Neoplasma 2020; 67(5): 1146–1156

doi:10.4149/neo_2020_191022N1076

Evaluation of the prognostic value of fibrinolytic elements in invasive breast carcinoma patients

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Received October 22, 2019 / Accepted January 22, 2020

Breast cancer (BrC) is one of the most serious oncological problems in the world. The aim of the study was to evaluate concentrations of tissue plasminogen activator (t-PA) and plasminogen activator inhibitor type 1 (PAI-1) and their ratios: t-PA/PAI-1 and PAI-1/t-PA in breast cancer patients and in healthy individuals and to estimate the ability of fibrinolytic parameters in predicting neoplasm disease and disease relapse. One hundred and five women were enrolled in the study, including 60 cases with primary BrC, (M0) and 45 healthy females. Follow-up was completed in all BrC patients with a 16.7% recurrence rate. An immunoassay of t-PA, PAI-1 in all cases was made as well as the immunohistochemistry of estrogen and progesterone receptors, human epidermal growth factor receptor 2, E-cadherin, and Ki-67 was performed in BrC subjects. A significantly higher PAI-1 concentration in breast cancer patients below the age of 55 than in controls was obtained. According to the ROC curve analysis, the PAI-1 concentration demonstrates the most accurate prognostic value with the cut-off point at 33.91 ng/ml, with 90% sensitivity and 36% specificity, which discriminates between controls and cancer patients. However, t-PA presents the highest area under the receiver-operating characteristic curves (AUC^{ROC})=0.634 in predicting disease relapse with the cut-off value of 5.3 ng/ml. According to the Kaplan-Meier curves, a high concentration of t-PA (>5 ng/ml) and a lower PAI-1/t-PA ratio (<7.5) are associated with shorter survival. Evaluation of plasma t-PA and PAI-1 concentrations may deliver relevant prognostic information for breast cancer patients.

Key words: invasive breast cancer, fibrinolytic system, thrombosis, metastasis

The neoplastic process is characterized by clonal, unrestrained cell proliferation. The balance between proliferation and apoptosis in cancer cells is disrupted. Most types of cancer cells proliferate as solid masses of tissue (solid tumors); one of its representatives is breast cancer (BrC) [1]. Breast cancer remains the most common neoplasm in women population with 2.0 million newly diagnosed cases in 2018 globally [2]. BrC was one of the first cancers investigated in antiquity. The first descriptions of nodular alterations come from ancient Egypt. The Edwin Smith Surgical Papyrus, dating back to 3000–2500 B.C., and presumably ascribable to Imhotep (the Egyptian physician, architect), delivers bona fide depiction of breast cancer [3, 4]. Breast cancer is strongly related to non-modifiable (age, genetic background, the occurrence of menarche and menopause, *in situ* breast carcinoma) and modifiable (application of oral contraceptives, menopausal hormonal therapy, parity status, lack of lactation, obesity, sedentary lifestyle) risk factors [1].

Histological classification divides breast cancer into two groups: non-invasive (*in situ* – limited to the epithelial layer of the mammary gland) and invasive (tumor cells infiltrate through the duct wall into stroma). Both carcinomas were categorized as ductal and lobular on grounds of the niche from which the tumor originated. In the invasive carcinoma group, 75% make up an invasive ductal carcinoma (IDC) no special type (NST), which displays a wide spectrum of morphological variations. Thus, it demonstrates a diverse aggressive nature or different response to therapy [5]. Subsequently, the immunohistochemical (IHC) analysis of estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER-2), Ki-67 (mitotic index), and E-cadherin were introduced to the clinical practice in order to stratified patients with BrC according to molecular subtypes as well as to assess future outcomes and to establish personalized therapy. In the last twenty years, there has been a prominent breakthrough in the diagnosis of breast

cancer including molecular methods such as a genomic hybridization or Sanger sequencing and NGS (Next-Generation Sequencing). The information obtained thanks to them is used to improve the classification of breast cancer and develop narrow tumor subdivisions in order to offer patients an adequate therapy scheme [6].

In the 19th century, Armand Trousseau noticed the link between cancer and hemostasis disorders. Thromboembolic events developing in patients with cancer are named as the Trousseau syndrome. It has been shown that in about 15% of patients with cancer, thromboembolic events occur during the clinical course of the disease, while in 50% of patients, evidence of venous thromboembolism (VTE) is confirmed during post-mortem examinations. The pathophysiology of cancer-related VTE is not completely elucidated. There are 3 classes of probability factors for VTE development: patient-, treatment- and cancer-associated. Patient-dependent factors include age, obesity, immobilization, infection, high white blood cells and platelets counts, previous incidents of VTE. Treatment-related such as chemotherapeutics, adjuvant hormonal therapy, intravenous catheters, and surgical procedures, and cancer-associated factors are primary site and advancement of the tumor, activation of platelets, pro-angiogenic agents, overexpression of tissue factor (TF), and plasminogen activator inhibitor type 1 (PAI-1) [7, 8].

Interestingly, abnormalities in the fibrinolytic system in cancer patients may influence an increase of cell detachment that favors metastasis of tumor cells and the progression of the disease [9]. Fibrinolysis is controlled by a series of cofactors, inhibitors, and receptors. The main elements constitute the tissue-type plasminogen activator (t-PA) and the plasminogen activator inhibitor type 1. t-PA is a serine protease, which initiates a transformation of plasminogen to plasmin. Additionally, it causes degradation of the basement membrane and extracellular matrix. These, in turn, cause the spreading of cancer cells throughout the entire body. t-PA is present on endothelial cells surface, indicating that it may participate in neoangiogenesis [10, 11]. t-PA presents the ability to directly induce the vascular endothelial growth factor (VEGF – the main pro-angiogenic agent) expression via transactivation of p38 by the ERK system [12]. However, the importance of t-PA in the neoplastic process has not yet been fully clarified, and the study results are still divergent [11, 13, 14]. Whereas PAI-1 is a main inhibitor of t-PA and/or urokinase, and it regulates adhesion and cellular migration (connected with vitronectin) [9, 15]. PAI-1 exerts paracrine and autocrine effects on cells including depletion of cancer cells apoptosis [15, 16], promotes angiogenesis by protecting the extracellular matrix against excessive degradation through suppression of proteolysis [15]. Elevated PAI-1 levels in patients with breast cancer are correlated with tumor aggressiveness, its ability to metastasize, and poor treatment response [9, 16]. Thus, the aim of the study was to evaluate concentrations of tissue plasminogen activator (t-PA) and plasminogen activator inhibitor type 1 (PAI-1), and their ratios: t-PA/PAI-1 ratio and PAI-1/t-PA in breast cancer patients and in healthy individuals, and to find associations of analyzed determinants with clinicopathological factors in BrC cases. An additional purpose was to assess the ability of fibrinolytic parameters in predicting of neoplasm disease and disease relapse.

Patients and methods

Patient samples and clinical data. In this a single-center, prospective study 105 Caucasian ethnicity women were enrolled. Sixty females with newly diagnosed, invasive, unilateral breast cancer without distant metastases (M0) were recruited from the Clinical Ward of Breast Cancer and Reconstructive Surgery, Oncology Centre in Bydgoszcz, Poland. The mean age of BrC cases was 52.9 years (range 41–67 years). The patients underwent a complete clinicopathological and post-surgical examination and were well-recognized with respect to the invasion standards. Invasive or infiltrative carcinomas were defined as malignant abnormal proliferation of neoplastic cells in the breast tissue, which has penetrated through the duct wall into the stroma. In order to classify the histological type of breast cancer, we used the classification of the World Health Organization. The median tumor size was 16 mm (range 5–35 mm). Seventeen subjects (28%) demonstrated lymph node metastasis (N1). Tumor stage was determined in all cases according to the AJCC 7th Edition Staging for Breast Cancer 2010 [17]. The tumor localization, diameter, histological, molecular, and clinical types as well as histological grade according to Elston-Ellis classification of BrC patients are presented in Table 1. There was no perioperative mortality and the average length of hospital stay was 7.0±1.1 days.

A total of 45 healthy volunteers (age range, 44–68 years) undergoing routine physical examination in January–June 2015 were also included. In those individuals, breast cancer was excluded by performing control mammography. All subjects were divided into two groups based on age: below 55 years and above 55 years. Menopausal status was determined with respect to natural menopause, which was defined as the permanent cessation of menstruation for at least 12 months. Parity was specified as the number of full-term pregnancies. Body mass index (BMI – kg/m^2) was categorized as normal (18.5–24.9), overweight (>25.0–29.9) according to WHO recommendations. Controls and breast cancer cases were age-, menopausal status-, BMI-, parity- matched (Table 2).

Inclusion and exclusion criteria for analyzed subjects. The major inclusion criteria for BrC patients were primary, invasive, unilateral, early-stage (IA–IIB) breast cancer, lack of application of neoadjuvant treatment, and absence of local or metastatic advancement, additionally stable clinical condition during the observational period. The exclusion criteria for all cases were as follows: age <40 years, male gender, uncompleted clinical information, uncontrolled cardiovascular disease, and visceral obesity, overt diabetes, using oral

Table 1. Clinical characteristics of the study group.

Feature	Number of patients (%)
Localization of the tumor	
Right breast	30 (50%)
Left breast	30 (50%)
Diameter of the tumor	
<20 mm	42 (70%)
≥20 mm <50 mm	18 (30%)
Lymph node status	
Negative (N0)	43 (72%)
Positive (N1)	17 (28%)
Histological type	
Invasive ductal carcinoma (IDC)	53 (88%)
Invasive lobular carcinoma (ILC)	7 (12%)
TNM staging classification	
IA	29 (48%)
IB	13 (22%)
IIA	14 (23%)
IIB	4 (7%)
Grade according to Elston-Ellis	
1	1 (1%)
2	49 (82%)
3	10 (17%)
Molecular type	
Luminal A (HR+/HER2-/Ki-67 <14%)	37 (62%)
Luminal B (HR+/HER2-/Ki-67 ≥14%)	12 (20%)
Luminal B HER2 (positive) (HR+/HER2+)	3 (5%)
Non-luminal HER2 (positive) (HR–/HER2+)	2 (3%)
Triple-negative (HR–/HER2–)	6 (10%)
Estrogen receptor (ER)	
Negative	8 (13%)
Positive	52 (87%)
Progesterone receptor (PR)	
Negative	10 (17%)
Positive	50 (83%)
HER2	
Negative	55 (92%)
Positive	5 (8%)
E-cadherin	
Negative	4 (7%)
Positive	56 (93%)
Ki-67 (%)	
<14	37 (62%)
≥15	23 (38%)

TNM classification of malignant tumors: T – tumor; N – node; M – metastasis; HR – hormonal receptors; HER-2 – human epidermal growth factor receptor 2; Ki-67 – proliferation marker

contraceptives or menopausal hormonal therapy as well as thrombolytic, anticoagulant, and antiplatelet agents.

Ethical approval. The study was performed under the appropriate Institutional ethics approvals (permission number: KB 547/2015) and in accordance with the principles embodied in the Declaration of Helsinki. Written informed consent was obtained from each participant.

Table 2. Baseline characteristics of the study group and controls.

Feature	Number of patients (%)	Number of controls (%)
Age		
<55 years	35 (58%)	27 (60%)
≥55 years	25 (42%)	18 (40%)
BMI (kg/m²)		
Normal (18.5 ≤ 24.99)	37 (62%)	27 (60%)
Overweight (25 ≤ 29.9)	23 (38%)	18 (40%)
Menopausal status		
Premenopausal	25 (42%)	21 (47%)
Postmenopausal	35 (58%)	24 (53%)
Parity status		
0	7 (12%)	3 (7%)
1–2	44 (73%)	35 (78%)
3 and more	9 (15%)	7 (16%)

Patient follow-up information. Follow-up was completed in all 60 BrC patients. For the relapse-free survival analysis, ten events occurred and follow-up ranged from 28 to 40 months (median follow-up was 33 months) with a 16.7% 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 as of the end of March 2019, and was expressed in months.

Blood sampling and laboratory tests. Venous blood samples from patients were drawn prior to any treatment, processed, and immediately analyzed for routine procedures. Blood samples were collected into cooled tubes (Becton Dickinson Vacutainer® System, Plymouth, UK) containing 0.13 mol/l trisodium citrate (final blood anticoagulant ratio 9:1). Samples were then mixed and centrifuged at 3000×g at 4 °C for 15 min, aliquoted and stored at –80 °C (as specified by the manufacturer) until assayed but no longer than six months. Storage conditions were carefully maintained, and aliquots were limited to one freeze-thaw cycle at the time of batch analysis.

Enzyme-linked immunosorbent assay (ELISA). t-PA levels were measured in citrated plasma samples by commercial enzyme-linked immunosorbent assay (AssayMax™ Human t-PA ELISA Kit, Assaypro LLC, St. Charles, MO, U.S.A). The cut-off level was set at 5.1 ng/ml on the basis of the 95th percentile noted in control women. PAI-1 levels were determined on citrated plasma samples using a commercially available enzyme immunoassay (IMUBIND Plasma PAI-1 ELISA test Kit 96-Well Plate Assay, Sekisui Diagnostics, LLC, Stamford, CT, USA) test. The reaction mixture was added to a 96-well plate. The cut-off level was set at 30 ng/ml on the basis of the 95th percentile observed in control women. All assays were run according to the manufacturer's specifications by personnel with no access to the clinical data of the patients.

Immunohistochemical detection of hormone receptors, Ki-67, and HER2. Determination of hormone

receptors including ER, PR as well as HER-2, Ki-67, and E-cadherin were assessed by immunohistochemistry according to a standard procedure, which had previously been published [18].

Statistical analysis. Statistical analysis was performed using the Statistical 13 program. The Shapiro-Wilk test was used to check compliance with the normal distribution. The Mann-Whitney U-test was used to analyze the difference in the expression of two independent variables. Data are expressed as median (Me) and the inter-quartile range (IQR) [lower quartile (Q1)/ upper quartile (Q3)]. Kaplan-Meier curves were used to express survival times, and the log-rank test was used to compare survival times. The progression-free survival (PFS) time was calculated from the date of enrolment until the relapse or progression of the disease. Receiver operating characteristic (ROC) analysis was performed to assess possible cut-off values. The area under the curve (AUC) was calculated in order to estimate diagnostic accuracy. The optimal cut-off points have been determined using the Youden index. A probability (p) <0.05 was considered to be statistically significant.

Results

Clinical summarization of the study population. The study included 105 Slavic descent females. Sixty of them suffered from invasive breast cancer and 45 women were free of breast cancer (control group), ranging in age from 41 to 68 (mean age of BrC subjects was 52.9 years; mean age of healthy individuals was 53.5 years). The BMI for BrC cases was 24.4 kg/m^2 and for controls was 24.7 kg/m^2. There were no significant differences between BrC patients and controls with respect to age, weight, BMI, parity, or menopausal status (all p>0.05). In breast cancer group 49 (82%) patients underwent breast-conserving surgery (BCS) and 11 (18%) had a modified radical mastectomy (MRM). In the histological differentiation of BrC, patients had differentiated invasive breast cancer in 50 cases (83%) including those with well- and moderate-differentiated breast carcinomas. There were also 10 patients with poorly differentiated cancers (17%). In terms of the tumor diameter, there were 42 T1 cases (70%) and 18 T2 cases (30%). In terms of the N stage, there were 43 N0 cases (72%) and 17 N1 subjects (28%). With respect to the TNM staging system, there were 42 stage I cases (70%) and 18 stage II cases (30%). The mean diameter of the tumor was 16 mm. Of the patients suffering from breast cancer, 59 (98.3%) survived, and nine patients (15.0%) developed disease recurrence due to systemic metastatic disease or metastasis to opposite axillary lymph nodes. Finally, the relapse-free survival (RFS) rate was 83.3%.

General comparison of the fibrinolytic profile in the study group and controls. In our study, we assessed the fibrinolytic and anti-fibrinolytic profiles by a division of the concentration of t-PA (the main fibrinolytic activator) by PAI-1 level (the most powerful inhibitor of t-PA activity) and in an opposite manner. t-PA/PAI-1 ratio expresses the fibrinolytic profile but the PAI-1/t-PA ratio demonstrates the anti-fibrinolytic potential. At the beginning of the statistical analysis, the examined parameters were compared between groups. A significantly higher PAI-1 concentration in the breast cancer group compared to the controls was obtained (p=0.0494; Table 3).

Fibrinolytic profile according to age and body mass index in the study group and controls. Apart from a general comparative analysis of the study and control groups, we have made statistical calculations in all females based on age and BMI criteria (Tables 4 and 5). In BrC patients below the age of 55, a significantly higher PAI-1 concentration was reported (p=0.0125) in relation to the age-matched healthy women. However, we did not observe any dependencies with respect to BMI.

Distribution of the fibrinolytic profile according to clinicopathological attributes in the study group. We hypothesized that the concentration of selected fibrinolysis parameters can vary according to clinicopathological determinants. Thus, the next analysis was made only in patients with breast cancer depending on tumor diameter, TNM staging system, tumor localization, and grade according to Elston-Ellis, nodal status, histological type, molecular type, and proliferation index – Ki-67 (Table 6). With respect to tumor diameter, BrC patients were divided into two subgroups T1 (tumor diameters <20 mm) and T2 (tumor diameter ≥20 mm and <50 mm). Also, based on the TNM staging system, the study group was split into IA+IB and IIA+IIB. Higher fibrinolytic potential in T2 and IIA+IIB cancers was observed but a higher anti-fibrinolytic profile in T1 and IA+IB tumors was noted (p=0.0171, p=0.0189, respectively). A substantial tendency to higher concentration of t-PA was noted in T2, stage IIA+IIB as well as in lymph nodes involvement tumors (p=0.0608, p=0.0624, p=0.0806, respectively). Additionally, a growing tendency to a higher

Table 3. Plasma levels of tissue plasminogen activator (t-PA), plasminogen activator inhibitor type 1 (PAI-1), as well as t-PA/PAI-1 and PAI-1/t-PA ratios in all cases.

Parameter [units]	Study group N=60	Control group N=45	p-value
t-PA [ng/ml]	4.51 3.23/6.19	4.45 2.65/5.79	0.5977
PAI-1 [ng/ml]	39.45 27.92 /53.13	33.91 22.63/45.69	**0.0494**
t-PA/PAI-1	0.12 0.06/0.18	0.13 0.08/0.20	0.2504
PAI-1/t-PA	8.49 5.48/16.82	7.92 5.07/12.64	0.2504

Data are expressed as median (Me) and the inter-quartile range (IQR) [lower quartile (Q1)/upper quartile (Q3)]; t-PA – tissue plasminogen activator; PAI-1 – plasminogen activator inhibitor type 1; a significant difference is denoted by bold p-value

t-PA/PAI-1 ratio and a lower PAI-1/t-PA ratio in lymph nodes involvement tumors (p=0.0738) was noted.

Fibrinolytic elements as predictors of breast cancer morbidity. The ROC curves for separate laboratory parameters were constructed and the areas under the curve with 95% confidence interval were established (AUC, 95% CI thresholds with sensitivity [SE] and specificity [SP]). We estimated the ROC curves in order to determine the diagnostic accuracies of fibrinolysis system elements: t-PA, PAI-1, and the ratios of t-PA/PAI-1 and PAI-1/t-PA in the prediction of breast cancer morbidity. The borderline of the diagnostic usefulness of the test, based on the area under the ROC curve (AUC^{ROC}) ≥0.5; p<0.05 was reached only for PAI-1. Although the AUC^{ROC} for t-PA and PAI-1/t-PA ratio was above 0.5, the p-values did not reach significances, thus the Youden Index cut-off points were not identified. Based on the AUC^{ROC}, the Youden Index cut-off value was established to maximize the sum of sensitivity and specificity. PAI-1 concentration presents the most accurate prognostic value with an AUC^{ROC}=0.606 (95% CI: 0.5087–0.7482). Using the Youden Index cut-off value, we identified a plasma PAI-1 concentration of 33.91 ng/ml with a sensitivity of 90% and a specificity of 36% as the best cut-off value to discriminate between healthy individuals from breast cancer cases (Figure 1).

Fibrinolytic elements as predictors of breast cancer recurrence. Furthermore, in order to evaluate the diagnostic power of plasma levels of t-PA, PAI-1, and t-PAI-1/PAI-1 and PAI-1/t-PA ratios for prediction of disease relapse among the invasive breast cancer patients, further analysis has been made. The highest value of AUC^{ROC} was obtained for the t-PA concentration and AUC^{ROC} was 0.634 (AUC ≥ 0.5; 95% CI: 0.5138–0.7665). The cut-off point for the t-PA concentration was set at 5.3 ng/ml with 66% specificity and 70% sensitivity, this value distinguishes disease recurrence patients and non-disease relapse subjects (Figure 2).

Cut-off points of t-PA concentration and PAI-1/t-PA ratio as the best predictors of breast cancer recurrence. Figure 3 demonstrates the Kaplan–Meier PFS curves for breast cancer patients stratified on the basis of plasma t-PA concentration. As shown, a baseline elevated plasma t-PA level had a negative prognostic impact in terms of PFS (PFS rates 75% vs. 91%; p<0.0377). The significant p-value indicates that patients with a pre-operative higher concentration of t-PA than 5 ng/ml present worse future outcomes. Twenty-eight patients (47%) demonstrated a baseline concentration of t-PA above 5 ng/ml, whereas 32 cases (53%) had a lower than 5 ng/ml concentration of t-PA. Recurrence of the disease in the group of patients with a higher concen-

Table 4. Plasma concentrations of tissue plasminogen activator (t-PA), plasminogen activator inhibitor type 1 (PAI-1), as well as t-PA/PAI-1 and PAI-1/t-PA ratios in the study group and controls according to age <55 years and ≥55 years.

Parameter [units]	< 55 years			≥55 years		
	Study group N=35	Control group N=27	p-value	Study group N=25	Control group N=18	p-value
t-PA [ng/ml]	4.42 3.15/5.87	4.45 3.20/5.42	0.9614	5.38 3.45/6.34	4.01 2.13/6.88	0.5053
PAI-1 [ng/ml]	39.50 28.93/64.65	32.69 18.82/45.98	**0.0125**	39.39 24.40/44.60	37.10 26.88/44.15	0.8176
t-PA/PAI-1	0.11 0.05/0.16	0.13 0.09/0.23	0.0554	0.13 0.07/0.25	0.11 0.06/0.18	0.5556
PAI-1/t-PA	9.28 6.20/19.78	7.88 4.30/11.70	0.0554	7.47 4.06/14.67	8.96 5.49/17.57	0.5556

Data are expressed as median (Me) and the inter-quartile range (IQR) [lower quartile (Q1)/upper quartile (Q3)]; t-PA – tissue plasminogen activator; PAI-1 – plasminogen activator inhibitor type 1; a significant difference is denoted by bold p-value

Table 5. Plasma concentrations of tissue plasminogen activator (t-PA), plasminogen activator inhibitor type 1 (PAI-1), as well as t-PA/PAI-1 and PAI-1/t-PA ratios in the study and control groups based on BMI <24.99 kg/m² and ≥24.99kg/m².

Parameter [units]	< 24.99 kg/m²			≥24.99kg/m²		
	Study group N=37	Control group N=27	p-value	Study group N=23	Control group N=18	p-value
t-PA [ng/ml]	4.46 3.44/6.16	4.32 2.13/5.95	0.5229	4.55 2.74/6.22	4.59 3.40/5.77	0.9686
PAI-1 [ng/ml]	36.50 26.42/45.48	33.91 21.17/46.27	0.3015	42.91 37.20/64.65	33.84 26.14/45.69	0.0902
t-PA/PAI-1	0.13 0.07/0.22	0.13 0.07/0.22	0.5497	0.10 0.05/0.16	0.13 0.09/0.20	0.2219
PAI-1/t-PA	7.82 4.56/14.85	7.99 4.48/13.47	0.5497	9.87 6.20/19.91	7.88 5.07/11.32	0.2219

Data are expressed as median (Me) and the inter-quartile range (IQR) [lower quartile (Q1)/upper quartile (Q3)]; BMI - body mass index; t-PA - tissue plasminogen activator; PAI-1 - plasminogen activator inhibitor type 1; a significant difference is denoted by bold p-value

Table 6. Concentrations of selected fibrinolytic parameters depending on clinicopathological determinants in all breast cancer cases.

Analyzed variables	t-PA concentration p-value	PAI-1 concentration p-value	t-PA/PAI-1 ratio p-value	PAI-1/t-PA ratio p-value
Tumor diameter T1 (<20 mm)	4.23 2.74/5.98	40.91 28.10/56.19	0.10 0.04/0.16	10.08 6.25/22.54
Tumor diameter T2 (≥20 mm and <50 mm)	5.38 3.92/7.47 p=0.0608	37.55 27.31/44.60 p=0.3054	0.16 0.12/0.25 **p=0.0171**	6.21 4.06/8.51 **p=0.0171**
Stage of disease IA+IB	4.26 2.74/5.98	40.86 28.10/56.19	0.10 0.04/0.16	9.98 6.25/22.54
Stage of disease IIA+IIB	5.54 3.92/7.47 p=0.0624	38.04 27.31/44.60 p=0.3837	0.17 0.12/0.25 **p=0.0189**	5.99 4.06/8.51 **p=0.0189**
Localization of the tumor – **left breast**	4.51 3.21/6.44	43.76 28.93/56.19	0.11 0.06/0.16	9.31 6.11/16.49
Localization of the tumor – **right breast**	4.49 3.24/6.01 p=0.6467	37.88 24.18/45.10 p=0.1260	0.13 0.06/0.25 p=0.5106	7.64 4.06/17.14 p=0.5106
Grade according to Elston-Ellis – **1+2**	4.44 3.24/6.01	39.70 27.31/53.24	0.12 0.06/0.18	8.49 5.67/17.14
Grade according to Elston-Ellis – **3**	5.51 3.15/7.25 p=0.5127	38.35 28.93/53.01 p=0.9763	0.13 0.06/0.19 p=0.7435	8.15 5.29/15.82 p=0.7435
Nodal status – **positive**	5.27 4.14/6.25	37.20 22.40/56.19	0.14 0.09/0.26	7.02 3.81/11.43
Nodal status – **negative**	4.28 2.62/6.01 p=0.0806	40.80 28.10/53.01 p=0.5439	0.11 0.05/0.16 p=0.0738	9.34 6.11/20.63 p=0.0738
Histological type – **IDC**	4.51 3.24/6.25	40.40 27.73/53.24	0.11 0.06/0.18	8.80 5.67/16.49
Histological type – **ILC**	4.49 2.74/5.26 p=0.5321	33.75 28.10/42.91 p=0.2151	0.13 0.05/0.22 p=0.8817	7.85 4.56/19.91 p=0.8817
Molecular type – **luminal A**	4.46 3.24/6.22	39.39 30.45/48.12	0.11 0.06/0.19	9.35 5.16/16.48
Molecular type – **other subtypes***	4.61 3.21/6.16 p=0.7960	39.50 27.73/64.65 p=0.8433	0.14 0.06/0.18 p=0.9515	7.03 5.67/17.14 p=0.9515
Ki-67 (%) **<14**	4.46 3.04/6.22	39.39 30.45/48.12	0.10 0.06/0.19	9.68 5.16/16.48
Ki-67 (%) **≥15**	4.61 3.44/6.16 p=0.4962	39.50 27.73/53.24 p=0.8014	0.14 0.06/0.18 p=0.6787	7.03 5.67/17.14 p=0.6787

Data are expressed as median (Me) and the inter-quartile range (IQR) [lower quartile (Q1)/upper quartile (Q3)]; t-PA – tissue plasminogen activator; PAI-1 – plasminogen activator inhibitor type 1; IDC – invasive ductal carcinoma; ILC – invasive lobular carcinoma; Other molecular subtypes include Luminal B HER2 (negative and positive); Non-luminal HER2 (positive); Triple-negative; Ki-67– proliferation marker; significant differences are denoted by bold p-value; the underlined p-value represents closeness to statistical significance

tration of t-PA occurred in seven out of 28 (25%). However, in the group with a lower baseline concentration of t-PA, 3 out of 32 (9%) cases had a recurrence of the disease.

Figure 4 presents the Kaplan–Meier PFS curves for the breast cancer patients analyzed on the basis of plasma PAI-1 concentration. According to this analysis, a PAI-1 does not predict disease relapse (p=0.2282).

Figure 5 presents the Kaplan–Meier PFS curves for the breast cancer patients analyzed on the basis of the PAI-1/t-PA ratio. As shown, a lower PAI-1/t-PA ratio characterized a negative prognostic influence with respect to PFS (PFS rates 74% vs. 91%; p<0.0377). A lower PAI-1/t-PA ratio than 7.5 is associated with a shorter survival rate. Thirty-three patients (55%) demonstrated a baseline PAI-1/t-PA ratio above 7.5, whereas 27 cases (45%) had a lower than 7.5 the PAI-1/t-PA ratio. Recurrence of the disease in the group of patients with a higher PAI/t-PA ratio occurred in three out of 33 (9%). However, in the group with a lower baseline PAI-1/t-PA ratio, 7 out of 27 (26%) cases had a recurrence of the disease.

Discussion

Breast cancer still remains an important socio-economic problem worldwide. The morbidity and mortality rates in

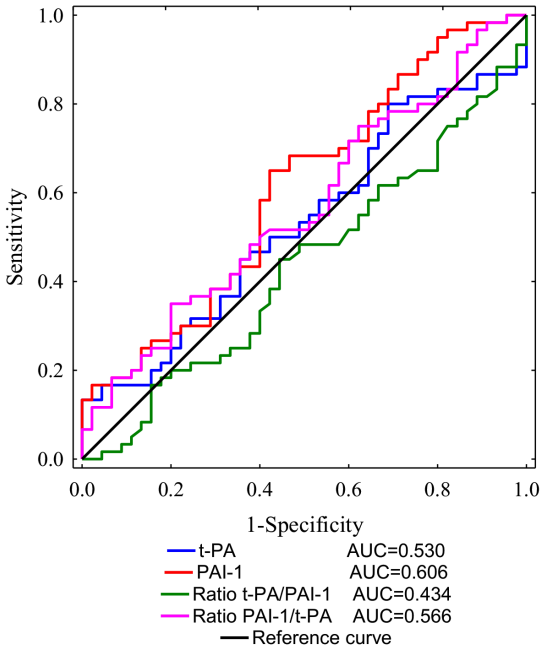

Figure 1. Four ROC curves and AUC values of examined parameters were designed in order to discriminate breast cancer cases from healthy individuals.

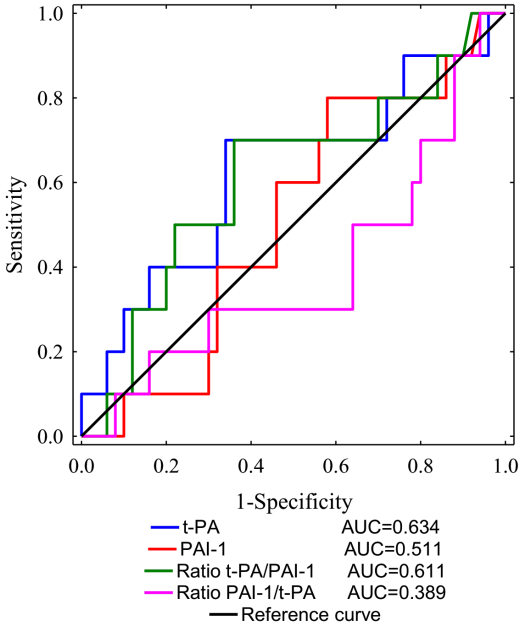

Figure 2. Four ROC curves and AUC values for plasma levels of t-PA, PAI-1, as well as t-PA/PAI-1 and PAI-1/t-PA ratios among BrC patients were established in order to distinguish disease recurrence patients and non-disease relapse subjects.

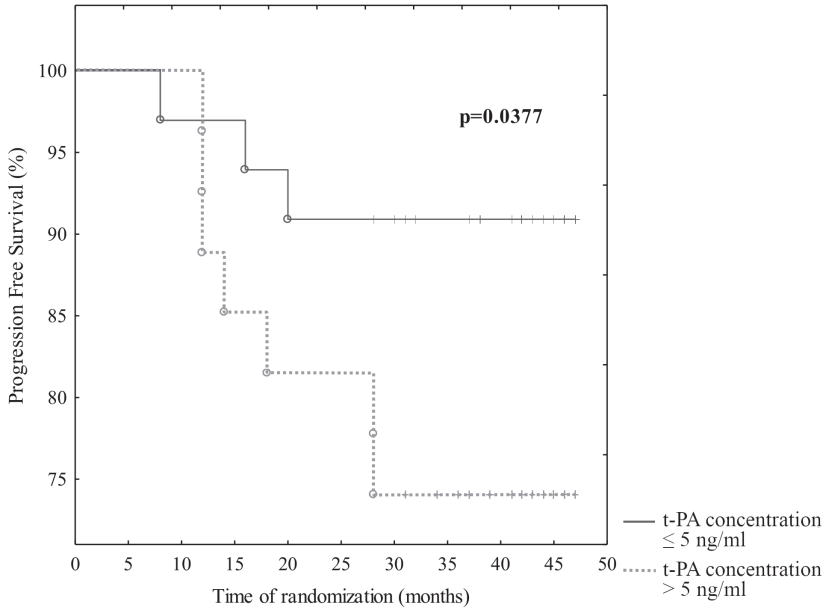

Figure 3. Kaplan-Meier curves of progression-free survival (PFS) of patients with breast cancer. Comparison between patients with low and high plasma t-PA levels (cut-off ≤5 ng/ml).

recent years have dramatically increased to over two million newly diagnosed cases, constituting of 25.4% the total number of new cases diagnosed in 2018 [1]. The development of new diagnostic and screening tests as well as modern therapeutic strategies led to enhance relative survival rates (over 85% of BC five-year survivors after diagnosis). A relevant amelioration of survival is associated with early diagnosis and application of adjuvant therapy. However, breast cancer is a remarkably heterogeneous neoplasm, and in order to optimize health care, the complete histopatho-

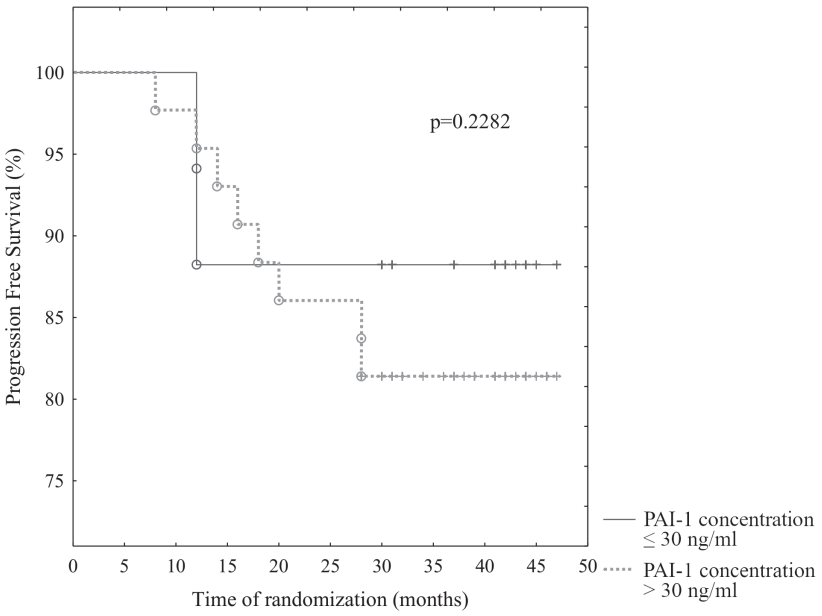

Figure 4. Kaplan-Meier curves of progression-free survival (PFS) of patients with breast cancer. Comparison between patients with low and high plasma PAI-1 levels (cut-off ≤30 ng/ml).

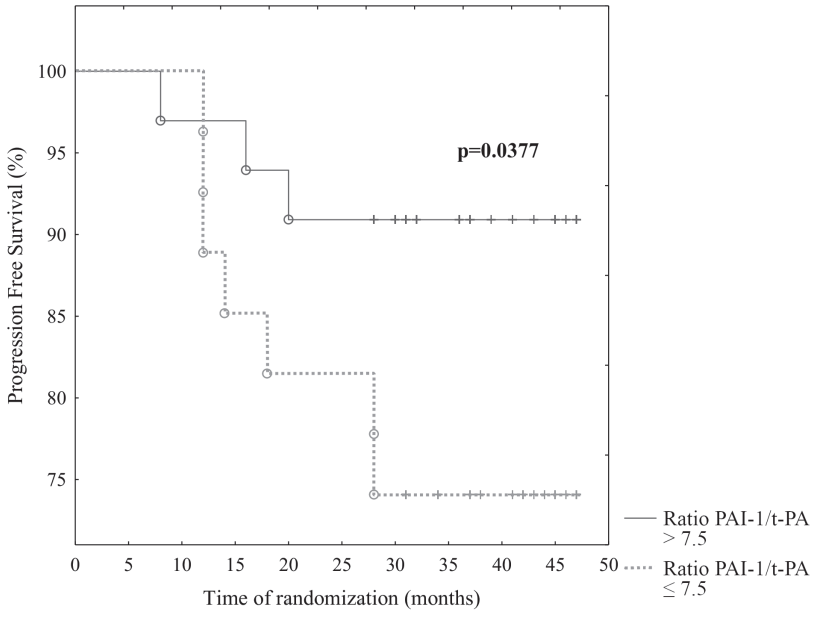

Figure 5. Kaplan-Meier curves of relapse-free survival (RFS) of patients with breast cancer. Comparison between patients with low and high PAI-1/t-PA ratio (cut-off ≤7.5).

logical and molecular examination should be performed to adequately categorize BrC [19]. Important risk factors such as age, BMI value, genetic susceptibility and family predispositions, use of menopausal hormone therapy or early menses, and late menopause play a fundamental role in breast cancer patients' treatment strategy [1, 19].

Reliable markers of recurrence and response to treatment are still sought out. Thus, we focused in this study on the analysis of two major compounds of the fibrinolytic process, which exert opposite function in clot lysis regulation: tissue plasminogen activator (t-PA) and plasminogen activator inhibitor type 1 (PAI-1) with respect to clinicopath-

ological factors and we estimated the diagnostic accuracy of examined parameters for the disease recurrence prediction in breast cancer cases. In our study, we observed that the concentration of PAI-1 was higher in breast cancer cases compared to controls. Further detailed analysis revealed that in BrC patients below the age of 55, a significantly higher PAI-1 concentration was noted than in healthy individuals. This observation may indicate the anti-fibrinolytic phenotype and a possible higher pro-thrombotic risk in breast cancer patients. In natural conditions the PAI-1 inhibits t-PA, thus should suppress cancer progression. However, in the malignant process paradoxical phenomenon is observed, indicating that a PAI-1 promotes cancer progression including tumor cell adhesion, migration, invasion, and angiogenesis, thus contributing to the increased cancer aggressiveness [9, 20]. A partial explanation for this paradox is based on the PAI-1 concentration. It means that a PAI-1 can be pro-angiogenic or anti-angiogenic depending on its concentration [21]. However, due to the non-hemostatic function of PAI-1, it may be a negative prognostic factor for those subjects. Interestingly, in our study only in healthy women, we observed age-dependent elevation of PAI-1, suggesting that in breast cancer patients a neoplastic process regulates PAI-1 level independently on well-defined factors. Since aging is associated with a progressive impairment of the fibrinolytic system expressed by increased releasing of plasminogen activator inhibitor-1 (PAI-1) [22].

Additionally, our results demonstrated that PAI-1 had the highest AUC^{ROC} from all the examined parameters, suggesting that PAI-1 may be a good diagnostic biomarker of breast cancer morbidity. The receiver-operating characteristic curve identified the PAI-1 concentration of 33.91 ng/ml, with 36% specificity and 90% sensitivity, as the best cut-off value to discriminate between healthy women and breast cancer patients. Our findings indicate the relevant usefulness of PAI-1 evaluation in the differentiation between breast cancer patients and non-cancerous cases. PAI-1 exerts a pro-angiogenic and anti-angiogenic activity and the ability to inhibit malignant cells apoptosis [19, 23]. The diminishing of the apoptotic process could thus increase the survival rate of cancer cells during dissemination, therefore increasing the possibility for the formation of secondary disease site [19]. The second perspective coming from overexpression of PAI-1 is the risk of VTE due to hypofibrinolysis and prolongation of clot lysis. It is well-known that cancer-related thrombosis is connected with worse clinical outcomes [24]. Despite the scientific consensus that PAI-1 expression presents prognostic and predictive value, still, the immunoassay evaluation of PAI-1 is not commonly applied in clinical practice [9].

It is well-established that the natural function of t-PA is the stimulation of the conversion of plasminogen into plasmin. The consequence of this reaction is an enhancement of plasmin generation, which can lead to direct induction of the disintegration of several connective tissues and extracellular matrix proteins including fibrin, laminin, fibronectin, and perlecan. All of these reactions lead to further activation of certain growth factors leading to the pro-angiogenic phenotype [19, 25]. Plasmin, as well as a t-PA, can activate VEGF, fibroblast growth factor-2 (FGF2), insulin-like growth factor 1 (IGF-1), hepatocyte growth factor (HGF) and so on, which promote new vessel network formation, subsequently support a tumor growth, invasion, and metastasis [12, 23]. Thus, t-PA is involved in the formation of metastases, by the degradation of the basal membrane and extracellular matrix allowing cancer cells to spread to distant sites. In addition, t-PA is attributed to the ability to stimulate cancer cell proliferation, motility, and metastasis [10, 11, 19]. Our study confirms mentioned role of t-PA in cancer biology since a higher fibrinolytic potential in T2 and IIA+IIB cancers was observed, but a higher anti-fibrinolytic profile (PAI-1/t-PA) in T1 and IA+IB tumors was noted. An essential tendency to a higher concentration of t-PA was noted in T2, stage IIA+IIB as well as in lymph nodes positive tumors. Additionally, a growing tendency to a higher t-PA/PAI-1 ratio and a lower PAI-1/t-PA ratio in lymph nodes involvement tumors was reported. Based on our study, we suggest that bigger and more advanced tumors demonstrate a higher t-PA concentration and a higher fibrinolytic potential as well as a lower anti-fibrinolytic profile. Furthermore, according to the Kaplan-Meier curves, we suggest that a higher concentration of t-PA >5 ng/ml as well as a lower PAI-1/t-PA ratio <7.5 may indicate a more aggressive character of the tumor and a high possibility of breast cancer dissemination. This suggestion is based on the relapse rates of 25% and 26%, respectively, in BrC cases. Furthermore, our study indicates that the baseline concentration of t-PA and PAI-1 may be used as appropriate, easy-applicable prognostic indicators next to tumor-related determinants such as tumor diameter, TNM staging system, or nodal status.

Apart from Kaplan–Meier curves, these results were supported by further statistical analysis (ROC curves), which also indicates that in patients with higher concentrations of t-PA, the higher rate of disease relapse occurs. t-PA concentration reached the highest AUC^{ROC}=0.634 (AUC≥0.5; 95% CI: 0.5138–0.7665). The cut-off point for the t-PA concentration was identified at 5.3 ng/ml with 66% specificity and 70% sensitivity, in order to discriminate between disease recurrence patients and those without disease relapse. To date, there are only a few trials focusing on the prognostic relevance of plasma t-PA in the early-stage of BrC, and consensus in cut-off points does not exist. However, our results related to the t-PA level are inconsistent with the study by Corte et al. They noted a lower concentration of tissue plasminogen activator in large size and low differentiated tumors, compared to T1 (tumor diameter <2 cm) and well-differentiated cancers. The authors claim that the low concentration of t-PA in breast cancer cases is associated with higher aggressiveness of cancer cells and poor future prognosis for patients [14]. This discrepancy seems surprising, but most

likely it is due to the fact that Corte et al. used a solid-phase enzyme immunoassay in tumor cytosol samples to measure of t-PA, whereas in the current study, we used 'liquid biopsy' as biological material. In our study longer PFS was associated with a lower than 5 ng/ml a pre-surgical plasma concentration of t-PA. Teliga-Czajkowska et al. analyzed 60 ovarian cancer cases. The authors divided patients into two subgroups with low t-PA concentration (37 subjects with below 6.5 mg/l) and with higher t-PA level (23 subjects with above 6.5 mg/ml). Patients with baseline t-PA >6.5 mg/l demonstrated a significantly lower probability of 5-year survival [26], which is in line with our results. However, in the era of reference values reduction, based on our study we postulate to narrow the cut-off point for t-PA concentration up to 5 ng/ml. Thanks to this action, it will be possible to earlier recognize patients with relapse disease.

There are some limitations to our study that need to be acknowledged. The main disadvantage of the present study is the size of the study population that might have weakened the statistical power. Thus, the present results limited the ability to generalize. On the other hand, the strength of our research is expressed by the use of samples collected and processed using standard operating procedures. Additionally, in late-stage cancer patients, numerous factors associated with cancer status might affect of fibrinolytic parameters. Hence, the elimination of patients with late-stage BrC allowed us to investigate specifically the association between stage IA–IIB of BrC and t-PA and PAI-1 levels, regardless of the crucial confounders. Also, we excluded cases with obesity, due to the fact that adipose tissue is a well-known source of PAI-1. Taken together, the investigation group is more homogenous.

In summary, the present prospective study demonstrated that pre-surgical t-PA and PAI-1 concentrations are able to serve as both predictive and prognostic markers in early-stage breast cancer.

Acknowledgments: This study has been supported by the Nicolaus Copernicus University in Toruń, Collegium Medicum in Bydgoszcz, Poland. We would like to thank all of the patients for participating in our study.

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