JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY 2017, 68, 1, 139-148
www.jpp.krakow.pl

P. RHONE[1,2], B. RUSZKOWSKA-CIASTEK[1], M. CELMER[1], A. BRKIC[1],
K. BIELAWSKI[1], J. BOINSKA[1], E. ZARYCHTA, D. ROSC[1]

INCREASED NUMBER OF ENDOTHELIAL PROGENITORS IN PERIPHERAL BLOOD AS A POSSIBLE EARLY MARKER OF TUMOUR GROWTH IN POST-MENOPAUSAL BREAST CANCER PATIENTS

[1]Department of Pathophysiology, Faculty of Pharmacy, Nicolaus Copernicus University w Torun, Collegium Medicum in Bydgoszcz, Bydgoszcz, Poland; [2]Clinical Ward of Breast Cancer and Reconstructive Surgery, Oncology Centre Prof. F. Lukaszczyk Memorial Hospital, Bydgoszcz, Poland

The aim of the study was to evaluate the number of circulating endothelial progenitor cells (circulating EPCs) in the blood of patients diagnosed with breast cancer and to make an attempt at finding associations with the number of circulating EPCs and selected clinic-pathological factors; TNM and histological grading, molecular subtype of breast cancer, hormonal status, the expression of Ki-67 and the size of tumour. The study involved 96 Caucasian ethnicity post-menopausal women. Sixty-six women aged 48 – 63 (mean age 55) with breast cancer diagnosis without distant metastases (M0). The median value of the tumour diameter was 1.51 cm. The control group consisted of 30 healthy, non-smoking, post-menopausal women, mean age 49, range 44 – 54 years of age. The exclusion criteria for all the participants were hypertension, hyperlipidaemia, and hyperglycaemia, acute and chronic infection. With regard to the fresh blood samples the number of circulating endothelial progenitors was determined using flow cytometry. The fluorescence of 100,000 cells was measured during the analysis. Circulating EPCs were identified with the immune-phenotype $CD45^-$, $CD34^+$, $CD133^+$, $CD31^+$. A significantly higher number of circulating EPCs in the study group, as compared to the controls ($P = 0.0001$) and a significantly higher number of circulating EPCs in women over 60 with breast cancer than in the younger women ($P = 0.0029$) were reported. A positive correlation was noted between circulating EPCs and age as well as between circulating EPCs and HER-2 ($P = 0.0231$, $P = 0.0414$, respectively), and a negative correlation between circulating EPCs and histological grading of breast cancer ($P = 0.0272$). The study has shown a higher number of circulating EPCs in breast cancer patients, which indicates stimulation of neovascularization. Additionally, since bone morrow-derived circulating EPCs are more intensively mobilised in older and overweight breast cancer patients, we can speculate that more aggressive neo-angiogenesis can occur in those patients.

Key words: *breast cancer, circulating endothelial progenitor cells, body mass index, oestrogen receptor, menopausal hormonal therapy*

INTRODUCTION

Malignant diseases are the second cause of morbidity, after heart disease, and mortality worldwide. Additionally, breast cancer is the most common female cancer globally diagnosed (1). According to the National Cancer Institute, in 2016 there was an estimated 246,660 new cases of invasive breast cancer in the United States with 40,450 deaths. The World Health Organization claimed that 1 in 8 women will develop, in their lifetime, breast cancer (WHO, 2015). The most common breast cancer risk factors represent: age, certain inherited genetic mutation, post-menopausal obesity (expressed by increased body mass index (BMI)), use of combined menopausal hormonal, oestrogen and progesterone, therapy, cigarette smoking or alcohol consumption (2).

Breast cancer is a patchy disease in respect to morphology, clinical nature and genetics as well as in terms of response to therapy (1). Apart from clinical parameters the breast cancer diagnostic procedure is based on molecular determinants; progesterone (PR) and oestrogen receptors (ER), human epidermal growth factor receptor 2 (HER-2), expression of Ki-67 (proliferation marker) and E-cadherin calcium-dependent transmembrane adhesion protein (E-cadherin). The expression of oestrogen receptors in breast cancer is an important prognostic and predictive indicator. Overexpression of HER-2 is associated with more aggressive tumour growth and metastasis (3).

The metastatic spread and growth of solid tumours are predominantly dependent on tumour vascularity *via* pro-angiogenic stimulation. Bone morrow-derived circulating endothelial progenitor cells (circulating EPCs) are necessary agents in post-natal angiogenesis. Circulating EPCs are considerable in early and late development, invasion and metastatic progression of selected malignant diseases. Such action runs according to an established pattern by migration and luminal incorporation to the site; where new vessels are created and differentiate into mature bona fide endothelial cells (4-6).

Circulating EPCs cannot be successfully defined with a single surface antigen, instead they require several markers including; *CD133⁺CD34⁺KDR⁺*; *CD34⁺KDR⁺*; *CD34⁺CD133⁺CD31⁺* (6, 7). However, on the endothelial progenitor's surface no specific antigens for hematopoietic stem cells, such as $CD14^+$, $CD45^+$ is found (6).

There are numerous physiological and pathophysiological aetiologies, as well as regulators, which have been described to influence the number of circulating EPCs and their functions. Some factors known to increase mobilization of EPCs are vascular endothelial growth factor A (VEGF-A), erythropoietin, oestrogens, stromal cell-derived factor 1 (SDF-1), as well as physical effort (6, 8). Furthermore, atherosclerosis and various cancers such as gliomas, non-small lung cancer, myeloid leukaemia, hepatocellular carcinoma, colorectal cancer, lymphoma and breast cancer have been discovered to have a similar increasing effect (6). Whereas diabetes mellitus, vascular diseases, hypertension and nicotine addiction are the factors that decrease the number of circulating EPCs (6, 9).

The creation of a vasculature is necessary for cancer growth. Nevertheless, it is well established that the phenotype of tumour vessels changes dramatically. Tumour vessels have an improper shape and diameter with irregular, immature, and extremely spiral capillaries. They also present incomplete differentiation of perivascular space and vessel permeability is on the rise (10). Suppression of development and spread of the tumour might be regulated and supported by reduced EPCs mobilization, whereas exact mechanisms of EPCs-mediated tumour angiogenesis are still unknown and should, therefore, be elucidated in further detailed experiments (2, 10, 11). This study aims at quantifying the number of circulating endothelial progenitors in the blood of patients diagnosed with breast cancer and it is an attempt at finding associations with the number of circulating EPCs and selected clinic-pathological factors; TNM and histological grading, molecular subtype of breast cancer, hormonal status, the expression of Ki-67 and the size of tumour.

PATIENTS AND METHODS

Patients

Ninety-six Caucasian ethnicity post-menopausal women were enrolled into this study. Sixty-six women aged 48 – 63 (mean age of 55) with breast cancer diagnosis without distant metastases (M0). The median value of patients' BMI was 25.15 kg/m^2. The median value of tumour diameter was 1.51 cm. The patients were supervised by the Clinical Ward of Breast Cancer and Reconstructive Surgery, Oncology Centre in Bydgoszcz, Poland. The patients were prepared to surgical procedure of either breast conserving surgery (BCS) or mastectomy. The subjects underwent a comprehensive clinico-pathological and post-surgical examination and were well-identified with regard to the invasion standards.

Thirty healthy, non-smoking, post-menopausal women who created the control group were invited to participate in the study while waiting for their routine medical visits; the mean age of 49, age range of 44 – 54, the average BMI value was 26.35 kg/m^2. The exclusion criteria for controls were hypertension, hyperlipidaemia, and hyperglycaemia, acute and chronic infection.

A medical history interview conducted by an oncologist revealed information about the general condition of all the subjects and the occurrence of co-existing diseases. Patients were free from history of cerebral-vascular diseases, congestive heart failure, overt diabetes mellitus, dyslipidaemia and others cancers. Additionally, 10 women underwent hysterectomy due to myocytoma, 11 women were obese and 17 suffered from hypertension. Hypertension was diagnosed according to the values of systolic blood pressure (BP) of 140 mmHg or higher and a diastolic BP of 90 mmHg or higher. The body mass index (BMI) was evaluated by dividing the weight expressed in kilograms (kg) by the height in square meters (m^2) and characterized according to the WHO criteria. The concentration of fasting glucose, lipid profile and C-reactive protein were measured using specific tests for Automated Immuno-Biochemical Analyzer, Cobas®6000, Roche Diagnostics, USA (*Table 1*).

The exclusion criteria for all the participants (study and control subjects) were as follows: premenopausal status, the age over 67 and below 40, surgery < 1 month. Neither did the respondents take any medication that could essentially affect the value of the results: anticoagulants, antiplatelet agents, thrombolytic drugs, nonsteroidal anti-inflammatory drugs, statins, metformin and insulin.

All the participants gave their written informed consent and the study was approved by the local Bioethics Committee (Collegium Medicum in Bydgoszcz, the Nicolaus Copernicus University in Torun, Poland (no KB/547/2015)).

Blood sampling and estimation of molecular determinants and circulating EPCs

The procedure for evaluation of Ki-67 expression is based on the immune-histochemical method with the use of monoclonal mouse antibody for the demonstration of the Ki-67 antigen in the specimens (Auto-stainer Link 48, Agilent Technologies, USA). Antibodies VENTANA anti-HER-2/neu (4B5) was applied for laboratory semi-quantitative detection of the antigen HER-2/neu with the use of VENTANA aperture for staining immune-histochemical microscopic slide, (Benchmark Ultra, Roche-Ventana).

The method for the determination of the level of circulating EPCs was based on a previous report (12, 13). 4.5 ml fresh blood with minimal stasis on the day before surgery procedure was collected into cooled tubes (Becton Dickinson Vacutainer® System, Plymouth, the UK) containing potassium ethylene-diaminetetraacetic acid (K2EDTA) and analysed within 2 hours. The subjects were after 30 min of rest between 7.30 and 9.30 am and after a 12 hour overnight fast. The approach of the current study has been to use four concurrent markers ($CD45^-$, $CD34^+$, $CD133^+$, $CD31^+$) to increase the accuracy of endothelial progenitor's detection.

Cells were further confirmed by fluorescent-activated cell sorting (FACS) Calibur flow cytometer (Becton Dickinson, San Diego, USA) using monoclonal antibodies directed against antigens specific for circulating endothelial progenitor cells. The data acquired was analysed by using CellQuest software (Becton Dickinson). Circulating EPCs counts were assessed with the four-colour flow cytometry according to the procedure provided by Mancuso *et al*. (12).

Fresh peripheral blood (50 µL) was incubated with a fluorescein isothiocyanate (FITC)-conjugated anti-CD31, PerCP-Cy5.5-conjugated anti-CD45, as well as APC-conjugated anti-CD34 antibody (all BD Biosciences, Pharmingen, San Diego, CA, USA), phycoerythrin (PE)-conjugated anti-CD133 (Miltenyi Biotec, Bergisch Gladbach, Germany) (*Fig. 1*). Circulating endothelial progenitor cells were defined as negative for hematopoietic marker CD45 and positive for endothelial progenitor marker CD133 and positive for endothelial cell markers CD31 and CD34 show expression on early hematopoietic and vascular-associated tissue. At least 100,000 events were measured in each sample. The total cell count was

Table 1. Patients'demographic and clinical characteristics at the time of breast cancer diagnosis.

Feature	Number of patients	%
Age at diagnosis (years)		
< 60	49	74
≥ 60	17	26
BMI kg/m^2 according to WHO criteria:		
Normal (< 24.99)	34	51
Overweight (≥ 25 – 29.99)	21	32
Obese (30 or more)	11	17
Menopausal status:		
Post-menopausal	66	100
MHT administration:		
Oral route of administration	10	15
Transdermal route of administration	5	7.5
Parity (number of full-term pregnancies):		
None	8	12
1 or 2	45	68
3 or 5	13	20
History of smoking habit:		
Yes	15	23
Tumour size:		
T1 (< 2 cm)	40	61
T2 (≥ 2 cm, but < 5 cm)	26	39
Nodal status:		
N0	47	71
N1	19	29
Histological grading:		
Grade I	3	4
Grade II	50	76
Grade III	13	20
Histological type of breast cancer:		
Invasive ductal carcinoma (IDC)	56	85
Invasive lobular carcinoma (ILC)	10	15
Molecular type of breast cancer		
Luminal A (*ER+/HER2−/Ki-67 < 14%)*	44	67
Luminal B HER positive (*ER+/HER2+/Ki-67 ≥ 14%)*	8	12
Luminal B HER negative (*ER+/HER2−/Ki-67 ≥ 14%)*	6	9
Triple-negative	8	12
Hormonal receptor:		
ER positive	58	88
PR positive	50	76
HER-2 positive	8	12
E-cadherin positive	62	94
Ki-67:		
≤ 14%	35	53
15 – 19%	8	12
≥ 20	23	35
Diameter (cm)		
≤ 1.9	40	61
≥ 2 – 3.5	26	39
Breast surgery:		
Breast conserving surgery (BCS)	52	79
Mastectomy	14	21
Localisation:		
Left breast	34	51
Right breast	32	49

BMI, body mass index; MHT, menopausal hormonal therapy; ER, oestrogen receptor; PR, progesterone receptor; HER-2, human epidermal growth factor receptor 2; Ki-67, proliferation marker.

calculated by TruCount tubes (BD Biosciences, San Jose, CA, USA) containing a calibrated number of fluorescent beads and; 'lyse-no-wash' procedures were used in the present study to improve the sensitivity (13).

Statistical analysis

Statistical analysis was performed with the use of Statistica v. 12.0 software (StatStoft®). The Shapiro-Wilk test was applied

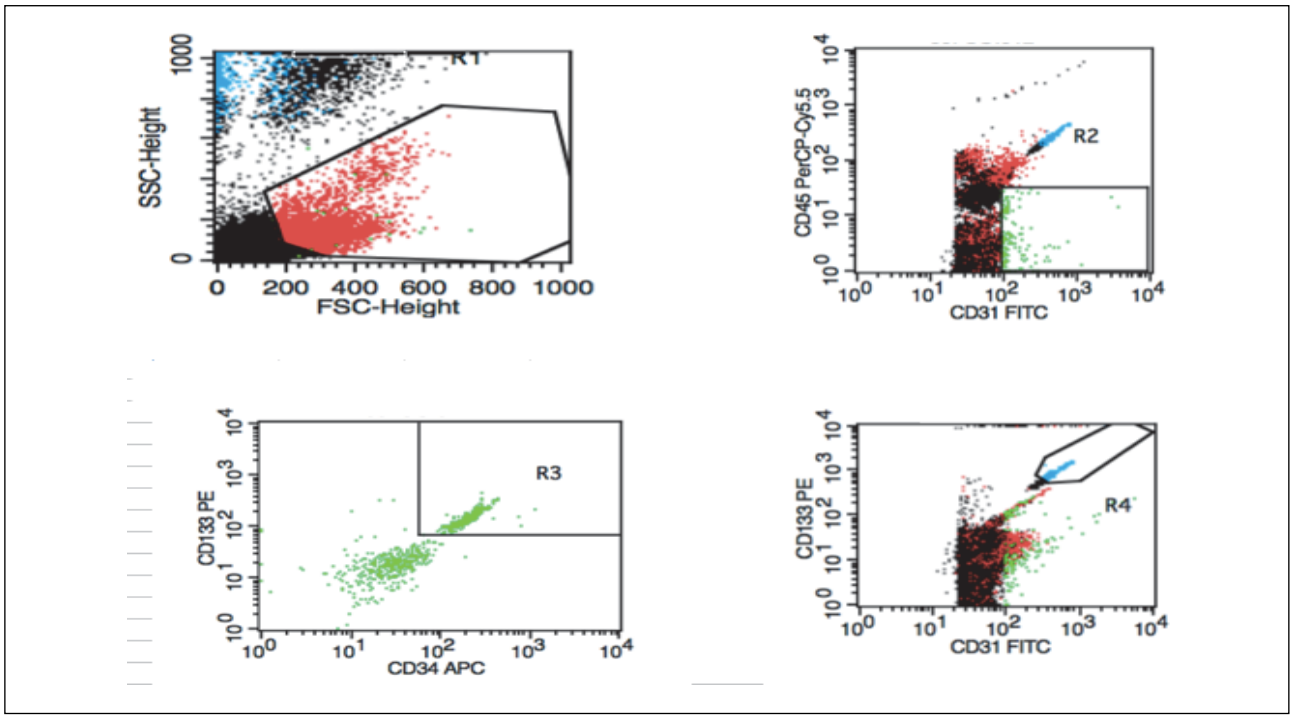

Fig. 1. Representative flow cytometric plots indicating the strategy for identification of circulating endothelial progenitor cells. EPCs were defined as $CD45^{-}$, $CD34^{+}$, $CD133^{+}$, $CD31^{+}$ using CD45-PerCP-Cy5.5, CD34-APC, CD133-PE, CD31-FITC conjugated antibodies. Flow cytometry was performed using sequential gating: (R1) side scatter (SSC) and forward (FSC) plot of cells and gating region R1 to include all the mononuclear cells while excluding cell debris; (R2) $CD45^{-}/CD31^{+}$ gating to exclude lymphocytes; (R3) $CD133^{+}/CD34^{+}$ gating identifies endothelial progenitor cells; (R4) is based on the number of events recorded in the absolute bead region count.

to assess the normality of data distribution. The data was distributed non-normally, the U-Mann-Whitney rank-sum test was used, and the variables were expressed as median (interquartile range (IQR)). The correlations were sought by the Spearmann's rank method. The P-values < 0.05 were considered significant.

RESULTS

The study group consisted of 96 post-menopausal women. Sixty-six patients with primary breast carcinomas and thirty healthy, non-smoking women were enrolled to the study as the controls. Forty-two were diagnosed with luminal-A-type breast cancer, and 51 subjects with confirmed breast cancer diagnosis were free of menopausal hormonal therapy (MHT). Histological grade and ER/PR/HER-2/Ki-67 characteristics were all evaluated in this cohort of patients using standard criteria and procedures. The other anthropometric, demographics and tumour data in those patients are presented in *Table 1*.

A significantly higher number of circulating EPCs in the study group as compared to healthy post-menopausal women (P = 0.0001) was noted (*Table 2*).

Furthermore, a significantly higher number of circulating EPCs in breast cancer patients over 60 than in the younger ones (P = 0.0029) was obtained. We made an interesting observation in all the patients from the study group. A significant growing tendency towards a higher number of circulating EPCs in both study and control groups was recorded in terms of the BMI. This analysis shows that increased BMI in post-menopausal women increase the number of circulating EPCs regardless of health status (*Table 3*).

Subsequently, the patients were divided into two groups on the basis of proliferation marker: Ki-67 expression. The first group consisted of women with expression of Ki-67 antigen lower than 14% and the second group included patients with Ki-67 expression above 15%. A markedly higher number of circulating EPCs was observed in patients with expression of Ki-67 lower than 14% (P = 0.0479). Additionally, there were differences in the number of circulating endothelial progenitors dependent on breast cancer localisation (left or right breast). Higher numbers of circulating EPCs were noted in patients with breast cancer in the left breast. However, those observations were revealed only in patients who had never taken menopausal hormonal therapy and in the subjects with luminal-A-type breast cancer (P = 0.0163, P = 0.0237, respectively) (*Table 4*).

At the next step of statistical analysis the correlation coefficient in all the breast cancer patients was calculated. A positive correlation was reported between circulating EPCs and age as well as between circulating EPCs and HER-2 (P = 0.0231, P = 0.0414, respectively), and a negative correlation between circulating EPCs and histological grading of breast cancer according to the Elston-Ellis classification (P = 0.0272) (*Table 5*).

Furthermore, the study group was very carefully extracted according to molecular determinants and this step led us to new observations. First of all, a positive correlation was identified between circulating EPCs and age as well as between circulating EPCs and parity (P = 0.0221, P = 0.0440, respectively), as well as a negative correlation between circulating EPCs and the diameter of the tumour as well as between circulating EPCs and histological grading of breast cancer (P = 0.0284, P = 0.0327-respectively), among luminal-A-type breast cancer patients (*Table 6*).

Table 2. Number of circulating endothelial progenitor cells in the blood of breast cancer patients and in the controls.

Parameters (units)	**Breast cancer patients** n = 66			**Controls free of breast cancer** n n = 30			**P-values**
	Q1	**Me**	**Q3**	**Q1**	**Me**	**Q3**	
Circulating EPCs (cells/μL)	4.82	10.57	23.02	0.20	0.36	0.82	**0.0001**
Age (years)	52.00	56.00	61.00	49.00	50.00	52.00	0.4651
Weight (kg)	62.00	66.50	7500	67.00	72.00	73.50	0.2051
BMI (kg/m^2)	22.31	25.15	28.72	24.74	26.35	28.01	0.7082

Data are expressed as median (Me) and the inter-quartile range (IQR), lower quartile (Q1), upper quartile (Q3).
Circulating EPCs, circulating endothelial progenitor cells; BMI, body mass index.

Table 3. Number of circulating endothelial progenitor cells in the blood of breast cancer patients and in the controls according to age and BMI.

Parameter	**Breast cancer patients**		**Controls free of breast cancer**	
	< 24.9 kg/m^2 n = 34	≥ 25.0 kg/m^2 n = 32	< 24.9 kg/m^2 n = 13	≥ 25.0 kg/m^2 n = 17
Circulating EPCs (cells/μL)	7.54 $^{\text{I vs. III}}$ *** 4.42/21.91	14.93 $^{\text{II vs. IV}}$ *** 5.42/28.09 $^{\text{I vs. II}}$*	0.20 $^{\text{III vs. IV}}$ * 0.10/0.31	0.70 0.40/1.53
Circulating EPCs (cells/μL)	< 60 years N = 49	≥ 60 years N = 17	< 60 years N = 18	≥ 60 years N = 12
	6.74 $^{\text{I vs. III}}$ *** 4.42/14.87	24.62 $^{\text{II vs. IV}}$ *** 13.02/35.78 $^{\text{I vs. II}}$ **	0.40 0.20/0.92	0.30 0.20/0.70

Data are expressed as median (Me) and the inter-quartile range (IQR), lower quartile (Q1), upper quartile (Q3). *P < 0.05, **P < 0.001, ***P < 0.0001.

Table 4. Number of circulating endothelial progenitor cells depending on clinical parameters in the study group.

Parameters	Circulating EPCs with the immune-phenotype CD45$^-$, CD31$^+$, CD34$^+$, CD133$^+$			**P-values**
	Q1	**Me**	**Q3**	
Localisation of tumour: left breast	5.70	12.06	33.57	
Localisation of tumour: right breast	4.42	8.20	17.79	0.1101
Localisation of tumour: left breast without MHT (N = 26)	6.09	13.68	33.84	
Localisation of tumour: right breast without MHT (N = 25)	3.96	6.18	14.77	**0.0163**
Localisation of tumour: left breast, luminal A type cancer (N = 20)	8.82	15.78	37.99	
Localisation of tumour: right breast, luminal A type cancer (N=22)	4.42	8.20	14.87	**0.0237**
Diameter of lump ≤ 1.9 cm	5.63	11.44	31.05	
Diameter of lump ≥ 2.0 cm	4.42	10.15	15.18	0.1969
Ki-67 ≤ 14 %	9.25	13.02	26.63	
Ki-67 ≥ 15%	3.51	6.33	21.91	**0.0479**
Clinical classification TNM - pT1	5.61	10.85	26.63	0.5958
Clinical classification TNM - pT2	4.42	10.15	25.33	
Histological type IDC	5.03	10.85	26.63	
Histological type ILC	7.54	12.06	23.02	0.7068

Data are expressed as median (Me) and the inter-quartile range (IQR), lower quartile (Q1), upper quartile (Q3).
MHT, menopausal hormonal therapy; Ki-67, proliferation marker, TNM, classification of malignant tumours; IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma.

Secondly, patients were selected only according to one criterion (*Table 7*); the subjects were free of menopausal hormonal therapy (MHT) and a positive correlation was noted between circulating EPCs and age as well as between circulating EPCs and HER-2 (P = 0.0041, P = 0.0171- respectively). A negative correlation between circulating EPCs and the diameter

Table 5. Correlations between circulating EPCs with selected clinical feature.

All patients with breast cancer n = 66	Circulating EPCs with the immune-phenotype $CD45^-$, $CD31^+$, $CD34^+$, $CD133^+$	
	R	P-values
Age (years)	0.2858	0.0231
BMI (kg/m^2)	0.0642	0.6201
Parity (number of full-term pregnancies)	0.1805	0.1568
Use of MHT	0.1563	0.2251
Smoking tobacco	0.0833	0.5267
Diameter of the tumour (cm)	–0.1639	0.1993
Histological grading	–0.2783	0.0272
Ki-67 expression	–0.1269	0.3241
Addressing nodes	–0.1219	0.3413
Oestrogen receptor	0.0599	0.6411
Progesterone receptor	–0.0409	0.7497
HER-2	0.2578	0.0414
E-Cadherin	0.0839	0.5129

Circulating EPCs, circulating endothelial progenitor cells; MHT, menopausal hormonal therapy; BMI, body mass index; Ki-67, proliferation marker; HER-2, human epidermal growth factor receptor 2.

Table 6. Correlations between circulating endothelial progenitor cells with selected clinical features among luminal A type of breast cancer patients.

Luminal A type breast cancer patients n = 42	Circulating EPCs with the immune-phenotype $CD45^-$, $CD31^+$, $CD34^+$, $CD133^+$	
	R	P-values
Age (years)	0.3139	0.0221
BMI (kg/m^2)	–0.0036	0.9796
Parity (number of full-term pregnancies)	0.2777	0.0440
Use of MHT	0.2382	0.0890
Smoking tobacco	0.1152	0.4256
Diameter of the tumour (cm)	–0.3425	0.0284
Histological grading	–0.2939	0.0327
Ki-67 expression	–0.0791	0.5736
Addressing nodes	–0.1102	0.4323
Oestrogen receptor	0.0739	0.5989
Progesterone receptor	–0.0315	0.8229
E-cadherin	0.1632	0.2431

Circulating EPCs, circulating endothelial progenitor cells; MHT, menopausal hormonal therapy; BMI, body mass index; Ki-67, proliferation marker.

Table 7. Correlations between circulating endothelial progenitor cells with selected clinical features in breast cancer patients with adjustment for menopausal hormonal therapy.

Breast cancer patients free of menopausal hormonal therapy n = 51	Circulating EPCs with the immune-phenotype $CD45^-$, $CD31^+$, $CD34^+$, $CD133^+$	
	R	P-values
Age (years)	0.4065	0.0041
BMI (kg/m^2)	–0.0307	0.8377
Parity (number of full-term pregnancies)	0.2652	0.0685
Smoking tobacco	–0.0639	0.6769
Diameter of the tumour (cm)	–0.3445	0.0165
Histological grading	–0.2918	0.0442
Ki-67 expression	–0.1830	0.2131
Addressing nodes	–0.1411	0.3386
Oestrogen receptor	–0.0492	0.7397
Progesterone receptor	–0.1431	0.3318
HER-2	0.3429	0.0171
E-cadherin	0.2068	0.1585

Circulating EPCs, circulating endothelial progenitor cells; BMI, body mass index; Ki-67, proliferation marker; HER-2, human epidermal growth factor receptor 2.

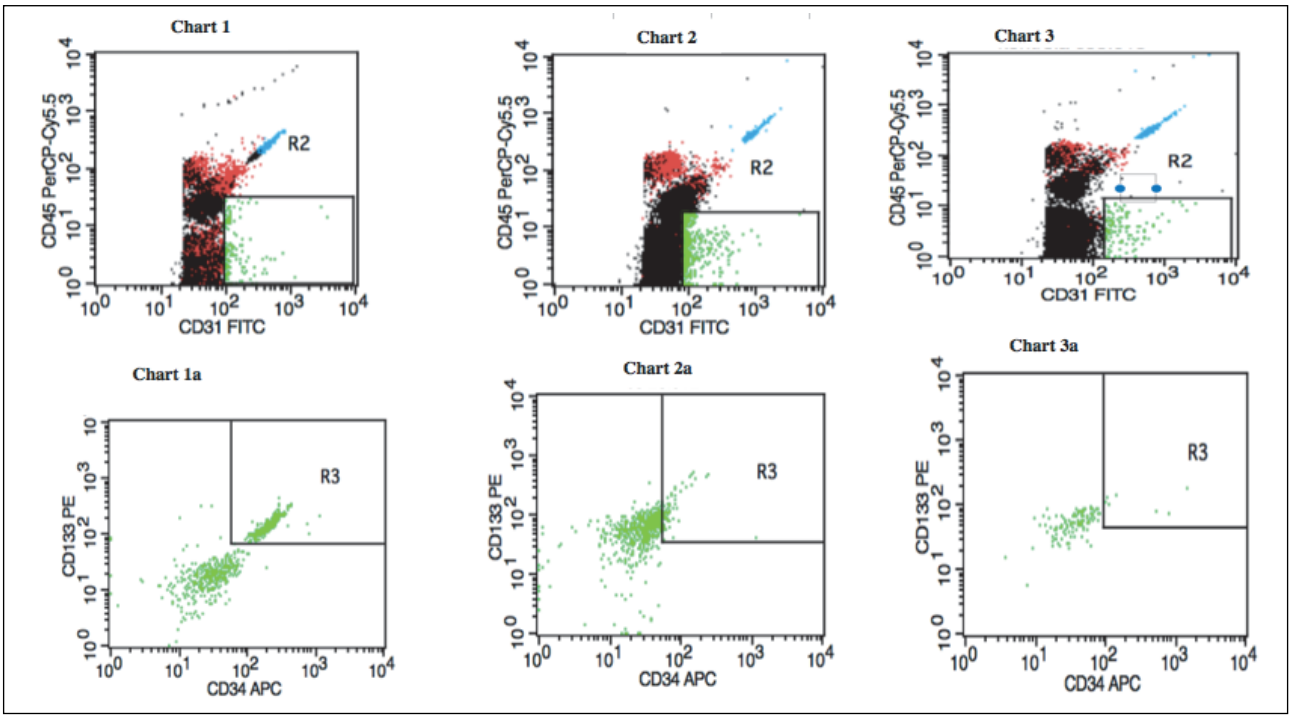

Fig. 2. Sample of selected flow cytometric plots for identification of circulating endothelial progenitor cells depending on breast cancer advancement and in healthy subjects.

of the tumour was found as well as between circulating EPCs and the histological grading of breast cancer (P = 0.0165, P = 0.0442, respectively).

To support our results, we have added *Fig. 2* demonstrating the number of circulating EPCs (green dots) depending on breast cancer advancement and in the healthy subjects *Fig. 2*. *Chart 1* and *1a* present the patient with a higher number of circulating EPCs with selected clinical, anthropometric determinants: BMI = 20.40 kg/m^2, Elston-Ellis = 1, Her-2-negative; Ki-67 = 5%, circulating EPCs = 32.56 cells/µL; *Fig. 2* panel *Chart 2* and *2a* shows a lower number of circulating EPCs in patients with a clinical profile: BMI = 23.14 kg/m^2, Elston-Ellis = 3, Her-2-positive; Ki-67 = 40%, circulating EPCs = 7.54 cells/µL; *Fig 2*, panel *Chart 3* and *3a* presents subject free of breast cancer with BMI = 24.68 kg/m^2 and circulating EPCs equal 0.70 cells/µL.

DISCUSSION

Our present experiments demonstrate a markedly higher count of circulating EPCs with the immune-phenotype CD45$^-$, CD31$^+$, CD34$^+$, CD133$^+$ as compared to the controls. This observation is consistent with Mancuso *et al*. and Botelho *et al*.; the authors confirmed that the endothelial progenitor cells are mobilised during breast cancer development due to their high proliferative potential (12, 14). Endothelial progenitor cells are recruited during malignant transformation and facilitate growth and metastasis of the tumours. Van't Hull *et al*. report on plasma from breast cancer patients stimulating both the kinetics of differentiation of CD34$^+$ progenitors into angiogenic CD11b$^+$ cells and the proangiogenic tendency of these cells (15). However, it is not an obvious observation as Goon *et al*. noted a significantly lower number of circulating progenitors with immune-phenotype CD34$^+$/CD133$^+$, CD45$^-$ in breast cancer subjects (16). Furthermore, Purhonen *et al*. postulated that circulating EPCs are unnecessary for tumour growth and metastasis, detected by various cancer-cell and angiogenesis models. The authors did not confirm endothelial progenitor's mobilisation to peripheral circulation during tumour development due to inadequate indications (17). These controversies may be due to discrepancies in their surface identification markers, methods and protocols, which were applied to endothelial progenitor cells enumeration (18).

In luminal-A-breast cancer patients and those who had never taken menopausal hormonal therapy, a significant negative correlation between the number of circulating EPCs and the histological grading as well as between circulating EPCs and the tumour diameter was observed. We may speculate that those correlations point out to the role of circulating EPCs at the tumour growth stage. This thesis is consistent with the report by Botelho *et al*. who suggest that breast tumour cells mobilize EPCs in a very specific limited manner and reveal that the tumour engages EPCs during malignant transformation and endothelial progenitors are no longer required when the tumour reaches a plateau of growth (14). However, Naik *et al*. observed a higher number of circulating EPCs at stage III-IV breast cancer patients than that of I-II subjects. They indicate the existence of a positive correlation between the number of circulating EPCs with the stage of breast cancer, which is inconsistent with our study (19), whilst Jain *et al*. noted an increase in EPCs with immune-phenotype CD45^{dim}, CD133$^+$, VEGFR2$^+$ in breast cancer patients which occurred immediately before a recurrence of cancer. The authors claim that circulating EPCs may be successfully used as an indicator to predict relapse or disease progression (20).

Subsequently, the present study reports on a markedly higher number of circulating EPCs in the group of women with expression of Ki-67 antigen below 14% than in the patients with Ki-67 expression above 15%. Antigen Ki-67 as a proliferation marker is a good indicator of the mitotic index, which is useful in determining the progress and intensity of the promotion phase of breast cancer (21). Taken together, based on clinical and

molecular determinants (expression of Ki-67, histological grading and diameter of the tumour), we can assume that circulating EPCs are necessary at tumour growth and can be used as a non-invasive biomarker to monitor the clinical state of patients. Nevertheless, our results are inconsistent with Inwald *et al*. as they suggest that higher tumour stages and a higher nodal status were associated with higher Ki-67 (21).

Besides, it is worth underlining that the present study demonstrates differences in the number of circulating endothelial progenitors dependent on breast cancer localisation (left or right breast). A higher number of circulating EPCs in the patients with breast cancer in the left breast was noted. Albeit, this observation was only revealed in the patients who had never taken menopausal hormonal therapy and in those with luminal A subtype of breast cancer. It is well known that women most frequently develop cancer in the left breast. However, the number of patients included for statistical analysis was similar in our study. Interestingly, Amer noted a comparable overall survival rate between patients with left and right breast cancer, also in those with or without a family history of cancer (22).

Presumably, the left breast is more intensively exposed to hypoxia and then disruption of endothelial integrity initiates pro-angiogenic switch *via* increased expression and/or activation of hypoxia-inducible factor-1α (HIF-1α). Breast density is associated with a variation of oestrogen level during a menstrual cycle. Some women are more sensitive to 'fluctuating asymmetry' and simultaneously one breast is more predisposed to cancer development (23). Oestrogens have an influence on EPCs function *via* their activation, migration and proliferation, and then they lead to increased neo-angiogenesis (24); such observation coincides with our study. The confirmation of this thesis is the study performed by Wilting and Hagedorn who observed that some growth factors, fibroblast growth factor (FGF), heparin-binding epidermoid growth factor (HB-EGF), and hepatocyte-growth factor (HGF), may act as molecular stimulators of left-right asymmetry. Therefore, those agents can activate cancer development (25). Rybicka *et al*. indicate that transforming growth factor-β (TGF-β) might also be involved in the maintenance of stem cell characteristics of canine mammary cancer stem cells and it may stimulate tumorigenesis (26).

The novel findings noted in our study are a positive correlation between the number of circulating endothelial progenitors and the age in breast cancer women; it is worth mentioning that a significantly higher number of circulating EPCs was noted in women over 60. Likewise, a significantly higher number of circulating EPCs was noted in overweight patients (BMI ≥ 25 kg/m^2) as compared to normal-weight patients. Those findings suggest that neo-vasculogenesis is more intensive and aggressive in older and overweight women due to chronic hypoxia or inflammation, the EPCs are successfully activated and mobilised (9). Indeed Chen *et al*. demonstrated that the number of circulating EPCs in the healthy population decreases with age (27). The ageing process influences the progenitors *via* suppressing their function and mobilisation, as well as accelerating apoptosis and depletion of the pool of bone marrow-derived EPCs (27, 28). However, there is less direct evidence indicating that biology of endothelial progenitors in breast cancer is completely out of control, which, most probably, can be associated with chronic hypoxia and thus low oxygenation influences the growth and survival of progenitors generating a 'defensive' autophagy and a slight apoptosis when exposed to cellular stress (9).

The study group was selected according to molecular subtypes and a positive correlation was clearly noticed between parity and circulating EPCs only in the subjects with luminal-A-breast cancer type, which means that multi-parity may contribute to aggressive neo-vessel formation due to their proliferation, migration and differentiation into endothelial cells in oestrogen receptors (ER) of positive patients. George *et al*. proved that oestrogen can molecularly mimic hypoxia by activating HIF-1α and directly leads to pro-angiogenic microenvironment and induces tumour vascularization (29). Nevertheless, those observations are inconsistent with Dall *et al*. who believed that parity reduces the risk of breast cancer due to a decreasing number of ovulation and thus extending the period of lactation. Albeit, the intensity of protection recognized by parity appears when the first full term birth occurs before the age of 20 (30). It is well established that long-term exposition to endogenous oestrogen during pregnancy or pro-inflammatory stimulation and tissue remodelling of the mammary gland during postpartum involution can increase susceptibility to intensive cancero-genesis (24, 29). Additionally, the explanation is more complicated due to spacing between pregnancies. A 1 – 3 year gap between successive births essentially increased the risk of breast cancer, as compared to the gap of less than one year and greater than 3 years (31).

Solid tumours are created by various populations of cells. Cancer stem-like cells (CSLCs) are able to self-renew and to differentiate into different types of cells. It is confirmed that CSLCs are necessary for tumour initiation, recurrence and drug resistance. Rybicka *et al*. indicate that the angiogenesis *in vitro* assay was performed to demonstrate that tumour-associated macrophages (TAMs) enhance pro-angiogenic properties of CSLCs (32).

Overexpression of HER-2 indicates more aggressive tumours due to their intensive proliferation, angiogenesis, and the reduction of apoptosis and a less effective response to chemotherapy (3). Dissemination occurs in advanced breast cancer, most frequently to bones, and also to visceral organs; such as liver, lungs and brain, which may accompany pain in these locations. Bone metastases are commonly the cause of pain and may lead to hyper-calcemia and more severe consequences (33). A positive correlation between the number of circulating EPCs and human epidermal growth factor receptor 2 in breast cancer subjects suggests a negative indication and that dependence insinuate a less favourable outcome for those patients. Goon *et al*. found a similar association between circulating progenitors and HER-2 (16). However, Naik *et al*. noted no connections between EPCs and age, hormone status and Her-2 status (19). A further study in this field is necessary to confirm this hypothesis.

In summary, the study has shown a higher number of circulating EPCs in breast cancer patients, which indicates a stimulation of the neovascularization process. Albeit, a negative correlation between circulating EPCs and the tumour diameter as well as between circulating EPCs and histological grading of breast cancer and a higher number of circulating EPCs in less proliferative tumours (expressed by lower level of Ki-67) suggest the role of circulating EPCs at the tumour growth phase. Additionally, since bone morrow-derived circulating EPCs are more intensively mobilised in older and overweight breast cancer patients, we can speculate that in those patients more stimulated neo-angiogenesis may occur. This might lead us to think that circulating EPCs may be used as an early diagnostic screening to recognize a potential of neo-angiogenesis or to define a tumour growth scenario.

Abbreviations: BMI, body mass index; EPCs, endothelial progenitor cells; ER, estrogen receptor; HER-2, human epidermal growth factor receptor 2; Ki67, proliferation marker; MHT, menopausal hormonal therapy; PR, progesterone

receptor; SDF-1, stromal cell-derived factor 1; VEGF-A, vascular endothelial growth factor A

Acknowledgements: This study has been supported by the Nicolaus Copernicus University in Torun, Collegium Medicum in Bydgoszcz, Poland.

Conflict of interests: None declared.

REFERENCES

1. Malhotra GK, Zhao X, Band H, Band V. Histological, molecular and functional subtypes of breast cancers. *Cancer Biol Ther* 2010; 10: 955-960.
2. Janssens JP, Vandeloo M. Breast cancer: a life-time disease. Direct and indirect age-related lifestyle risk factors. *J Oncol* 2009; 59: 159-167.
3. Parise CA, Caggiano V. Breast cancer survival defined by the ER/PR/HER2 subtypes and a surrogate classification according to tumor grade and immunohistochemical biomarkers. *J Cancer Epidemiol* 2014; 2014: 469251. doi: 10.1155/2014/469251
4. Plummer PN, Freeman R, Taft RJ, *et al*. MicroRNAs regulate tumor angiogenesis modulated by endothelial progenitor cells. *Cancer Res* 2013; 73: 341-352.
5. Le Bourhis X, Romon R, Hondermarck H. Role of endothelial progenitor cells in breast cancer angiogenesis: from fundamental research to clinical ramifications. *Breast Cancer Res Treat* 2010; 120: 17-24.
6. de la Puente P, Muz B, Azab F, Azab AK. Cell trafficking of endothelial progenitor cells in tumor progression. *Clin Cancer Res* 2013; 19: 3360-3368.
7. Hagensen MK, Vanhoutte PM, Bentzon JF. Arterial endothelial cells: still the craftsmen of regenerated endothelium. *Cardiovasc Res* 2012; 95: 281-289.
8. Cheng CC, Chang SJ, Chueh YN, *et al*. Distinct angiogenesis roles and surface markers of early and late endothelial progenitor cells revealed by functional group analyses. *BMC Genomics* 2013; 14: 182.
9. Jiang WG, Lane J, Cui YX. Significance and therapeutic implications of endothelial progenitor cells in angiogenic-mediated tumour metastasis. *Crit Rev Oncol Hematol* 2016; 100: 177-189.
10. Furuya M, Nishiyama M, Kasuya Y, Kimura S, Ishikura H. Pathophysiology of tumor neovascularization. *Vasc Health Risk Manag* 2005; 1: 277-290.
11. Mellick AS, Plummer PN, Nolan DJ, *et al*. Using the transcription factor inhibitor of DNA binding 1 to selectively target endothelial progenitor cells offers novel strategies to inhibit tumor angiogenesis and growth. *Cancer Res* 2010; 70: 7273-7282.
12. Mancuso P, Antoniotti P, Quarna J, *et al*. Validation of a standardized method for enumerating circulating endothelial cells and progenitors: flow cytometry and molecular and ultra-structural analyses. *Clin Cancer Res* 2009; 15: 267-273.
13. Ruszkowska-Ciastek B, Sokup A, Leszcz M, *et al*. The number of circulating endothelial progenitor cells in healthy individuals - effect of some anthropometric and environmental factors (a pilot study). *Adv Med Sci* 2015; 60: 58-63.
14. Botelho MC, Alves H. Endothelial progenitor cells in breast cancer. *Int J Immunother Cancer Res* 2016; 2: 1-2.
15. Van't Hull EF, Bron S, Henry L, *et al*. Bone marrow-derived cells are implicated as a source of lymphatic endothelial progenitors in human breast cancer. *Oncoimmunology* 2014; 3: e29080.
16. Goon PK, Lip GY, Stonelake PS, Blann AD. Circulating endothelial cells and circulating progenitor cells in breast cancer: relationship to endothelial damage/ dysfunction/apoptosis, clinicopathologic factors, and the Nottingham Prognostic Index. *Neoplasia* 2009; 11: 771-779.
17. Purhonen S, Palm J, Rossi D, *et al*. Bone marrow-derived circulating endothelial precursors do not contribute to vascular endothelium and are not needed for tumor growth. *Proc Natl Acad Sci USA* 2008; 105: 6620-6625.
18. Danova M, Comolli G, Manzoni M, Torchio M, Mazzini G. Flow cytometric analysis of circulating endothelial cells and endothelial progenitors for clinical purposes in oncology: a critical evaluation. *Mol Clin Oncol* 2016; 4: 909-917.
19. Naik RP, Jin D, Chuang E, *et al*. Circulating endothelial progenitor cells correlate to stage in patients with invasive breast cancer. *Breast Cancer Res Treat* 2008; 107: 133-138.
20. Jain S, Ward MM, O'Loughlin, *et al*. Incremental increase in $VEGFR1^+$ hemaotopoietic progenitor cells and $VEGRF^+$ endothelial progenitor cells predicts relapse and lack of tumor response in breast cancer patients. *Breast Cancer Res Treat* 2012; 132: 235-242.
21. Inwald EC, Klinkhammer-Schalke M, Hofstadter F, *et al*. Ki-67 is a prognostic parameter in breast cancer patients: results of a large population-based cohort of a cancer registry. *Breast Cancer Res Treat* 2013; 139: 539-552.
22. Amer MH. Genetic factors and breast cancer laterality. *Cancer Manag Res* 2014; 6: 191-203.
23. Chen JH, Chan S, Yeh DC, Fwu PT, Lin M, Su MY. Response of bilateral breasts to the endogenous hormonal fluctuation in a menstrual cycle evaluated using 3D MRI. *Magn Reson Imaging* 2013; 31: 538-544.
24. George AL, Bangalore-Prakash P, Rajoria S, *et al*. Endothelial progenitor cell biology in disease and tissue regeneration. *J Hematol Oncol* 2011; 4: 24. . doi: 10.1186/1756-8722-4-24
25. Wilting J, Hagedorn M. Left-right asymmetry in embryonic development and breast cancer: common molecular determinants? *Curr Med Chem* 2011; 18: 5519-5527.
26. Rybicka A, Mucha J, Majchrzak K, *et al*. Analysis of microRNA expression in canine mammary cancer stem-like cells indicates epigenetic regulation of transforming growth factor-beta signaling. *J Physiol Pharmacol* 2015; 66: 29-37.
27. Chen CH, Cheng BC, Leu S, Sun CK, Chua S. Circulating level of endothelial progenitor cells in healthy Taiwanese. *Acta Cardiol Sinica* 2010; 26: 94-101.
28. Altabas V, Altabas K, Kirigin L. Endothelial progenitor cells (EPCs) in ageing and age-related diseases: How currently available treatment modalities affect EPC biology, atherosclerosis, and cardiovascular outcomes. *Mech Ageing Dev* 2016; 159: 49-62.
29. George AL, Rajoria S, Suriano R, Mittleman A, Tiwari RK. Hypoxia and estrogen are functionally equivalent in breast cancer-endothelial cell interdependence. *Mol Cancer* 2012; 11: 80. doi: 10.1186/1476-4598-11-80
30. Dall G, Risbridger G, Britt K. Mammary stem cells and parity-induced breast cancer protection- new insights. *J Steroid Biochem Mol Biol* 2016; Feb 22: S0960-0760(16)30029-2. doi: 10.1016/j.jsbmb.2016.02.018. [Epub ahead of print]
31. Dall G, Anderson R, Britt K. The role of stem cells in parity induced protection against breast cancer. *J Cancer Biol Res* 2014; 2: 1049.
32. Rybicka A, Eyileten C, Taciak B, *et al*. Tumour-associated macrophages influence canine mammary cancer stem-like cells enhancing their pro-angiogenic properties. *J Physiol Pharmacol* 2016; 67: 491-500.
33. Leppert W, Zajaczkowska R, Wordliczek J, Dobrogowski J, Woron J, Krzakowski M. Pathophysiology and clinical

characteristics of pain in most common locations in cancer patients. *J Physiol Pharmacol* 2016; 67: 787-799.

Received: November 29, 2016
Accepted: February 24, 2017

Author's address: Dr. Barbara Ruszkowska-Ciastek, Department of Pathophysiology, Faculty of Pharmacy, Nicolaus Copernicus University in Torun, Collegium Medicum in Bydgoszcz, 9 Sklodowskiej-Curie Street, 85-094 Bydgoszcz, Poland.
E-mail: ruszkowska.basia@gmail.com