Plasma Plasminogen Activator Inhibitor-1 (PAI-1) Levels in Breast Cancer – Relationship with Clinical Outcome

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Abstract. *Background: Signaling pathways triggered by increased thrombin or plasminogen activator inhibitor-1 (PAI-1) expression drastically alter the tumor microenvironment, contributing to an adverse outcome. This study aimed to evaluate the prognostic value of coagulation/fibrinolytic activities in breast cancer (BC). Materials and Methods: Coagulation/fibrinolytic activities were investigated in 187 patients with breast cancer, with respect to possible associations with clinicopathological features and survival outcomes. Results: Levels of plasma PAI-1 (p<0.001), D-dimer (p=0.037) and activated protein C-dependent thrombin generation (p=0.003) were higher in women with breast cancer compared to 187 healthy women. PAI-1 directly correlated with D-dimer levels (p=0.009) and Ki67 expression (p=0.027), which were both predictors of elevated PAI-1 levels at multivariate regression analysis. Cox analysis demonstrated that an elevated plasma PAI-1 level had a negative prognostic impact in terms of relapse-free (hazard ratio=2.5, p=0.021) and overall survival (hazard ratio=2.7, p=0.002). Conclusion: Determination of plasma PAI-1 levels might provide important prognostic information in risk stratification and survival outcomes for patients with breast cancer.*

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Key Words: Plasminogen activator inhibitor-1, D-dimer, breast cancer, relapse-free survival, overall survival.

The acquisition of a prothrombotic state by patients with cancer suggests a causative role for abnormal coagulation in the natural history of cancer (1). Altered coagulation typically results from up-regulation of tissue factors, enhancing thrombin generation. Thrombin is a multi-functional serine protease which, besides its role in coagulation, is capable of stimulating tumor cell proliferation and of releasing pro-angiogenic cytokines (2).

Alterations of the fibrinolytic pathway in the tumor pericellular *milieu* may also result from an imbalance between urokinase-like plasminogen activator (uPA, responsible for extracellular membrane proteins degradation) and plasminogen activator inhibitor-1 (PAI-1, responsible for the inhibition of plasminogen activation) (3). In particular, signaling pathways triggered by increased PAI-1 expression have been shown not only to alter tumor microenvironment by remodeling cell–cell and cell–matrix attachments, but also to enhance tumor growth through inhibition of apoptosis and promotion of angiogenesis, ultimately contributing to adverse outcome in multiple cancer types, including those of the breast (4).

Up-regulation of uPA/PAI-1 has been correlated with poor prognosis in patients with breast cancer, suggesting that their abnormal expression on tumor tissues could contribute to tumor metastatic potential (5-10) and that they might be used as potential tissue tumor markers for breast cancer (11). Nonetheless, tumor uPA/PAI-1 expression is rarely used in clinical decision making, mainly because it necessitates fresh/frozen tumor samples and enzyme immunoassays, thus limiting its determination to specialist research Centers and clinical trials (12). To overcome these limitations, use of plasma antigen levels has been proposed, but available data are scarce and often discordant (13-16).

On this respect, we demonstrated that an elevated plasma PAI-1 level could be predictive of shorter relapse–free survival (RFS), with 70% of node-positive breast cancer cases with low

PAI-1 levels surviving free of disease after a 5-year follow-up compared to 20% of those with high PAI-1 levels (16). These results were in agreement to those obtained in a pooled analysis by the European Organization for Research and Treatment of Cancer – Receptor and Biomarker Group (EORTC-RBG) showing that tumor PAI-1 expression had a strong and independent prognostic value in both node-negative [hazard ratio (HR)=2.77) and node-positive (HR=2.4) primary breast cancer (6).

Despite this agreement with literature data, results on the prognostic value of plasma PAI-1 observed in our former study were limited by the small number of recruited patients and by the fact that RFS analysis represented a secondary aim, since the study was specifically designed to address the role of genetic polymorphisms of PAI-1 in breast cancer progression (16). For these reasons, we broadened the analysis of the prognostic value of plasma PAI-1 to a larger series of patients. Moreover, in order to discriminate the relative contribution of elevated plasma PAI-1 levels in the context of sub-clinical coagulation activation (17), activated protein C (APC)-dependent thrombin generation and highly sensitive (HS) D-dimer were used as markers of abnormal coagulation/fibrinolysis. Possible associations of these markers with clinico-pathological features of patients with breast cancer are here reported, with particular emphasis on the potential prognostic value of plasma PAI-1 in terms of RFS and overall survival (OS).

Patients and Methods

Patient data. One hundred and eighty-seven women with primary breast cancer were included in this study. Breast cancer was pathologically staged according to the TNM classification (18). One hundred and eighty-one women had primary breast cancer underwent radical surgery. The remaining six patients had relapsing disease and entered the study prior to the start of chemotherapy. Neoadjuvant chemotherapy regimens were instituted in 63 (34%) women. Adjuvant chemotherapy regimens were instituted in 126 (63%) women, 78 with and 48 without lymph node involvement. Adjuvant chemotherapies were anthracycline-containing (n=111) and non-anthracycline (n=15)-containing. Thirty-nine women with node-negative disease underwent adjuvant endocrine therapy only (tamoxifen or aromatase inhibitor). First-line chemotherapy was instituted in all patients with metastatic disease. Follow-up was completed in 152 patients, with a median time of 48 months and a 26% recurrence rate (Table I). The study was performed under the appropriate Institutional ethics approvals and in accordance with the principles embodied in the Declaration of Helsinki. Written informed consent was obtained from each participant. Clinical and pathological characteristics of patients are summarized in Table I.

Table I. *Patients' characteristics*.

	Whole series (n=187)	Followed-up patients (n=152)
Age (years)		
Mean±SD (range)	55±11 (28-80)	54±11 (28-80)
Menopausal status		
Pre	80 (43%)	69 (45%)
Post	107 (57%)	83 (55%)
ECOG performance status		
0	178 (95%)	150 (99%)
1	9 (5%)	2 (1%)
Diagnosis		
Ductal	146 (78%)	122 (80%)
Lobular	32 (17%)	26 (17%)
Other[a]	9 (5%)	4 (3%)
Grading		
1	7 (4%)	7 (5%)
2	75 (40%)	56 (37%)
3	105 (56%)	89 (58%)
Stage of disease		
T1-2N0M0	71 (38%)	59 (39%)
T3-4N0M0	8 (4%)	4 (3%)
T1-2N1M0	53 (28%)	45 (29%)
T3-4N1M0	10 (5%)	10 (6%)
T1-4N2M0	16 (9%)	12 (8%)
T1-4N3M0	7 (4%)	4 (3%)
TxNxM1	16 (9%)	12 (8%)
Relapsing	6 (3%)	6 (4%)
Hormone receptor expression		
ER+	130 (70%)	109 (72%)
PR+	122 (65%)	104 (68%)
HER2+	112 (60%)	93 (61%)
Ki67 expression	125 (67%)	100 (66%)
Length of follow-up (months)		
Median (IQR)		48 (33-66)
Type of recurrence		
Skin		5
Lymph node		6
Bone		13
Lung		4
Liver		3
Brain		1
Multiple metastases		8

[a]Including medullary, tubular, papillary, mucinous and mixed histotypes. ECOG: Eastern Cooperative Oncology Group; ER: estrogen receptor; PR: progesterone receptor; HER2: human epidermal growth factor receptor 2; IQR: interquartile range.

Blood sampling and laboratory tests. Blood samples from patients were drawn prior to any treatment, processed and immediately analyzed for routine blood chemistry (Accelerator Total Lab Automation; Abbott Laboratories, Abbott Park, IL, USA), routine coagulation studies (ACL-TOP automated coagulometer; Instrumentation Laboratories, Lexington, MA, USA) and complete and differential blood cell counts (Coulter LH 750 routine hematology analyzer; Beckman Coulter, Miami, FL, USA). Samples were then aliquoted and stored at –80˚C in the facilities of the Inter-Institutional Multidisciplinary Biobank (BioBIM) of the Scientific Institute for Research, Hospitalization and Health Care (IRCCS) San Raffaele Pisana, Rome, Italy. Storage conditions were carefully maintained and aliquots were limited to one freeze-thaw cycle at the time of batch analysis.

Table II. *Spearman rank correlation coefficients among clinical and laboratory variables analyzed in 187 patients with breast cancer.*

	HS D-dimer	ThromboPath	ER	PR	Ki67	CA15.3
PAI-1	0.256*	0.095	–0.062	–0.069	0.224†	0.145†
HS D-dimer		–0.322*	–0.011	–0.011	0.232†	0.334*
ThromboPath			–0.194†	–0.160	0.106	–0.016
ER				0.632‡	–0.355‡	0.062
PR					–0.419‡	0.050
Ki67						0.214†

PAI-1: Plasminogen activator inhibitor-1; HS D-dimer: highly sensitive D-dimer; ER: estrogen receptor; PR: progesterone receptor; CA15.3: cancer antigen 15.3. Ki67, ER and PR expression was entered into the analysis as a continuous variable. *$p<0.01$, †$p<0.05$, ‡$p<0.001$.

Activated Protein C (APC)-dependent thrombin-generation (HemosIL ThromboPath; kindly provided by Instrumentation Laboratory) and highly sensitive (HS) D-dimer levels (HemosIL D-Dimer HS 500; kindly provided by Instrumentation Laboratory) were analyzed on citrated plasma samples using an ACL TOP automated coagulometer, as previously reported (19). Locally defined cut-off values for both assays were determined on the basis of the distribution of values recorded for 187 unrelated healthy women (mean age 52±16 years, 53% in post-menopausal status) recruited among donors of the BioBIM. The cut-off for ThromboPath was set at 81% protac-induced coagulation inhibition (PICI%), as previously reported, and all samples with values below this cut-off were categorized as pathological (19). HS-D-dimer cut-off was set at 500 ng/ml (fibrinogen equivalent units, FEU), in agreement with previous observations (20).

PAI-1 levels were determined on citrated plasma samples using a commercially available enzyme immunoassay (Asserachrom PAI-1; Diagnostica Stago, Asnières sur seine, France) according to the manufacturer's instructions. Intra- and inter-assay variation coefficients for PAI-1 determination were below 6% and 8%, respectively. The cut-off level was set at 30 ng/ml on the basis of the 95th percentile of the values observed in control women.

Serum cancer antigen 15.3 (CA15.3) levels were measured using a commercially available immunoassay on an ARCHITECT i2000 System (Abbott Laboratories, Abbott Park, IL, USA). The analytical sensitivity of the CA15.3 assay was calculated to be better than 0.5 U/ml. The cut-off limit chosen for sample evaluation was 30 U/ml, as recognized in international literature.

Measurements were ascertained while blinded to the sample origin. All samples were assayed in duplicate and those with values above those of the standard curve were re-tested with appropriate dilution.

Immunohistochemical detection of Ki67 and scoring method. Formalin-fixed, paraffin-embedded tumor sections were used for immunohistochemical detection of Ki67. Briefly, breast tumor sections were immunostained with the CONFIRM anti-Ki67 (30-9) rabbit monoclonal primary antibody (Ventana Medical Systems, Inc. Tucson, Arizona, USA). Staining was performed on the Ventana BenchMark XT automated staining platform with the UltraView Universal DAB Detection Kit (Ventana) according to manufacturer's instructions. Ki67 proliferative index in surgical specimens was assigned by the pathologist on the basis of the percentage positive on at least 500 neoplastic cells counted in the peripheral area of the nodule. A cut-off value of 20% was used in all association analyses.

Statistical analysis. Data are presented as percentages, mean±SD, or median and interquartile ranges (IQR). Student's unpaired *t*-test and ANOVA test were used for normally distributed variables. Appropriate non-parametric tests (Mann–Whitney *U*-test and Kruskal–Wallis ANOVA and median test) were employed for all the other variables. RFS time was calculated from the date of enrollment until relapse or progression of disease. OS was calculated from the date of enrollment until disease-related death or study end. RFS and OS curves were calculated by the Kaplan–Meier method and the significance level was assessed according to the log-rank test. Cox proportional hazards analysis was used to evaluate the association between clinical variables and survival times. All tests were two-tailed and only *p*-values lower than 0.05 were regarded as statistically significant. Calculations were made using a computer software package (Statistica, StatSoft Inc., Tulsa, OK, USA) or free web-based applications (http://statpages.org/).

Results

Plasma PAI-1 ($p<0.0001$), HS D-dimer ($p<0.0001$) and ThromboPath ($p<0.0001$) levels were higher in breast cancer compared to healthy controls (Figure 1). Univariate association analysis showed a significant correlation between PAI-1 and HS D-dimer, but not ThromboPath levels (Table II). PAI-1 and HS D-dimer both directly correlated with Ki67 and CA15.3 (Table II). HS D-dimer ($\beta=0.283$, $p=0.015$) and Ki67 ($\beta=0.247$, $p=0.033$) were independent predictors of elevated PAI-1 level at multivariate regression analysis, independently of age, menopausal or receptor status, diagnosis, stage, ECOG performance status, grading, APC-dependent thrombin generation and CA15.3 level.

The associations between PAI-1, HS D-dimer, ThromboPath and clinico-pathological features were further analyzed after variable categorization. Cross-tabulation analyses are reported in Table III, confirming the association between elevated plasma PAI-1 and stage of disease ($p=0.037$), Ki67 expression ($p=0.045$) and CA15.3 positivity ($p=0.010$). Elevated HS D-dimer levels were associated with PAI-1 ($p<0.001$), ThromboPath ($p<0.001$) and serum CA15.3 ($p=0.007$), but not with clinico-pathological features of breast cancer, although a trend towards higher values was observed in metastatic disease (Table III). APC-dependent thrombin

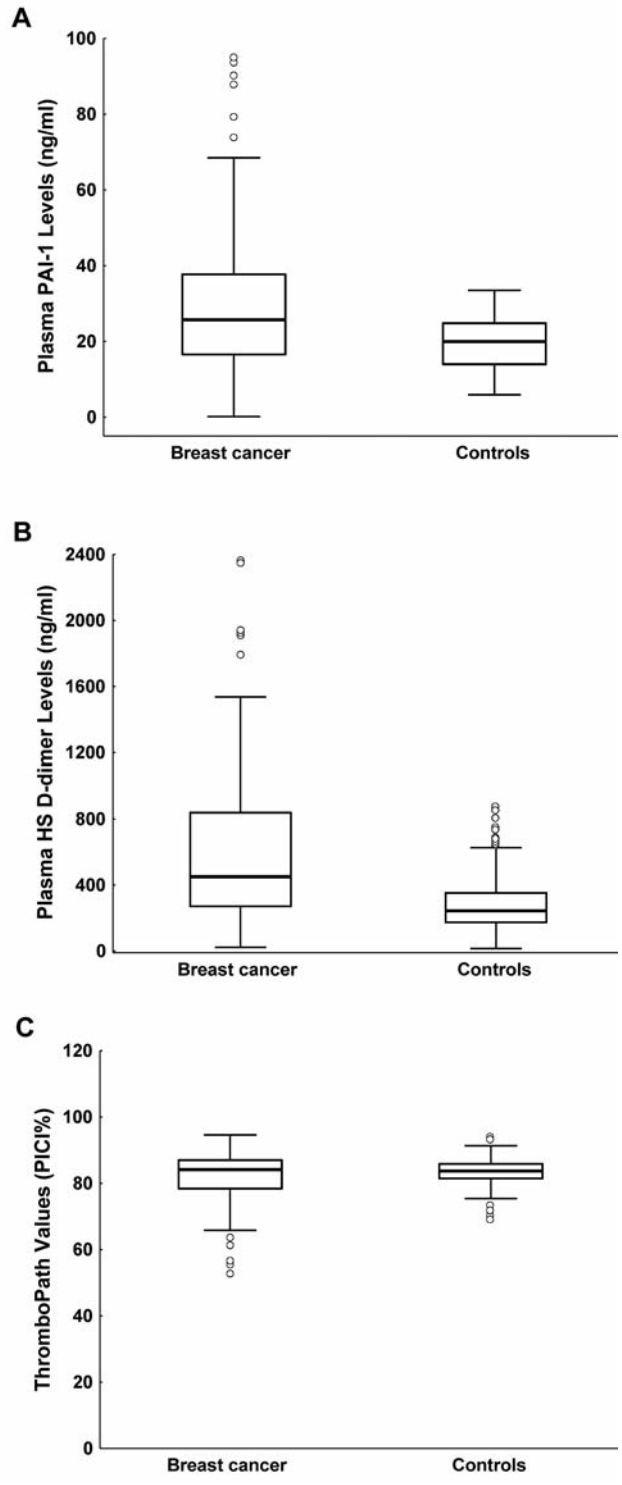

Figure 1. *Box-plot analysis of plasma plasminogen activator inhibitor-1 (PAI-1) (A), highly sensitive (HS) D-dimer (B) levels and activated protein C (APC)-dependent thrombin generation (ThromboPath) (C). Comparison between patients with breast cancer and control women. Data are presented as median values (solid lines), interquartile ranges (columns) and non-outlier ranges (whiskers). Open circles indicate outliers.*

generation (measured by ThromboPath) was not associated with plasma PAI-1 levels, nor with clinico-pathological features of breast cancer.

One hundred and fifty-two patients were followed-up for a median period of 48 months (ranging from 12 to 180 months). One hundred and twelve (74%) patients remained clinically free of disease, while 40 (26%) patients had relapsing disease (Table I), eight of them (20%) were alive with disease at the time of study end. Two (1%) patients had a second primary breast carcinoma after 52 and 53 months, respectively; they were included in the analysis as being free of disease at the time of clinical diagnosis.

Twenty-six (65%) out of 40 patients with relapse had a high PAI-1 level (>30 ng/ml) compared to 14 (35%) with a low PAI-1 level (≤30 ng/ml) (Chi-square analysis: p=0.002). No association was observed between relapse rates and HS D-dimer or APC-dependent thrombin generation (data not shown). To further assess the possible determinants of disease progression among the clinical and laboratory features of breast cancer, logistic regression analyses were performed including age, receptor or menopausal status, tumor stage, adjuvant treatment, PAI-1, HS D-dimer, ThromboPath and CA15.3 levels as predictor variables of RFS or OS. The results obtained are summarized in Table IV, demonstrating that stage of disease, adjuvant treatment and PAI-1 levels were independent predictors of both RFS (overall model fit: p=0.004) and OS (overall model fit: p=0.0001). These results were confirmed in multivariate Cox proportional hazards analyses using the same predictor variables in which disease stage (HR for RFS=2.1, 95% CI=1.2-3.4, p=0.006; HR for OS=2.7, 95% CI=1.5-4.9, p=0.002), adjuvant treatment (HR for RFS=3.2, 95% CI=1.3-7.7, p=0.011; HR for OS=2.9, 95% CI=1.1-7.9, p=0.038] and PAI-1 levels [HR for RFS=2.5, 95% CI=1.2-5.5, p=0.021; HR for OS=2.7, 95% CI=1.5-4.9. p=0.002] significantly predicted disease outcome (overall model fit: RFS: p=0.006; OS: p=0.019).

Figure 2A demonstrates the Kaplan–Meier RFS curves for the 152 patients with breast cancer stratified on the basis of plasma PAI-1 level. As shown, an elevated plasma PAI-1 level had a negative prognostic impact in terms of RFS (5-year RFS rates 53% *vs*. 79%; log-rank=3.4, p<0.001) with a median relapse time of 23 months. Survival analysis performed after exclusion of patients with metastatic disease did not differ from that obtained in the whole population (Figure 2B). This result was further confirmed only when node-positive cases were included in the survival analysis (5-year RFS rates 32% *vs*. 75%; log-rank=2.1, p=0.037) or considering the sub-group of women treated with adjuvant chemotherapy (5-year RFS rates 59% *vs*. 84%; log-rank=3.1, p=0.003).

Kaplan–Meier OS curves for the 152 patients with breast cancer stratified on the basis of plasma PAI-1 level are reported in Figure 3A. As shown, an elevated plasma PAI-1 level had a negative prognostic impact in terms of OS both in

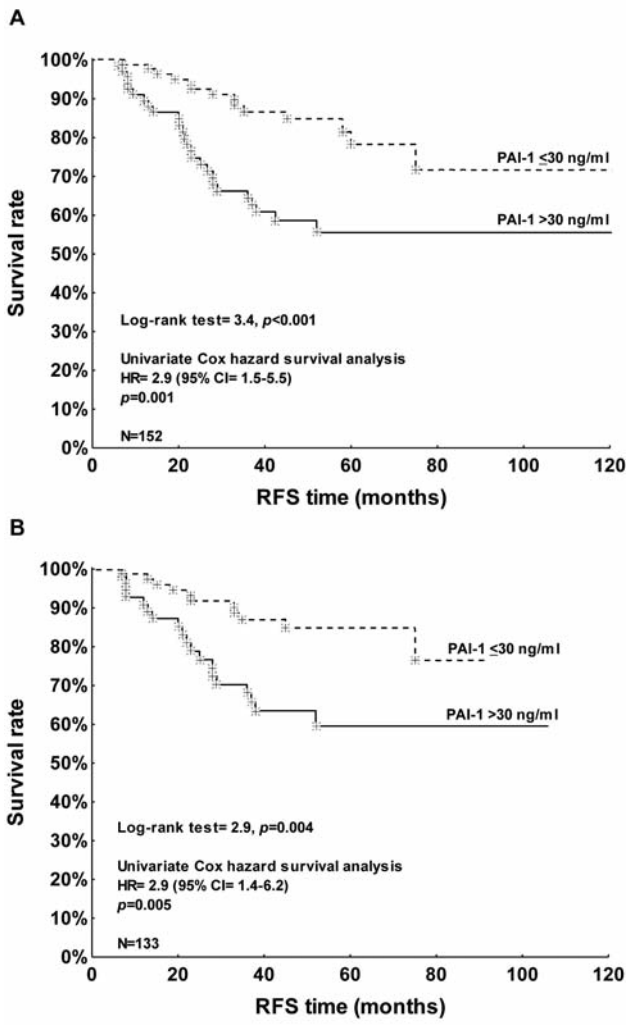

Figure 2. *Kaplan–Meier curves of relapse-free survival (RFS) of patients with breast cancer. Comparison between patients with low (dotted line) and high (solid line) plasma PAI-1 levels (cut-off ≤30 ng/ml). A: Kaplan–Meier curves for the entire population (n=152, 5 year RFS rates 53% vs. 79%). B: Kaplan-Meier curves for non-metastatic breast cancer (n=134, 5-year RFS rates 58% vs. 84%).*

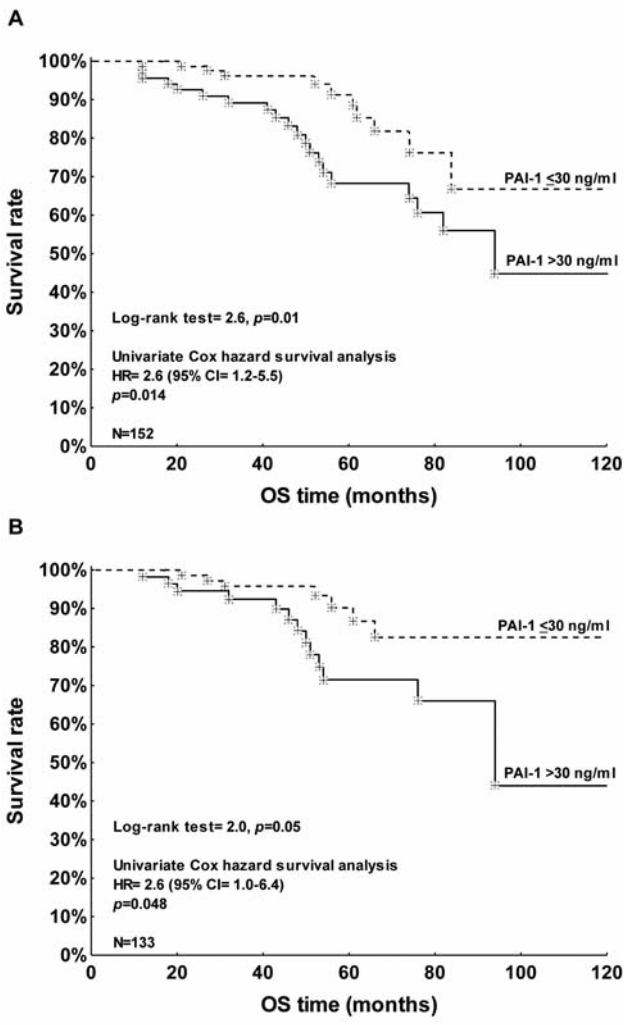

Figure 3. *Kaplan–Meier curves of overall survival (OS) of patients with breast cancer. Comparison between patients with low (dotted line) and high (solid line) plasma PAI-1 levels (cut-off ≤30 ng/ml). A: Kaplan–Meier curves for the entire population (n=152, 10 year OS rates 45% vs. 67%). B: Kaplan–Meier curves for non-metastatic breast cancer (n=134, 10-year OS rates 58% vs. 84%).*

the overall population (10-year OS rates 45% *vs*. 67%; log-rank=2.6, p=0.01) and in the group with non-metastatic disease (Figure 3B).

Discussion

The coagulation/fibrinolytic systems include a large spectrum of proteolytic enzymes with pathophysiological functions in a variety of processes, such as hemostasis, wound repair and tissue remodeling, tumor invasion and angiogenesis. Although only poorly-understood, up-regulation of coagulation or fibrinolytic factors has been associated with an unfavorable prognosis in breast cancer (1, 2, 21). Tumor PAI-1 expression, for example, was shown to have a strong and independent prognostic value in both node-negative and node-positive primary breast cancer (6). Moreover, in patients with node-negative breast cancer, those with low levels of uPA/PAI-I in their primary tumor were shown to have a very good prognosis, and may, thus, be candidates for being spared the burden of adjuvant chemotherapy (5, 6, 10). Based on these observation, the American Society of Clinical Oncology (ASCO) stated in the 2007 guideline update that "uPA/PAI-1 measured by ELISAs on a minimum of 300 mg of fresh or frozen breast cancer tissue may be used for the determination of prognosis in

Table III. *Association analysis among levels of plasminogen activator inhibitor-1 (PAI-1), highly sensitive (HS) D-dimer and ThromboPath and clinico-pathological features of 187 patients with breast cancer.*

		PAI-1		HS D-Dimer		ThromboPath	
Variable	N	≤30 ng/ml	>30 ng/ml	≤500 ng/ml	>500 ng/ml	≥81% PICI	<81% PICI
Menopausal status							
Pre-	80	50 (63%)	30 (37%)	42 (53%)	38 (47%)	59 (74%)	21 (26%)
Post-	107	57 (52%)	51 (48%)	52 (49%)	55 (51%)	73 (68%)	34 (32%)
Grading							
1	7	4 (57%)	3 (43%)	7 (100%)	0 (0%)	4 (57%)	3 (43%)
2	75	45 (60%)	30 (40%)	39 (52%)	36 (48%)	48 (64%)	27 (36%)
3	105	57 (54%)	48 (46%)	47 (45%)	58 (55%)	83 (79%)	22 (21%)
ECOG performance status							
0	178	101 (57%)	77 (43%)	93 (52%)	85 (48%)	126 (71%)	52 (29%)
1	9	5 (56%)	4 (44%)	5 (56%)	4 (44%)	5 (56%)	4 (44%)
Diagnosis							
Ductal	146	86 (59%)	60 (41%)	72 (49%)	74 (51%)	104 (71%)	42 (29%)
Lobular	32	18 (56%)	14 (44%)	23 (72%)	9 (28%)	18 (56%)	14 (44%)
Other	9	2 (22%)	7 (78%)	4 (44%)	5 (56%)	9 (100%)	0 (0%)
Estrogen receptors							
Positive	130	77 (59%)	53 (41%)	72 (55%)	58 (45%)	83 (64%)	47 (36%)
Negative	57	29 (51%)	28 (49%)	21 (37%)	36 (63%)	51 (89%)	6 (11%)*
Progesterone receptors							
Positive	122	71 (58%)	51 (42%)	59 (48%)	63 (52%)	78 (64%)	44 (36%)
Negative	65	35 (54%)	30 (46%)	36 (55%)	29 (45%)	54 (83%)	11 (17%)
HER2							
Positive	112	65 (58%)	47 (42%)	58 (52%)	54 (48%)	83 (74%)	29 (26%)
Negative	75	41 (55%)	34 (45%)	36 (48%)	39 (52%)	52 (69%)	23 (31%)
Ki67							
Negative	62	44 (71%)	18 (29%)	35 (56%)	27 (44%)	38 (61%)	24 (39%)
Positive	125	67 (54%)	58 (46%)*	61 (49%)	64 (51%)	98 (78%)	27 (22%)
Stage of disease							
Early	124	78 (63%)	46 (37%)	64 (52%)	60 (48%)	91 (73%)	33 (27%)
Advanced	41	19 (46%)	22 (54%)	24 (58%)	17 (42%)	33 (80%)	8 (20%)
Metastatic	22	9 (41%)	13 (59%)*	8 (38%)	14 (62%)	11 (50%)	11 (50%)
CA15.3							
Negative	152	93 (61%)	59 (39%)	90 (59%)	62 (41%)	108 (71%)	44 (29%)
Positive	35	13 (37%)	22 (63%)†	9 (26%)	26 (74%)*	24 (69%)	11 (31%)

PICI: Protac-induced coagulation inhibition; ECOG: Eastern Cooperative Oncology Group; HER2: human epidermal growth factor receptor 2; CA15.3: cancer antigen 15.3. *$p<0.05$, †$p<0.001$.

patients with newly-diagnosed, node-negative breast cancer" (11). Further support for these recommendations was recently provided by the final analysis of the prospective multicentre Chemo-N0 trial (10) and by two cost-effectiveness studies demonstrating that uPA/PAI-1 testing is beneficial for patients with breast cancer with no lymph node involvement (22, 23), with a return-on-investment ratio of 8.4:1, which was further increased by indirect cost savings (23).

These recommendations, however, are far from being introduced into clinical practice since, despite the introduction of novel assays needing very little amounts of tissue (24), tumor uPA/PAI-1 expression still necessitates fresh/frozen tumor samples in order to perform the analysis, limiting its determination to particular research settings (12). Thus, the possibility of using a plasma matrix to determine the prognostic value of PAI-1 is appealing, as it might allow a more widespread use of this marker. Unfortunately, available data are scarce and controversial (13-16), probably because of incorrect sample choice and processing (choice of anticoagulant, temperature conditions and elapsed time from sampling to centrifugation), ultimately leading to *in vitro* platelet activation and uncontrolled release of PAI-1. The latter, in fact, is mainly produced by endothelial cells and megakaryocytes, as well as smooth muscle cells, and is then stored in platelets (3). Most studies on plasma PAI-1 distribution in breast cancer were performed on samples using EDTA or heparin as anticoagulants, which may induce platelet changes over time (24). For example, the independent prognostic role of plasma PAI-1 in breast cancer was rejected by some authors on the basis of a lack of correlation between

Table IV. *Logistic regression analysis of predictors of relapse-free and overall survival in 152 patients with breast cancer.*

		Relapse-free survival		Overall survival	
Variable	Code	OR (95% CI)	*p*-Value	OR (95% CI)	*p*-Value
Age, years	≤60 >60	0.97 (0.91-1.04)	0.417	0.96 (0.90-1.04)	0.3066
Estrogen receptor status	Negative Positive	1.25 (0.41-3.78)	0.696	1.51 (0.43-5.39)	0.5248
Progesterone receptor status	Negative Positive	0.78 (0.26-2.39)	0.670	1.52 (0.42-5.51)	0.5206
Menopausal status	Pre Post	1.16 (0.28-4.88)	0.836	1.70 (0.35-8.63)	0.4935
Histological diagnosis	Ductal Lobular Others	0.88 (0.49-1.60)	0.678	0.55 (0.18-1.63)	0.2805
Stage of disease	Early Advanced Metastatic	2.48 (1.33-4.64)	0.005	4.67 (2.22-9.81)	<0.0001
Adjuvant treatment	No Yes	4.43 (1.50-13.1)	0.007	4.82 (1.34-17.4)	0.0161
ThromboPath level, PICI%	>81 ≤81	2.20 (0.51-9.42)	0.289	1.06 (0.17-6.81)	0.9481
PAI-1 level, ng/ml	≤30 >30	3.11 (1.37-7.05)	0.007	3.74 (1.46-9.59)	0.0061
HS D-dimer level, ng/ml	≤500 >500	0.27 (0.06-1.26)	0.096	0.74 (0.12-4.45)	0.7436
CA15.3, U/ml	≤30 >30	0.99 (0.36-2.67)	0.998	0.45 (0.13-1.50)	0.1947

CI: Confidence interval; OR: odds ratio; PICI: protac-induced coagulation inhibition; PAI-1: plasminogen activator inhibitor-1; HS: highly sensitive; CA15.3: cancer antigen 15.3.

plasma and tumor tissue levels of PAI-1, suggesting that determination of this factor in plasma does not reflect its concentration in tumor tissue (15). The use of EDTA-anticoagulated samples in this study might have affected plasma PAI-1 levels, also dependent on the elapsed time between blood withdrawal and processing (25). This effect appears to be unpredictable and must be controlled, either with the use of different anticoagulants or by standardizing the duration between sampling and analysis (26).

In our study, samples were collected as part of a biobank project, the BioBIM institutional project, using standard operating procedures and information and communications technology (ICT) tools to monitor temperature conditions (27) and in which all preanalytical variables, including elapsed time between blood withdrawal and processing, are encapsulated in a standard code calculated by means of a dedicated software tool (28). This ensures relative homogeneity among the samples used in a given study and minimizes the differences in sample analyses. The results obtained here using such a controlled set of samples support the idea that plasma PAI-1 levels might be used as a prognostic indicator in women with breast cancer. In our analysis, in fact, elevated plasma PAI-1 levels were associated with increasing tumor stage and disease relapse, and were predictive of shorter RFS and OS. This prognostic value was fully retained in node-positive cases, in agreement with the results obtained in the EORTC pooled analysis showing that tumor PAI 1 expression had a strong and independent prognostic value in node-positive primary breast cancer and reporting HRs similar to those found in our study (6). Moreover, the prognostic value of plasma PAI-1 was confirmed in the subgroup of women treated with adjuvant chemotherapy, with a 5-year RFS rate of 59% in women with PAI-1 levels above 30 ng/ml *vs*. 84% in those with PAI-1 levels below this cut-off, suggesting a benefit from adjuvant therapy in patients with high plasma PAI-1 levels, as previously shown for tumor PAI-1 expression (7).

Tumor heterogeneity, however, complicates this picture and PAI-1 production can be increased by a wide array of soluble factors released into the tumor microenvironment (*e.g*., vascular endothelial growth factor and inflammatory cytokines). In this respect, we must also take into consideration the fact that an elevated plasma PAI-1 level might be the result of a hypercoagulable state (17), which is a common finding in patients with solid cancer. The possibility of an involvement of coagulation activation in determining PAI-1 plasma levels could, at least partially, explain the lack of correlation between

plasma and tumor tissue levels of PAI-1 discussed above (15). In this study, APC-dependent thrombin generation and HS D-dimer determination were used as markers of abnormal coagulation/fibrinolysis in order to discriminate the relative contribution of coagulation activation to the prognostic value of elevated plasma PAI-1. Of interest, plasma PAI-1 correlated with HS D-dimer level, but no association was found with APC-dependent thrombin generation, although a hypercoagulable state was present in patients as evidenced by impaired ThromboPath values. Moreover, neither HS D-dimer nor ThromboPath were associated with survival outcomes, further suggesting that the tumor-associated plasminogen activator pathway, not the coagulation pathway, is a key distinguishing feature of more aggressive breast cancer phenotypes, as recently demonstrated in an *in vitro* model (29). This finding is of importance given that both coagulation and fibrinolytic pathways have been explored for therapeutic purposes (30, 31) and that blockade of PAI-1 has recently been reported to exert anticancer effects by modulating the function of uPA receptor (32). Of particular interest is the recent demonstration that SK-216, a specific PAI-1 inhibitor, was capable of reducing tumor size and extent of metastases in an animal model (33). Given that PAI-1 can be produced by host and tumor cells in the tumor microenvironment, a PAI-1-deficient mouse model was employed in that study to determine whether host or tumor PAI-1 was more crucial in tumor progression and angiogenesis. The results showed that host, not tumor, PAI-1 levels were able to control both processes, further suggesting that administration of SK-216 PAI-1 inhibitor exerts its antitumor actions through interaction with host PAI-1, regardless of tumor PAI-1 level (33).

On this context, the possibility of gaining prognostic information from plasma PAI-1 determination is of clinical relevance as it might provide the basis for a tailored therapeutic approach. There are, of course, some limitations to our study that need to be acknowledged. The most obvious resides in the small sample size that might have weakened the statistical power. Moreover, the study was a retrospective analysis, although all eligible consecutive patients within the designated timeframe were included and all measurements were performed while blinded to the patient outcome. On the other hand, the strength of our analysis is represented by the use of samples collected and processed using standard operating procedures, which ensures relative homogeneity among the samples used and minimizes the difference in sample analyses.

In conclusion, enhanced PAI-1 expression contributes to outcome measures in breast cancer patients and its determination might provide important information in risk stratification of patients with breast cancer. The potential limitations described above suggest that these results should be regarded with caution and that detailed experimental evaluation is needed before the ultimate prognostic significance of PAI-1 in breast cancer can be established. Additional studies are required to prospectively evaluate the clinical value of plasma PAI-1 in breast cancer. Nevertheless, we believe that its determination might provide important information in risk stratification, and encourage future investigations addressing the role of plasma PAI-1 levels in the management of patients with breast cancer, and in providing the rationale for new therapeutic strategies.

Acknowledgements

This work was partially supported by the Italian Ministry of Health Grant MERIT RBNE08NKH7.

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Received October 12, 2013
Revised November 9, 2013
Accepted November 12, 2013