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HEPARANASE LINK BETWEEN VASCULOGENESIS AND ANGIOGENESIS AS WELL AS A PREDICTIVE FACTOR OF A SHORTER SURVIVAL RATE

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Heparanase concentration is low in normal epithelia cells but its overexpression is reported in many carcinomas, including sarcomas and haematological malignancies. The purpose of this study was to investigate the association with selected angiogenic parameters as well as in the number of circulating endothelial progenitors (EPCs) in respect to low, moderate and high concentrations of heparanase. Also, we estimated the diagnostic usefulness of the heparanase concentration for disease recurrence prediction in breast cancer cases. Eighty-six patients with IA-IIB stage invasive breast carcinoma who passed a comprehensive clinicopathologic evaluation were included in the study. The median tumour diameter was 1.5 cm. Twenty cases showed lymph node metastasis (N1). Follow-up was completed in all patients a median follow-up was 33.5 months with a 11.6% recurrence rate. An immunoassay of selected angiogenic parameters, heparanase, as well as an immunohistochemistry of oestrogen and progesterone receptors, human epidermal growth factor receptor 2 (HER2), Ki67 and E-cadherin was performed in all cases. Circulating EPCs were determined by flow cytometry. Higher levels of heparanase in oestrogen and progesterone receptor negative cancers than in positive ones were noted. A higher concentration of heparanase was observed in T2 cases than T1 subjects. Significant positive associations between circulating EPCs, soluble forms of VEGF receptors and increasing plasma levels of heparanase were obtained. Follow-up revealed a significantly higher incidence of disease relapse in breast cancer patients with high baseline concentrations of heparanase. Heparanase was the most accurate biomarker with an $AUC^{ROC} = 0.72$. The cut-off value of 213.74 pg/mL was identified in order to discriminate between disease recurrence patients and those without disease relapse. We suggest, that a high concentration of heparanase next to tumour size and oestrogen and progesterone receptor expression may serve as an indicator of a more an aggressive character of tumour cells and a shorter survival rate.

Key words: *heparanase, invasive breast cancer, angiogenesis, vasculogenesis, tumour progression, circulating endothelial progenitors, growth factor receptors*

INTRODUCTION

According to the World Health Organization, global cancer morbidity is estimated to have risen to 18.1 million new cases and 9.6 million deaths in 2018. Breast cancer (BC) is the most frequently diagnosed female cancer in the vast majority of the countries and, additionally, is also the leading cause of cancer death due to still its recognition in unfavourable stages. In 2018, almost 2.1 million newly diagnosed breast cancer cases were identified worldwide (1). This accounts for 24.2% of cancer patients among women (1, 2).

In recent years, studies have focused on the genetic and biochemical components involved in tumour progression and its microenvironment. This has enabled understanding of the biology of breast tumour cells, improved treatment patterns and an increase in the survival rates of patients with breast cancer (3). Several breast cancer classifications are used. However, the most common classification, in order to determine survival rates, is the molecular one, which is based on the immunohistochemical (IHC) estimation of oestrogen receptors (ER), progesterone receptors (PR), human epidermal growth factor receptor 2 (HER2) and Ki67, E-cadherin expression (4, 5). Premenopausal patients are more likely to develop ER-negative cancers than postmenopausal cases. According to the Surveillance, Epidemiology and End Results (SEER) database, women with ER and PR positive tumours had statistically longer survival rates than those with negative hormonal receptor (ER-PR-) tumours. This effect is visible in all age groups and it is independent of disease stage, tumour grade, tumour histological subtype or ethnicity (5). The one of a main indicator of potential responses to hormonal therapy is ER expression and the best established a selective ER modulator (SERM) is tamoxifen. Thus, interesting seem resveratrol' anti-oxidant, anti-inflammatory and anti-carcinogenic properties. This is natural polyphenol present both estrogenic and antiestrogenic features when bound to the ER and therefore has potential SERMs-like (6).

The tumour microenvironment, encircled by the extracellular matrix (ECM), is a mosaic of crucial various cell types that are

responsible for the complex pathways of tumourigenesis, including cell proliferation, motility, apoptosis, and formation new blood vessels. Also, in BC progression, invasion and metastasis, the ECM plays a crucial role (2, 7). The two main classes of ECM macromolecules consist of fibrous proteins and polysaccharide chains belonging to the glycosaminoglycan class (GAG). The fibrous proteins include proteins with a mainly structural function (collagen and elastin) and proteins with a principally adhesive ability (laminins, nidogens, fibronectin and vitronectin) (8). When GAGs bind to proteins, they form proteoglycans, which can be rich in sulphate groups (3). Heparan sulphate proteoglycans (HSPGs) can be anchored to the cell surface and are important proteoglycans present in the ECM (3, 8-10). HSPGs inhibit cancer cell invasion by promoting the interaction between cells or between cells and the ECM. Additionally, HSPGs have a role in maintaining the structural integrity and self-assembly of the ECM (8, 10, 11). Heparan sulphate demonstrates an important role in building the cellular microenvironment and in cell signalling in the basement membrane and ECM through, among others, binding to growth factors: basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), keratinocyte growth factor, and hepatocyte growth factor (HGF), hence, its supporting function in angiogenesis and the distal spreading of the cancer (12).

In eukaryotic organisms there is only one enzyme that is able to enzymatically cleave and degrade the heparin sulphate side chains from HSPGs; this reaction is strongly involved in tumour growth, metastasis, and inflammation (7, 13, 14). This enzyme is named heparanase. It is an endo-B-D-glucuronidase and presents two activities: enzymatic and non-enzymatic, with both functions having an influence on a decrease in the structural integrity of the basement membrane and ECM. The enzymatic activity of the heparanase (58 kDa) dimer consists of 50 kDa and 8 kDa subunits non-covalently linked causing the remodelling of the ECM. Thus, it leads to diffusion of cytokines, growth factors and lipoproteins which can enhance angiogenesis, activation of the immune system, coagulation, acceleration of autophagy and exosome composition (3, 8, 10). Interestingly, an ideal enzymatic action of heparanase is observed in acidic milieu (pH = 5.8 – 6.2), which occurs in tumour microenvironment (14). Additionally, the pro-enzyme heparanase (65 kDa) has non-enzymatic properties and is able to induce the signalling cascade that enhances phosphorylation of selected proteins such as Akt, ERK, p38 and Src. Those activities promote tumour invasion and metastasis (3, 8, 11, 14). Furthermore, non-enzymatic function of heparanase is associated with hypercoagulability state. There is some evidence that heparanase is a cofactor of tissue factor. This function works as a vicious cycle, since tissue factor leads to thrombosis in tumour by elevation of thrombin and fibrin deposition and finally platelets activation. It is well-established that expression of heparanase is limited principally to the platelets, thus in this manner a more heparanase is realized from platelets (8, 14). Moreover, heparanase non-enzymatic property enhances a pro-inflammatory cell adhesion and signal transduction (8).

Additionally, studies indicate that up-regulation of heparanase correlates with enhanced vascular density, and shorter postoperative survival of cancer patients (15, 16). There is no data presenting the impact of heparanase on the number of endothelial progenitors (EPCs) which are involved in vasculogenesis. Mutual interactions between both components could be an insight into the early steps of tumour development, since circulating bone marrow-derived EPCs contribute to tumour neovascularisation (17). The EPCs characterized high proliferative potential and are mobilised during breast cancer development due (4). The aim of the study was to analyse fluctuations in selected angiogenic parameters as well as in the number of circulating endothelial progenitors in respect to low, moderate and high concentrations of heparanase. Additionally, we assessed the diagnostic accuracy of heparanase concentration for disease relapse prediction in breast cancer subjects.

MATERIALS AND METHODS

Patient samples and clinical data

The present study was approved by the Bioethics Committee Collegium Medicum in Bydgoszcz, the Nicolaus Copernicus University in Torun, Poland (reference number: KB/547/2015) and the study was conducted in accordance with the principles embodied in the Declaration of Helsinki. All patients provided written informed consent to participate in this study after a full explanation of the study had been given.

The current study comprised of 86 cases aged 40 to 71 (mean age of 55 years) with newly diagnosed breast malignancy with stage IA-IIB post partial or radical mastectomy, who underwent a comprehensive histopathological confirmation of the diagnosis. All patients also provided a full history and underwent general clinical examinations. Eligible women were recruited between November 2015 and January 2018 by the oncologist from the Clinical Ward of Breast Cancer and Reconstructive Surgery, Oncology Centre Professor F. Lukaszczyk Memorial Hospital, Bydgoszcz, Poland.

The medical records of all the invasive breast cancer (IBrC) patients were reviewed to obtain anthropometric, demographic data, which included age (dichotomised according to a median age of 55 years), menopausal status, parity (defined as full-term pregnancies), smoking history (non-smokers or ever-smoked), weight, height, alcohol intake history, past medical history including any previous history of thromboembolic events, other disorders and any drug intake. In this group, 30 patients (34%) were recognised as overweight and 15 (17%) as obese. The median value for the patients' body mass index (BMI) was 25.08 kg/m^2. Twenty-eight patients (33%) were premenopausal and 58 (67%) were postmenopausal. Baseline patient characteristics are presented in *Table 1*. Full details about the study's recruitment and procedures have been reported in a previous paper (18).

Tumour localisation and size, lymph node status, histological type, histological grade, and molecular subtypes have been obtained from all study participants. TNM (T - tumour, N - node, M - metastasis) classification was performed by a histopathological specimen in all cases. The breast cancer patients were free of distant metastasis (M0). The median tumour diameter was 1.5 cm (range 0.5 – 3.5 cm). Twenty cases (23%) showed lymph node metastasis (N1). The clinical staging was performed according to the AJCC 7th Edition Staging for Breast Cancer. There were 41 (48%) patients with stage IA (T1N0M0 - T1 tumours without nodal micrometastases) and 39 (45%) cases with stage IIA (T1N1M0 - T1 tumours with nodal micrometastases or T2N0M0 - T2 without nodal micrometastases), while six cases (7%) had stage IIB, which was defined by T2N1M0 - T2 with nodal micrometastases. Histological grading according to Elston-Ellis classification was available for all patients, consisting of five cases in grade 1, 65 subjects developed grade 2 and 16 patients had grade 3. The most common histological IBrC type was invasive ductal carcinoma, accounting for 88% (n = 76), followed by invasive lobular carcinoma (n = 10; 12%). Patients were stratified by molecular subtype according to an immunohistochemical marker profile including oestrogen and progesterone receptors (ER, PR, respectively), human epidermal growth factor receptor 2 (HER2), Ki67, and E-cadherin expressions. The positive expression rates for ER, PR and HER2 were established in 87%, 80% and 12% of cases, respectively. The clinical and

Table 1. The heparanase concentration according to demographic and anthropometrical determinants in study population.

Feature	Number of patients (%)	Heparanase concentration (pg/mL)	P-values
	86 (100%)	176.98 137.80/268.29	
Age			
< 55 years	45 (52%)	174.74 135.68/270.91	0.8471
≥ 55 years	41 (48%)	179.21 137.80/245.32	
Menopausal status			
Premenopausal	28 (33%)	165.88 124.13/241.82	0.3567
Postmenopausal	58 (67%)	185.97 137.80/268.29	
Body mass index			
≤ 24.9 kg/m^2	41 (48%)	170.30 135.68/252.87	0.5597
25 – 29.9 kg/m^2	30 (35%)	209.05 137.80/286.91	
30 – 39.9 kg/m2	15 (17%)	157.13 137.80/257.96	

pathological characteristics of the patients are summarised in *Tables 2* and *3*.

Inclusion and exclusion criteria for study participants

The major inclusion criteria were primary, invasive, unilateral, early-stage breast cancer and good general health and wellbeing. Exclusion criteria were male gender, carcinoma in situ, T3-T4 tumours, stage after IIB, neoadjuvant treatment and locally advanced or metastatic cancer. Additional exclusions were as follows: the presence of uncontrolled cardio-vascular disease, lung diseases, liver diseases, thyroid impairment, overt diabetes mellitus, dyslipidaemia, cerebral-vascular diseases, pre-existing chronic autoimmune or inflammatory disease, recent bleeding or thrombotic events.

Patient follow-up information

Follow-up was completed in 86 patients. For the relapse free survival analysis ten events occurred and follow-up ranged from 19 to 41 months (median follow-up was 33.5 months) with a 11.6 % recurrence rate. Follow-up times were calculated from the date of the initial visit until the earliest event of interest, *i.e*. disease spread, death or the last date of contact as of the end of March 2019, and were expressed in months.

Immunohistochemistry analysis

Formalin-fixed and paraffin-embedded tumour sections were used for the immunohistochemistry detection of oestrogen receptors (ER), progesterone receptors (PR), human epidermal growth factor receptor 2 (HER-2) and Ki67 expression. The ER and PR status were defined in accordance with the recommendations of the ASCO/CAP using SP1 and 1E2 primary antibodies (Ventana Medical Systems, Tucson, Arizona, USA), respectively. Receptor status is assessed as positive if there are at least 1% of tumour cells observed with nuclear staining and negative if the nuclear staining is completely absent. For the semi-quantitative detection of HER2, the rabbit monoclonal primary antibody VENTANA anti-HER2/neu (4B5) was used with a VENTANA aperture for staining the IHC microscopic slide (Benchmark Ultra, Roche-Ventana). HER2 scores were assessed using the standard ASCO/CAP guideline reporting system on a scale of 0, 1+, 2+ and 3+. Tumours with 0 or 1+ scores were considered as HER2-negative and those with 3+ scores were considered as HER2-positive. Tumours with 2+ scores were regarded as equivocal and tested by fluorescence in situ hybridisation (FISH). The Ki-67 antigen was scored as a percentage of nuclei-stained cells of all the cancer cells by using a monoclonal mouse antibody (Auto-stainer Link 48, Agilent Technologies, USA). The cut-off value for the Ki-67 proliferation index was 14% in all statistical analysis.

Blood collection and laboratory measurements
Enzyme-linked immunosorbent assay (ELISA)

Venous blood (4.5 ml) for testing for heparanase, vascular endothelial growth factor (VEGF-A), and its soluble forms of receptor types 1 and 2 and for the determination of the number endothelial progenitors (EPCs) was collected under standard operating procedures and conditions into tubes (Becton Dickinson Vacutainer® System, Plymouth, UK) containing potassium ethylenediaminetetraacetic acid (K_2EDTA). Whole blood samples were processed within 2 h after collection in order to estimate the accurate number of circulating EPCs. The remaining blood samples were immediately mixed and centrifuged at 3000 × *g* at +4°C for 15 min and then frozen at –80°C (as specified by the manufacturer) until assayed within six months.

Table 2. The heparanase concentration according to molecular characteristics in breast cancer cases.

Feature	Number of patients (%)	Heparanase concentration (pg/mL)	P-values
Expression of Ki 67			
< 15%	45 (52%)	165.88 135.68/213.74	0.0961
≥ 15%	41 (48%)	192.80 142.05/284.21	
Expression of HER2			
Negative	76 (88%)	183.71 137.80/266.99	0.6762
Positive	10 (12%)	159.31 137.80/378.48	
Hormone receptor status			
ER+	75 (87%)	165.88 135.68/257.96	**0.0196**
ER–	11 (13%)	268.29 213.74/378.49	
PgR+	69 (80%)	165.88 135.68/240.35	**0.0163**
PgR–	17 (20%)	265.69 197.40/378.49	
E-cadherin status			
Positive	81 (94%)	183.71 137.80/268.29	0.3145
Negative	5 (6%)	152.79 133.57/179.21	

ER, oestrogen receptor; HER2, human epidermal growth factor receptor 2; Ki67, proliferation marker; PR, progesterone receptor; significant differences are denoted by bold P-values; an underlined P-value represents closeness to statistical significance.

ELISA kits were used to quantify the EDTA-plasma concentrations of VEGF-A, the soluble form of VEGF receptors type 1 and 2 (VEGF-A; sVEGFR1/Flt-1; sVEGFR2/KDR Immunoassay Test, Quantikine, R&D systems, USA - respectively), and heparanase (ELISA Kit for Heparanase; HPA; Cloud-Colne Corp., TX, USA). The reaction mixture was added to a 96-well plate (18).

Flow cytometry

Determination of circulating endothelial progenitor cells with the immunophenotype CD34+CD133+CD31+CD45- were made by flow cytometry according to the methodological procedures, which had previously been published (18). All assays were run according to the manufacturer's specifications by personnel with no access to the clinical data of the patients.

Statistical analysis

All statistical analyses were conducted using Statistica v. 13.1 (StatStoft®, Cracow, Poland). The normality of the data set was tested by the Shapiro-Wilk test. Data are presented as percentages or medians and interquartile ranges (IQR) for non-normally distributed variables. Statistical differences between subgroups were determined with the U Mann-Whitney test or the ANOVA Kruskal-Wallis test. Receiver operating characteristic (ROC) curves were used to assess the diagnostic predictive capacity of the selected analysed biomarkers. The area under the curve (AUC) was calculated in order to estimate the diagnostic accuracy. The optimal cut-off points were determined according to Youden criteria. The multivariate Cox's regression analysis was used to determine the predictors for progression-free survival. The progression-free survival (PFS) time was calculated from the date of enrolment until the relapse or progression of the disease. PFS curves were calculated by the Kaplan-Meier method and the significance levels were assessed according to the log-rank test. P values less than 0.05 were considered statistically significant in all analyses.

RESULTS

Clinical summarisation of the study population

The mean patient age was 55 years. There were patients who underwent breast conserving surgery in 69 cases (80%) and who underwent mastectomy in 17 (20%). In the histological differentiation of IBrC, patients had differentiated invasive

Table 3. The heparanase concentration in respect to clinical and pathological characteristics in breast cancer subjects.

Feature	Number of patients (%)	Heparanase concentration (pg/ml)	P-values
Tumour localisation			
Left breast	44 (52%)	190.52 138.86/281.53	0.3464
Right breast	42 (48%)	165.88 135.68/252.87	
Molecular subtypes			
Luminal A	50 (58%)	165.88 133.57/257.96	0.3618
Luminal B HER2-	17 (20%)	188.24 157.13/265.69	
Luminal B HER2+ and non-luminal HER2+	10 (12%)	159.31 137.80/378.48	
Triple negative	9 (11%)	245.32 213.74/284.21	
Tumour diameter			
T1 (2 cm)	60 (70%)	165.88 135.68/242.84	**0.0222**
T2 (≥ 2cm < 5cm)	26 (30%)	219.71 157.13/286.91	
Nodal status			
N0	66 (77%)	179.23 137.80/270.91	0.5363
N1	20 (23%)	169.26 133.57/255.51	
Stage of disease			
IA	41 (48%)	165.88 137.80/228.10	0.3285
IIA + IIB	45 (52%)	190.52 135.68/268.29	
Elston and Ellis grade			
G1	5 (6%)	240.35 154.96/286.91	0.5264
G2	65 (76%)	174.74 135.68/268.29	
G3	16 (18%)	219.71 131.49/274.95	
Histological type			
Ductal	76 (88%)	174.74 137.80/268.29	0.9023
Lobular	10 (12%)	184.87 135.68/240.35	

A significant difference is denoted by bold P-value.

breast cancer in 70 cases (82%) including those with well- and moderate-differentiated breast carcinomas. There were also 16 patients with poor differentiated cancers (18%). In terms of the T stage, there were 60 T1 cases (70%) and 26 T2 cases (30%). In terms of the N stage, there were 66 N0 cases (77%) and 20 N1 subjects (23%). In terms of the TNM staging system, there were

Table 4. Vasculogenic and angiogenic biomarkers according to heparanase concentration.

Parameter [units]	**Heparanase low concentration** (< 140 pg/mL) n = 25	**Heparanase moderate concentration** (140 – 200 pg/mL) n = 27	**Heparanase high concentration** (> 200 pg/mL) n = 34	**P-values**
Circulating EPCs [cell/uL]	5.63 2.85/10.55	12.06 5.62/26.63	12.11 6.34/23.02	**0.0458**
VEGF-A concentration [pg/mL]	54.98 28.16/87.85	59.70 38.68/102.82	93.6 41.02/150.08	0.2037
sVEGFR1 concentration [pg/mL]	38.84 20.67/89.14	52.35 22.46/97.24	22.59 12.86/30.57	**0.0011**
sVEGFR2 concentration [pg/mL]	8181.14 7470.37/8967.11	9868.01 8358.41/12426.22	10486.90 8962.15/13107.40	**0.0108**
sVEGFR1/VEGF-A	0.95 0.48/1.27	0.69 0.37/2.32	0.23 0.12/0.50	**0.0006**
sVEGFR2/VEGF-A	152.14 94.78/242.12	200.64 107.65/241.18	128.63 72.59/219.31	0.4393
sVEGFR2/sVEGFR1	152.76 97.51/392.68	205.94 98.54/493.39	492.15 324.98/1016.78	**0.0018**

Circulating EPCs, circulating endothelial progenitor cells; VEGF-A, vascular endothelial growth factor A; sVEGFR1, soluble form of vascular endothelial growth factor receptor type 1; sVEGFR2, soluble form of vascular endothelial growth factor receptor type 2. Significant differences are denoted by bold P-values.

41 stage I cases (48%) and 45 stage II cases (52%). The mean diameter of the tumour was 1.50 cm. Of the patients suffering from breast cancer, 84 (97.7%) survived, and eight patients (9.3%) developed disease recurrence. Finally, the relapse-free survival rate was 88.4%.

Interestingly, among breast cancer patients the heparanase concentrations were similar in respect to age, menopausal status and body mass index (BMI) (*Table 1*). It may suggest that all of these factors did not have an influence on the heparanase concentration.

Tables 2 and *3* presents variabilities in the heparanase concentration in respect to molecular and clinical characteristics. According to the molecular determinants measured by the IHC method there were higher levels of heparanase in oestrogen and progesterone receptor negative cancers (P = 0.0196 and P = 0.0163, respectively) than in positive ones. Furthermore, based on clinical classification (TNM), a higher concentration of heparanase was noted in T2 cases (tumour diameters ≥ 2 < 5 cm) than T1 subjects (< 2 cm) (P = 0.0222). In addition, there was tendency towards a significance value in respect to the expression of Ki67. Tumours with an overexpression of Ki67 demonstrate a higher concentration of heparanase than tumours with a lower expression of Ki67 (P = 0.0961).

Furthermore, we carried out an analysis of VEGF-A, sVEGFR1, sVEGFR2 concentrations, sVEGFR1/VEGF-A and sVEGFR2/VEGF-A ratios as well as the number of circulating endothelial progenitors according to heparanase concentrations. We divided breast cancer patients into three subgroups - with low (< 140 pg/ml), moderate (> 140 and < 200 pg/ml) and high (> 200 pg/ml) concentrations of heparanase. Statistically significant positive associations between circulating EPCs, sVEGFR2 and increasing plasma levels of heparanase were noted (P = 0.0458, P = 0.0108, respectively). A statistically significant association between the sVEGFR1 concentration and increasing plasma levels of heparanase was reported. For low and moderate concentrations of heparanase the level of sVEGFR1 increases respectively, but surprisingly for high concentrations of heparanase, the level of sVEGFR1 essentially dropped in regard to low and moderate concentrations of heparanase (P = 0.0011). The juxtaposition of the sVEGFR1 and VEGF-A in the ratio (sVEGFR1/VEGF-A) indicates a negative link with heparanase (P = 0.0006), since sVEGFR1 presents strong anti-angiogenic properties while VEGF-A is a powerful pro-angiogenic agent. Thus, the anti-angiogenic potential expressed by sVEGFR1/VEGF-A decreased with a simultaneous increase in heparanase concentration. Based on the juxtaposition of both receptors in the ratio sVEGFR2/sVEGFR1, we have shown a positive association of the sVEGFR2/sVEGFR1 ratio with heparanase concentration (P = 0.0018) which indicates that in more advanced processes sVEGFR2 is more involved then sVEGFR1 (*Table 4*).

The ROC curves for separate laboratory determinants were designed and the areas under the curve with 95% confidence interval were specified (AUC, 95% CI thresholds with sensitivity (SE) and specificity (SP)). We appraised the ROC curves in order to estimate the diagnostic accuracies of the investigated variables for the prediction of disease relapse. The borderline of diagnostic usefulness of the test, according to the area under the ROC curve (AUC^{ROC}) ≥ 0.5; P < 0.05 was reached only for heparanase and sVEGFR1. Although the AUC^{ROC} for circulating EPCs and VEGF-A were above 0.5, the P values were > 0.05, thus the Youden Index cut-off points were not determined. Based on the AUC^{ROC}, the Youden index cut-off values were identified to maximise the sum of sensitivity and specificity. Heparanase was the most accurate biomarker with an AUC^{ROC} = 0.72 (95% CI: 0.5573 – 0.8754). Using the Youden

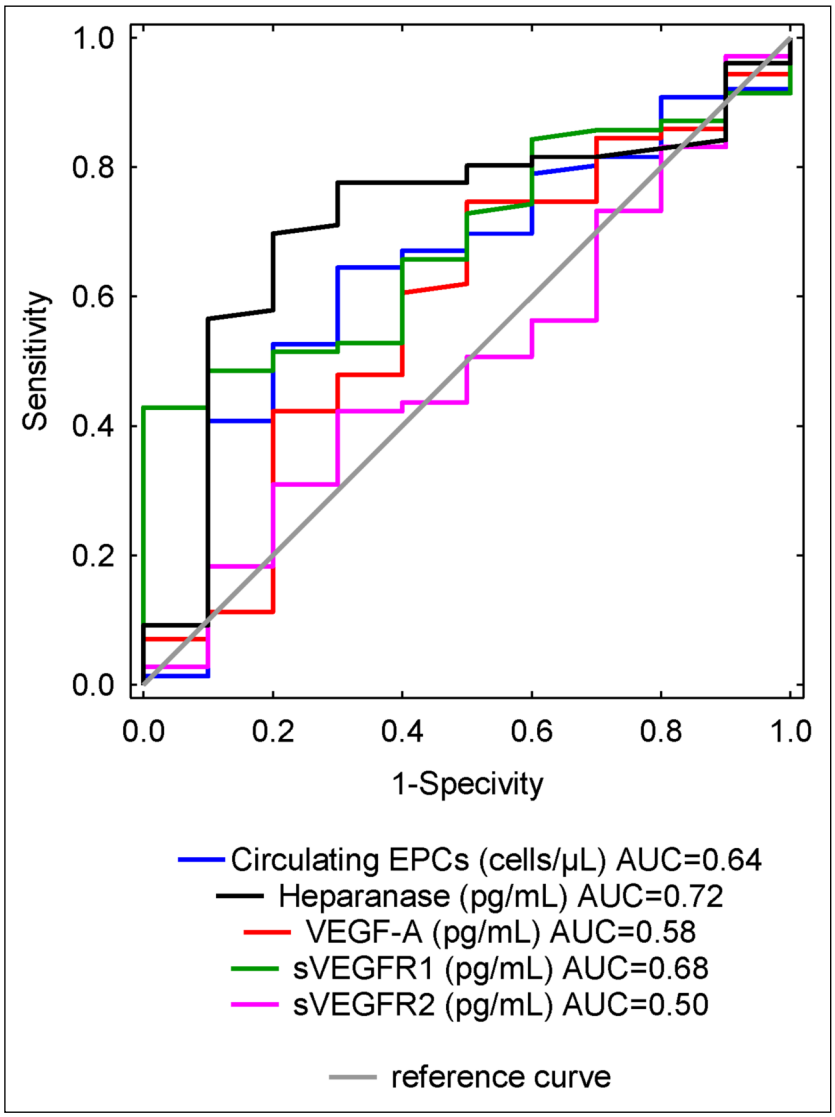

Fig. 1. ROC curves of measured parameters in patients with versus without progression free survival.

index cut-off value, we identified a plasma heparanase concentration of 213.74 pg/mL with a sensitivity of 70% and a specificity of 80% as the best cut-off value to discriminate between disease recurrence patients and those without disease relapse. Additionally, we noticed that the heparanase area under the ROC curve was higher than areas for sVEGFR1 (AUC^{ROC} = 0.68, 95% CI: 0.5487 – 0.8213). According to the receiver operating characteristic curve, we defined the Youden index cut-off values for sVEGFR1 in order to distinguish disease recurrence patients and non-disease relapse subjects. Thus, for sVEGFR1 concentrations, the Youden index cut-off value was 37.81 pg/mL with 100% specificity and 43% sensitivity (*Fig. 1*).

Thus, based on the ROC curves for separate laboratory parameters we designed a ROC curve for the tested model consisting of heparanase, VEGF-A, sVEGFR1, sVEGFR2 concentrations and the number of circulating EPCs. For all the analysed parameters creating this model, the area under the curve was 0.8132 and P = 0.0064 (*Fig. 2*). This model points to a strong diagnostic potential for the prediction of disease progression.

Finally, we performed a Cox's regression model in order to predict which analysed parameters present the diagnostic power to estimate the recurrence-free survival rate, which takes into account the function of time (*Table 5*). The strongest predictor values of disease relapse were demonstrated by VEGF-A and the soluble VEGF receptor type 2 in our study population (P = 0.0303 and P = 0.0488 respectively).

All patients in this study received regular follow-up for 19 to 41 months (median follow-up 33.5 months) after discharge. For the progression-free survival analysis, ten events occurred with a PFS rate of 11.6%. Two (2.3%) patients died during the follow-up period due to systemic metastatic disease. One subject presented a low baseline concentration of heparanase and the second one had a high baseline heparanase level (106.58 pg/mL and 265.69 pg/mL, respectively). More importantly, follow-up revealed a significantly higher incidence of disease relapse in breast cancer patients with high baseline concentrations of heparanase compared with those with low and moderate concentrations of heparanase (P = 0.0304). Twenty-five patients (29%) demonstrated a low baseline concentration of heparanase (< 140 pg/mL), whereas 27 patients (31.4%) had a moderate heparanase level (140 – 200 pg/mL) and 34 cases (39.6%) had a high concentration of heparanase (> 200 pg/mL). Recurrence of the disease in the group of patients with a low concentration of heparanase occurred in one out of 25 (4%),

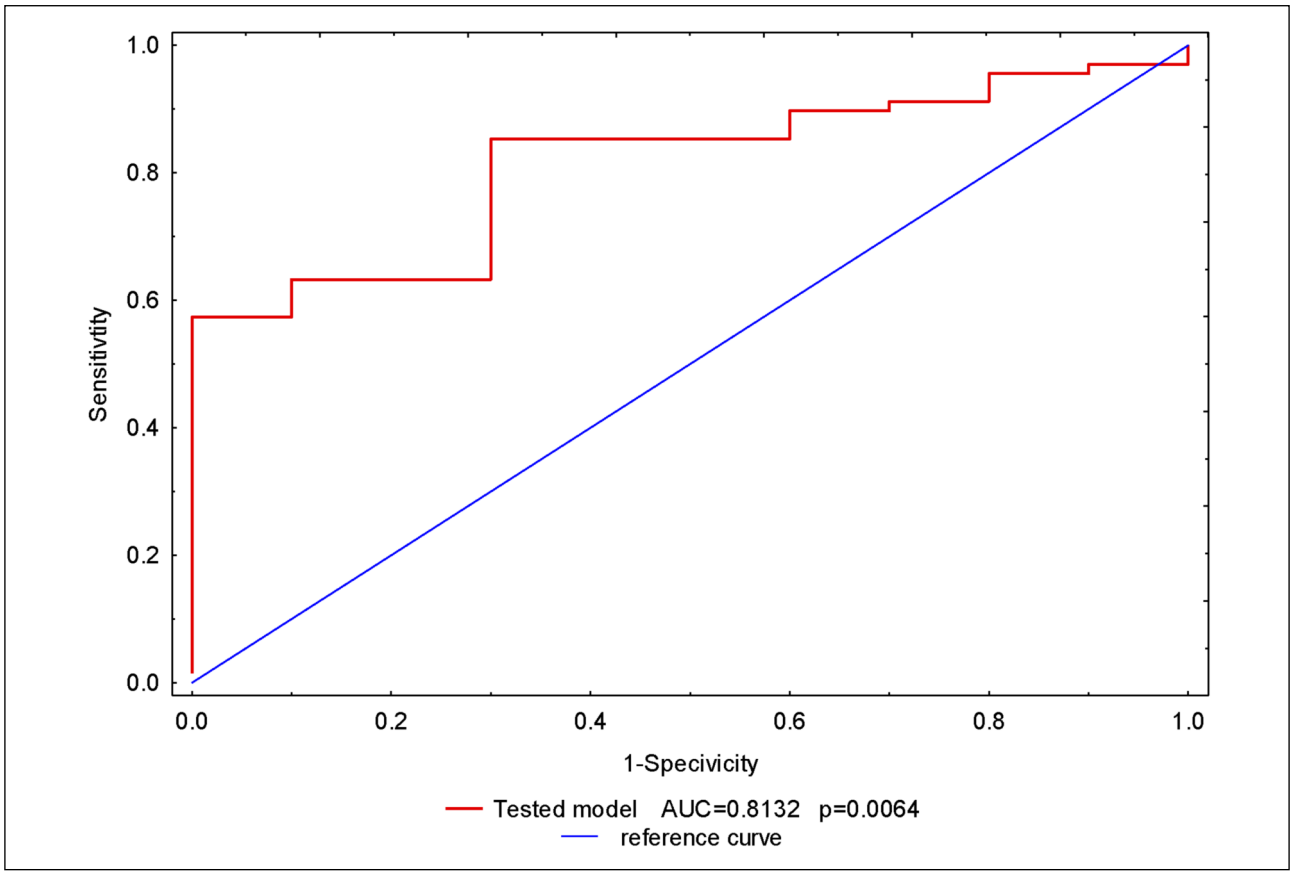

Fig. 2. ROC curves for tested model in patients with versus without disease progression.

Table 5. COXs regression models foir progression free survival.

Characteristics	HR	95% CI		P
Heparanase	0.9966	–0.0111	0.0042	0.3793
Circulating EPCs	1.0038	–0.0035	0.0111	0.3026
VEGF-A	1.0087	0.0008	0.0165	**0.0303**
sVEGFR1	0.9764	–0.0572	0.0095	0.1611
sVEGFR2	0.9996	–0.0007	–0.0001	**0.0488**

Circulating EPCs, circulating endothelial progenitor cells; VEGF-A, vascular endothelial growth factor A; sVEGFR1, soluble form of vascular endothelial growth factor receptor type 1; sVEGFR2, soluble form of vascular endothelial growth factor receptor type 2. Significant differences are denoted by bold P-values.

with a similar observation noted in patients with a moderate heparanase concentration, where disease relapse happened in one out of 27 (3.7%). However, in the group with a high baseline concentration of heparanase, eight out of 34 (23.5%) cases had a recurrence of the disease. We postulate that a high concentration of heparanase could be predictive of a shorter progressive-free survival (*Fig. 3*).

DISCUSSION

Breast cancer characteristics are extremely complex in respect to the genetic, molecular, histopathological, prognostic and treatment approaches. In conventional clinical practice, tumour size, histological subtype, tumour grade, stage, lymph node metastasis, and molecular determinants are applied as prognostic markers (19, 20). However, all mentioned tumour-related features do not successfully predict disease relapse. Interaction between metastasis tumour cells and vascular endothelial cells, lead to alterations in the haemostatic system, disruption of vascular endothelium and finally to tumour cells spread to distant organs (21). The dissemination of tumour cells throughout the body leads to the majority of cancer patient deaths and poses an essential clinical barrier to the solid malignant neoplasm treatment approach.

Heparan sulfate proteoglycans, throughout their co-operations as co-receptors with several bioactive compounds including bFGF, HGF, IL-8, and VEGF control their signalling and distribution. Heparanase is an endo-β-D-glucuronidase with the ability to cleavage the heparan sulfate side chains of the ECM. This property is associated with disintegration of the extracellular matrix by formation of oligosaccharides. Importantly, these 10 – 20 sugar residues exert stronger bioactivity than the native heparan sulphate, leading to stimulation of growth factors,

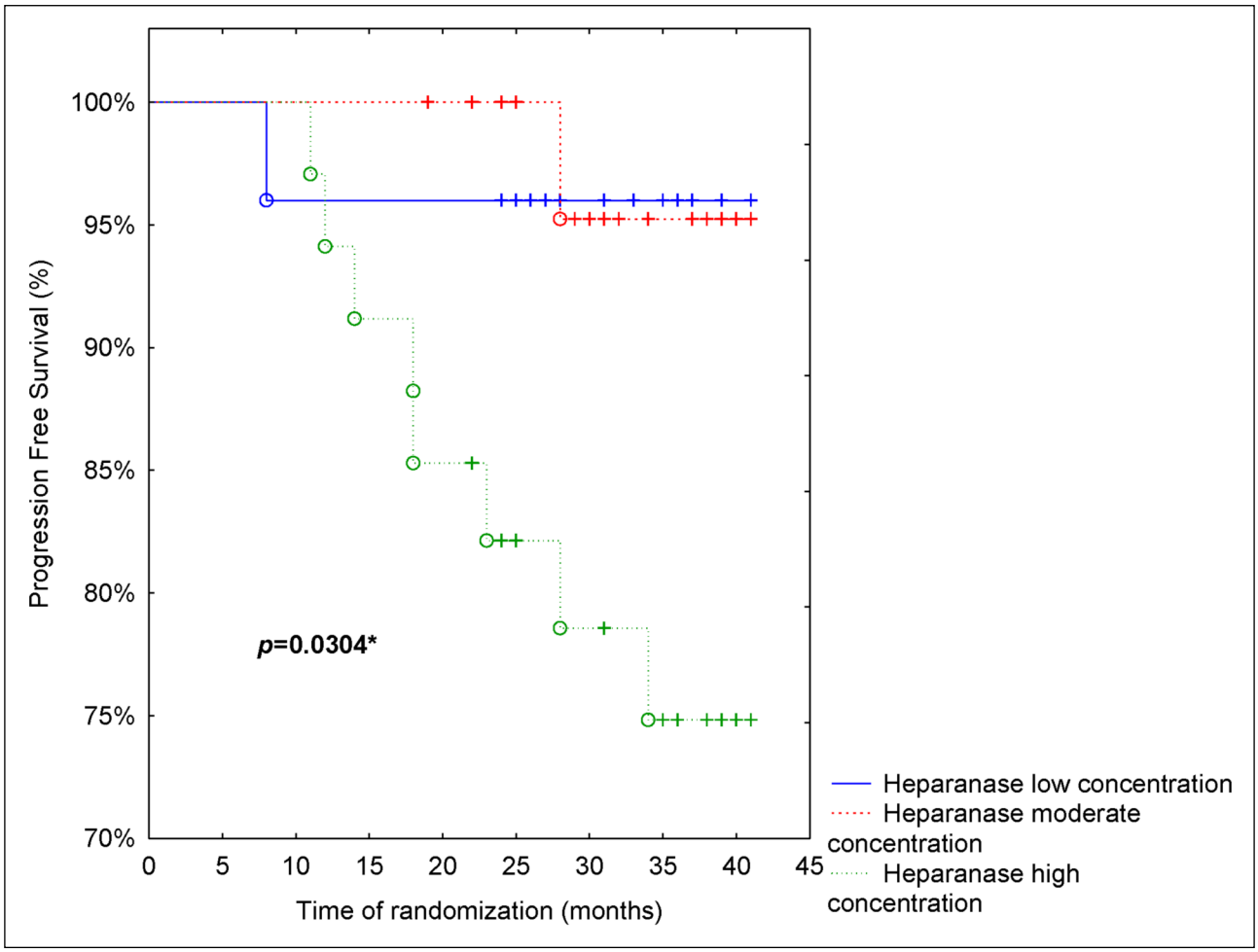

Fig. 3. Kaplan-Meier curve for the progression-free survival (PSF) analysis of the entire population with IBrC according to low, moderate and high heparanase concentration.

cytokines, and angiogenic factors, triggering cell proliferation, cell differentiation and angiogenesis, which induce tumour development as well as tumour cells dissemination (22-24). A high concentration of heparanase is considered to be a negative prognostic biomarker in selected types of cancer (11). There were established that patients with heparanase-positive tumours demonstrated a significantly higher probability of local and distant metastases as well as shorter post-operative survival, in respect to heparanase-negative cases (11, 14).

In our study we observed that the concentration of heparanase varies in accordance to tumour diameter, oestrogen and progesterone receptor expression and slightly with the percentage of Ki67. In our patient population, the mean tumour diameter was 1.5 cm: only 30% of patients had tumours over 2 cm but less than 3.5 cm and in those patients a higher concentration of heparanase was reported, which is consistent with the studies reported by Tang *et al*. (25, 26). Also, they observed in their previous study that heparanase expression was positively connected with a large size of breast cancer tumour, high clinical stage, and lymph node metastasis (26). The authors claim that a high level of heparanase is associated with tumour progression and metastatic ability. Additionally, the authors observed a higher concentration of heparanase in metastatic breast cancer compared with M0 breast cancer, benign disease, and healthy controls. They suggest that heparanase could be used as a potential biomarker for breast cancer recognition, the early phase of tumour spread and worse future outcomes (25).

Furthermore, our studies have shown that the heparanase concentration in hormonal receptor-positive (ER+, PR+) tumours is significantly lower than in tumours with hormone receptor-negative tumours (ER–, PR–). These results confirm the thesis that positive hormone receptor status is associated with a more favourable prognosis in breast cancer patients, due to its lower histological grade, lower degree of nuclear pleomorphism, lower proliferation index and, as a result of our study, lower heparanase concentration and diminished pro-metastatic phenotype. Indeed, hormone receptor-negative breast tumours are less frequently diagnosed, approximately in 25% of all cases. In our study, the ER-negative subgroup consisted of only 11 patients (12.8%) and 17 cases (19.3%) made up the PR-negative subgroup, which is in line with Yersal and Barutca's analysis (20). Negative hormone receptor status with a higher concentration of heparanase is associated with more advanced clinical characteristics and a worse prognosis since the ER/PR negative subgroup of breast cancer is created by a non-luminal HER2+ and a triple negative molecular subtype. Both of these confer more aggressive biological and clinical cancer behaviour. These tumours are highly proliferative, show poor tubule formation, and present a higher histological and nuclear grade (20). Thus, a high concentration of heparanase next to tumour size and oestrogen and progesterone receptor expression may serve as an indicator of a more aggressive character of tumour cells and a shorter survival rate. However, further study of this issue is needed, as Elkin *et al*. (27) determined homologous sequences of an oestrogen response element in the

heparanase gene promoter region and they reported that oestrogen induces transcriptional activity of the heparanase promoter. The authors observed that the oestrogen-dependent increase in heparanase levels led to enhanced neovascularisation. The oestrogen impact on heparanase expression was reported by Elkin *et al*. only in oestrogen receptor-positive, but not in oestrogen receptor-negative, breast carcinoma cell lines (27). Moreover, Cohen *et al*. (28) observed a significant up-regulation of heparanase in primary ER+ breast cancer in respect to the ER- subgroup. The authors claim that the ER-dependent heparanase regulation mechanism works *in vivo*, but the 15% incidence of heparanase expression in ER-negative tumours implies alternative molecular mechanisms are accountable for the increase of heparanase expression in the subgroup of ER-negative breast cancers (28). Most likely, the divergent and controversial results are due to the wide variety of detection methods of heparanase (immunoenzymatic, immunohistochemical staining, RT-PCR analysis) as well as the application of different biological materials (plasma, serum, tissue). Thus, this complicates adequate interpretation of published results.

Additional purpose of the current study was to analyse different plasma heparanase levels (low, moderate, high) in the context of VEGF-A, sVEGFR1, sVEGFR2 concentrations and sVEGFR1/VEGF-A, sVEGFR2/VEGF-A ratios and the number of circulating EPCs in primary, invasive breast cancer patients with early stage (IA-IIB) and demonstration of dependencies between them. It is well-established that heparanase stimulates carcinogenesis, angiogenesis, and cancer cell dissemination. The molecular mechanism of these heparanase properties is complex but most likely is based on heparan sulphate proteoglycans degradation, thereby eliminating its activity as an extracellular matrix protector. Moreover, growth factors that are bound by heparan sulphate, such as fibroblast growth factor and VEGF, are released, thus promoting tumour growth and spread. Finally, biologically active heparan sulphate fragments secreted by heparanase action set up a microenvironment that supports tumour cell survival (3, 7, 28). A significant increase in the number of EPCs with a heparanase concentration elevation was noted. Masola *et al*. pointed out that heparanase releases a combination of heparan sulphate-bound growth factors especially VEGF, which support neoangiogenesis. Also, increase of EPCs mobilization, differentiation could be caused by VEGF-dependent enhancement, since VEGF is a powerful EPCs promoter. Neovascularisation is also increased by the non-enzymatic action of heparanase by overexpression of VEGF *via* p38-phosphorylation and Src kinase (9). From our data, we can suggest that heparanase may mediate the interaction between vasculogenesis and angiogenesis, facilitating cell invasion in the cancer microenvironment. This observation is in line with Vlodavsky *et al*., (24) which claimed that heparanase not only support tumour spread, but also involves in the angiogenic switch and further cancer growth.

Extremely interesting is the fact that we observed a relevant association between sVEGFR1 concentration and increasing plasma levels of heparanase, but this dependence only persisted for low and moderate concentrations of heparanase. For high concentrations of heparanase, the level of sVEGFR1 essentially was lower in regard to low and moderate concentrations of heparanase. Soluble VEGFR1 presents a high affinity for VEGF-A and acts as a suppressor of the proliferation and migration of endothelial cells and the formation of new blood vessels in the course of malignant tumours (9, 29). Sela *et al*. (9) demonstrated that the soluble form of VEGFR1 is stored in the proximity of the produced cell, despite being a protein secreted outside the cell. Local accumulation of sVEGFR1 is mediated by cell-bound or ECM deposited heparan sulphate, and thus prevention of its systemic release may also be required to avoid massive neutralisation of VEGF in distant organs. Thus, heparan chains of the extracellular matrix may serve as a natural pool of sVEGFR1 (30). Sela *et al*. noticed that heparanase exerts a specific role in sVEGFR1 release (9). Eddy *et al*. suggest that increase in heparanase level and activity may lead to increased sVEGFR1 in the maternal circulation in preeclampsia cases (30). Our results indicate that lost of sVEGFR1 increasing trend with an elevation of heparanase could be due to the sVEGFR1 overconsumption. Since, heparanase stimulates angiogenic switch, thus a more VEGF is released from endothelial cells as well as cancer cells, thus enhancing angiogenesis, tumour cell dissemination and increases in vessel density. With the elevation of heparanase the over-activation of angiogenesis with a simultaneous reduction of anti-angiogenic properties has been observed. Additionally, we speculate that low sVEGFR1 level in heparanase-high patients is caused by lost of anti-angiogenic capacity by sVEGFR1 due to creation of VEGFR-sVEGFR1 or VEGF-sVEGFR1 complexes.

Additionally, the anti-angiogenic ability expressed by the sVEGFR1/VEGF-A ratio was negatively associated with the heparanase concentration, which indicates on heparanase-dependent negative impact on anti-angiogenic activity of the sVEGFR1. Juxtaposition of both anti- and pro-angiogenic agents may serve as a marker of anti-angiogenic activity. Interestingly, Saito *et al*. (29) observed the stimulation of the sVEGFR1 expression by VEGF-A occurs by the PKC-MEK pathway. Since it is well-established that the primary angiogenic activity of VEGF-A is mainly mediated through VEGFR2 and its downstream PKC-MEK-ERK pathways, these concepts indicate that VEGF-A-induced sVEGFR1 expression may work as a negative feedback axis in order to control angiogenic processes (29). Therefore, it is reasonable to juxtapose these factors with each other, since mutual interactions between these agents have been proven. Saito *et al*. (29) discovered the mechanism through which VEGF-A induces sVEGFR1 in endothelial cells *via* controlling the alternative splicing of the VEGFR1 gene. They suggest a lucid mutual interaction between VEGF-A and its inhibitor - sVEGFR1. Moreover, the lower plasma level of sVEGFR1 may be associated with the creation of the VEGF/sVEGFR1 complexes and may reflect the decreased plasma sVEGFR1 levels. Furthermore, the lower plasma VEGFR1 level with a simultaneous higher concentration of heparanase could be a possible mechanism for VEGF-mediated vascular permeability, due to less VEGF suppression.

Based on the juxtaposition of both receptors in the ratio: sVEGFR2/sVEGFR1, we have shown a positive association of the sVEGFR2/sVEGFR1 ratio with heparanase concentration ($P = 0.0018$), which indicates that in more advanced processes sVEGFR2 is more involved then sVEGFR1, most likely due to sVEGFR1 consumption or by limiting its secretion, since both soluble type 1 and 2 receptors exert anti-angiogenic functions and physiologically prevents vascular permeability (31). It is well-known that the affinity of VEGF for VEGFR2 is 10-fold less than its affinity for VEGFR1. On the other hand, in the case of a higher concentration of heparanase it may be sVEGFR2 takes advantage due to its role in lymph angiogenesis regulation (32). Taken together, our results suggest that vasculogenesis, angiogenesis and tumour cell spread are tightly regulated by the balance between pro- and anti-angiogenic agents as well as heparanase concentration, and that any fluctuations between them have the potential to induce cancer progression.

The Cox regression model consisting of all analysed variables, taking into account the function of time, revealed that VEGF-A and sVEGFR2 have a strong predictive value for disease relapse. However, according to the Kaplan-Meier curves, we can suggest that a high concentration of heparanase above 200 pg/mL may indicate the aggressive character of the tumour and a high possibility of breast cancer metastasis. This suggestion is based on the recurrence rate of 23.5% in those cases. Tang *et al*.'s studies

confirmed our observation that the up-regulation of heparanase concentration is associated with increases in tumour cell metastasis and poor prognosis due to chemo-resistance (25, 26). Our study indicates that the baseline concentration of heparanase may be used as a suitable, non-invasive prognostic biomarker next to tumour size and oestrogen and progesterone receptor expression. Besides that, these results were supported by further statistical analysis (ROC curves), which also indicate that in patients with higher concentrations of heparanase, the higher rate of disease relapse events or death occurs. Heparanase concentration reached the best AUC^{ROC} = 0.72 (95% CI: 0.5573 – 0.8754) from all analysed variables. The cut-off value of 213.74 pg/mL with 70% sensitivity and 80% specificity was identified in order to discriminate between disease recurrence patients and those without disease relapse. Furthermore, we performed a ROC curve for the tested model consisting of heparanase, VEGF-A, sVEGFR1, sVEGFR2 concentrations and the number of circulating EPCs. For all the analysed parameters creating this model, the area under the curve was 0.8132. This result points out that pro-vasculogenic, angiogenic agents as well as heparanase may exhibit strong diagnostic power in disease progression prediction.

Taken together, the link between heparanase and vasculogenesis and angiogenesis is crucial in breast cancer progression. Based on our study, we postulate that the concentration of heparanase can predict disease relapse. According to the Yuden index we point out that the cut-off point of 213.74 pg/mL of heparanase concentration assessed in plasma by an immunoenzymatic method may serve as a value which discriminates between disease recurrence patients and those without disease relapse. Additionally, a high concentration of heparanase next to tumour size and oestrogen and progesterone receptor expression may serve as an indicator of a more an aggressive character of tumour cells and a shorter survival rate.

Abbreviations: BC, breast cancer; bFGF, basic fibroblast growth factor; BMI, body mass index; ECM, extracellular matrix; EPCs, endothelial progenitor cells; ER, oestrogen receptors; GAG, glycosaminoglycan; HER2, human epidermal growth factor; HGF, hepatocyte growth factor; HSPGs, heparan sulfate proteoglycans; IBrC, invasive breast cancer; IHC, immunohistochemical; KGK, keratinocyte growth factor; ki67, proliferative marker; PR, progesterone receptor; SERM, selective oestrogen receptor modulator; sVEGFR1, soluble form of vascular endothelial growth factor receptor type 1; sVEGFR2, soluble form of vascular endothelial growth factor receptor type 2; VEGF-A, vascular endothelial growth factor A; TNM classification: T- tumour, N- node, M- metastasis.

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