_3<0.001*,p_4=0.211,p_5<0.001*p_6<0.001*					

F, p: F and p values for ANOVA test, Sig. bet. grps was done using Post Hoc Test (Tukey)



Area	Std. Error[a]	Asymptotic Sig.[b]	Asymptotic 95% Confidence Interval		Sensitivity	Specificity	Cutoff point
			Lower Bound	Upper Bound			
100%	0.0	0.0	1.0	1.0	100	100	52850

Figure 2. ROC curve analysis for calculation of cutoff values of circulating endothelial progenitor cells for detection of metastasis

On using receiver operating characteristic (ROC) curve analysis, the area under the curve (AUC) was 100% and the best cutoff point for CEPCs, which can detect the metastatic breast cancer patients, was 52850 /5x10^6 PBMC, with sensitivity of 100%, specificity of 100 % as shown in Figures (2). In this study, the level of CEPCs showed positive significant correlation with tumor markers (CEA and CA15.3) and alkaline phosphatase in breast cancer patients as shown in Table (2). In this study, there was statistically significant increase between control and cases there as regard tumor markers (CEA, CA15.3) but between stages there was significant increase only in stage IV Table (3). In current study, there was statistically significant increase between control and cases as regard alkaline phosphatase but between stages there was significant increase only in stage IV.

DISCUSSION

Breast cancer is a heterogeneous group of neoplasms that is characterized by abnormal proliferation and accumulation of cells that appear malignant and related to breast tissue (Ufen *et al*., 2014). Breast cancer is the most frequent cancer in women and represents the second leading cause of cancer death among women after lung cancer (Zervoudis *et al*., 2014). Deaths due to breast cancer are caused by metastatic spread of the tumor from the primary site to other parts of the body. Although some women who will ultimately have metastatic breast cancer present with it at time of diagnosis, the majority have localized disease at diagnosis (Lord *et al*., 2012). In Egypt, breast cancer ranks as the first malignancy affecting females, contributing 33% of all female cancers. It affects 1 in 14 women during their lifetime considered second cause of cancer deaths in females after lung cancer. The median age of incidence Egyptian patients is 46 years; one decade younger than the corresponding age in countries 60.5% of these patients are pre-menopausal (age 50 years and younger). The female to male ratio is 44:1 (Marino *et al*., 2013). Early detection of breast cancer can reduce the morbidity and mortality of breast cancer occult at the time of primary diagnosis. Because undetected micro metastasis can contribute to the failure of primary treatment, their identification may have a substantial impact on prognosis and treatment choice for these patients. Thus, improved direct identification of these occult metastases in blood offers a critical opportunity to optimize management of breast cancer patients (Caplan, 2014). Endothelial cell proliferation in the tumor vasculature has been studied in order to show evidence that the endothelial compartment is involved in tumor growth. CEPCs play a major role in prediction of metastasis so; they can be used as a prognostic marker and target for chemotherapy that act on angiogenesis (Verdegem *et al*., 2014). The identification of markers to distinguish between normal cells, tumorigenic cells and different stages of cancer is of critical importance for cancer diagnosis and prognosis. Detection of cancer-associated cellular markers is difficult due to the very low numbers of the circulating cancer cells in the blood. Humoral cancer markers, however, are characterized by their appearance in body fluids in amounts exceeding the normal physiologic concentrations. These markers can be released on tumor disintegration or may be secreted by tumors (Chen *et al*., 2017). Inconsistent data suggest protective effects from parity and lactation various age groups and a higher risk associated with consumption during pregnancy. Physical activity has emerged as a factor which reduces the incidence of breast cancer by 20% for each hour increase in physical activity per week during adolescence. The breast cancer risk drops with 3% due to delaying the onset of menarche and modifying the bioavailable hormone levels (Nabon, 2016). Patients with breast cancer may exhibit systemic manifestation such as cachexia, jaundice, lower limb edema and lymphadenopathy. Also, they may exhibit local as manifestation such as breast lump, skin changes, nipple retraction and discharges (Eccles *et al*., 2013).

EPCs differentiate from haemangioblasts during the development of mesodermal precursors, and further differentiate into mature endothelial cells to form the lining of blood vessels in a process known as vasculogenesis. In the course of vasculogenesis, EPCs mobilize from bone marrow to peripheral tissue sites in response to endogenous or exogenous signals, and autocrine/ paracrine activation results in differentiation, proliferation and vascular growth (George *et al*., 2012). EPCs can be detected in the peripheral and cord blood, and play important roles in physiological processes such as neovascularization, wound healing, and tissue regeneration following ischemia (E.g. myocardial infarction) and tissue remodeling (Asahara *et al*., 1999). The aim of this study was the measurement and comparison between CEPCs level in breast cancer patients and correlate them with the main parameters of primary tumor (size, grade), metastasis (site and size), tumor markers carcinoembryonic antigen and cancer 15.3, and also with other clinical variables. In tumor pathogenesis, however, EPC recruitment is thought to mediate aberrant vasculogenesis, and facilitate tumor growth and metastasis (Dong and Ha, 2012). CEPCs cannot be successfully defined with a single surface antigen, instead requiring the use of several markers including VEGFR2, CD14 and CD133 for their detection in peripheral blood (Khan *et al*., 2005). The majority of studies concerning the correlation of CEPCs with breast cancer have used double positive flow cytometry analysis e.g.CD133/VEGFR2 (Raida *et al*., 2006) CEPCs (CD133/VEGFR2) were shown to be significantly more numerous in advanced breast cancer than in early stage disease (Naik *et al*., 2008).

In this study, there was statistically significant increase CEPCs numbers in breast cancer patients when compared with control ($2.05 \pm 0.6x10^3$ CEPCs/$5x10^6$ PBMC). The level of CEPCs was significantly correlated with the stage of breast cancer. The least significant was stage I < II < III < IV ($7.71 \pm 1.40 < 8.67 \pm 1.65 < 30.01 \pm 9.74 < 76.19 \pm 13.2510^3$ CEPCs/$5x10^6$ PBMC) this is due to production of CEPCs by breast cancer tissue and is then secreted in blood. Also, there was highly statistically significant increase of CEPCs mean fluorescence intensity (Mx, My) with disease progression respectively ($31.18 \pm 3.38 < 37.80 \pm 8.02 < 65.78 \pm 9.51 < 85.50 \pm 9.15$) ($54.24 \pm 8.81 < 65.0 \pm 11.44 < 101.28 \pm 19.83 < 190.40 \pm 45.92$) as compared with controls (27.70 ± 5.54) (42.20 ± 8.61) .MFI assessing the reliability and repeatability of surface markers expression and reduce inter laboratory discrepancies so, high MFI mean strong positivity and good expression of receptors this explained increase MFI with disease progression these results were in agreement with Dome& and Goodale & colleagues as they found that MFI of CEPCs was statistically significant higher in breast cancer patients than controls .these results were supported by Dome and colleagues and Goodale and colleagues as they found that CEPCs were significantly higher in breast cancer patients than controls (Dome *et al*., 2016 and Goodale *et al*., 2015). In this study, there was statistically significant increase between control and cases ($P<0.001$) and statistically significant increase with disease stage progression as regard tumor size. So, the level of CEPCs can be used as a prognostic factor these results were also supported by Furstenberg *et al*., 2016 and Werner *et al*., 2015., since CEPCs may reflect the ability of a tumor to recruit the vascular infrastructure required to grow and metastasize, they may serve as a surrogate marker for disease recurrence and prognosis in a way similar to the evolution of use of tumor signatures.

In contrary Liu and colleagues found that the level of CEPCs had no role in clinical and pathological stage or primary tumor size as his study did not exclude patient who treated with chemotherapy which act on these cells (Liu *et al*., 2002). In this study, there was statistically significant increase between control and cases and statistically significant increase with disease stage progression as regard metastasis (size and site). Metastasis only found in stage IV (100%), according to size of metastasis in stage IV 50% was ≤1.5 cm and 50% was ≥1.5 cm, 80% of them were bone metastasis, 10% were liver and10% were brain as cancer cells invade nearby healthy cells, cancer cells penetrate into the circulatory or lymph system, migration through circulation, cancer cells lodge in capillaries and new small tumors grow. These results were in agreement with results of Parkinson *et al*., 2015 and Dong *et al*., 2012. In this study, On using receiver operating characteristic (ROC) curve analysis, the area under the curve (AUC) was 100% and the best cutoff point for CEPCs, which can detect the metastatic breast cancer patients, was 52850 /$5x10^6$ PBMC, with sensitivity of 100%, specificity of 100 %. In contrary Nguyen and Massague, 2007 found that no significant association between patients and control as regard as tumor metastasis site, size of metastasis because their study was on different study groups (without staging) and didn't put in concern if there was metastasis or not. This study showed that the level of CEPCs was significantly correlated with the tumor markers (CEA and CA15.3). These results were in agreement with that of Leitzel *et al*., 2011 and Vogel *et al*., 2012. As known tumor markers used as prognostic factors in breast cancer and increase with staging. In contrary Dome *et al*., 2016 found that the level of CEPCs had no relation to tumor markers between patients and control as they used another study design and tumor markers increase only in advanced stages. Also the level of CEPCs was significantly correlated with alkaline phosphatase as these cells increase with metastasis and the most common metastasis in breast cancer is bone metastasis. These results were also supported by Windrichova *et al*., 2016 and Zhang *et al*., 2016. In contrary Acar *et al*., 2005 found that the level of CEPCs had no relation to alkaline phosphatase because it can be increased in other many causes.

Conclusion

CEPC elevation in the blood of breast cancer patients established as a useful marker of tumor angiogenesis and progression. From existing research data, it is apparent that CEPCs monitoring is efficient, specific and reproducible as early predictors of metastatic potential in breast cancer patients with sensitivity 100 and specificity 100% at 52850 /$5x10^6$ PBMC as a cutoff point.

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