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ORIGINAL ARTICLE

Protein Z (PZ) and plasminogen activator inhibitor-1 (PAI-1) plasma levels in patients with previously untreated Hodgkin lymphoma (HL)

Georgios Boutsikas[1], Evangelos Terpos[2], Athanasios Markopoulos[3], Athanasios Papatheodorou[4], Angeliki Stefanou[3], George Georgiou[1], Zacharoula Galani[1], Veroniki Komninaka[1], Vasileios Telonis[1], Ioannis Anagnostopoulos[5], Lila Dimitrakopoulou[5], Konstantinos Anargyrou[6], Konstantinos Tsionos[6], Dimitrios Christoulas[6], Maria Gonianaki[6], Theophanis Giannikos[1], Styliani Kokoris[7], Kostas Konstantopoulos[1], John Meletis[1], Maria K. Angelopoulou[1], Anthi Travlou[7], Theodoros P. Vassilakopoulos[1]

[1]Department of Hematology, National and Kapodistrian University of Athens; [2]Department of Clinical Therapeutics, National and Kapodistrian University of Athens; [3]1st Propaedeutic Department of Internal Medicine, National and Kapodistrian University of Athens; [4]Department of Biomedical Research, 251 Air Force General Hospital of Athens; [5]Immunology Laboratory, Laikon General Hospital of Athens; [6]Department of Hematology, 251 Air Force General Hospital of Athens; [7]Hematology Laboratory, National and Kapodistrian University of Athens, Athens, Greece

Summary

Purpose: *The role of Protein Z (PZ) in conditions, such as thrombosis, inflammation or cancer, is under investigation. Plasminogen Activator Inhibitor-1 (PAI-1) is an acute phase reactant that promotes thrombosis and tumorigenesis. Subject of this work was to study PZ and PAI-1 in patients with Hodgkin Lymphoma (HL), a malignancy with inflammatory background and relatively low incidence of thrombosis.*

Methods: *Newly diagnosed patients were enrolled in the study. Healthy individuals were used as controls.*

Results: *PZ levels were higher in patients compared to controls (not significantly), while PAI-1 levels were significantly higher in patients. Both PZ and PAI-1 concentrations did not correlate with most of patients' characteristics. Lower PZ levels at diagnosis were associated with presence of B symptoms and positive final positron emission tomography (PET) and higher baseline PAI-1 levels with positive final PET, too. PZ had a declining trend, but PAI-1 increased initially and decreased thereafter, during the treatment period.*

Conclusions: *Conclusively, PAI-1, but not PZ, seems to be an acute phase protein in HL. Lower PZ and higher PAI-1 levels at diagnosis may be indicative of aggressive disease. These results need further verification.*

Key words: *Hodgkin lymphoma, plasminogen activator inhibitor-1, protein Z*

Introduction

Hemostasis is a protective process that has evolved to maintain homeostasis. It interacts with other defence mechanisms, such as inflammatory response, but it is also associated with pathological conditions, such as malignancy.

Inflammation is considered as a prothrombotic stimulus. Chronic inflammation is associated with diverse hemostatic disorders [1]. Cancer is a prothrombotic state and venous thrombosis is the most common hemostatic disorder observed in cancer patients affecting prognosis [2]. Cancer patients have high levels of factors related with thrombin generation, fibrin formation and fibrinolysis [3,4]. Tumor cells produce hemostatic fac-

Correspondence to: Georgios Boutsikas, MD, MSc. Department of Hematology and Bone Marrow Transplantation, National and Kapodistrian University of Athens, Laikon General Hospital, 17, Agiou Thoma Street, Athens 11527, Greece
Tel: +30 2132061702, Fax: +30 2132061498, E-mail: gboutsikas@yahoo.gr
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tors [5-7], while it is believed that prothrombotic conversion of tumor cells is related with oncogene expression [8-10]. Moreover, coagulation system has a prominent role in tumor progression [11-25]. HL is a lymphoproliferative disease usually affecting younger adults and presenting mainly with lymphadenopathy. It is usually characterized by intense inflammatory response. Hodgkin/Reed-Sternberg (HRS) cells release cytokines, promoting their proliferation and their immunologic escape and attracting other inflammatory cells which contribute to the inflammatory background by further cytokine production. This microenvironment is responsible for B symptoms and elevated inflammatory markers frequently observed in HL [26-38]. Furthermore, the incidence of thrombosis in HL patients is about 4.7% [39].

PZ is a vitamin-K-dependent glycoprotein synthesized by the liver. It was identified in bovine plasma in 1977 [40], consists of 360 amino-acids [41] and its plasma concentration is in the order of 2.9 pg/ml [42], although normal range varies widely among studies. PZ functions as cofactor for Protein Z dependent Protease Inhibitor (PZPI) for inhibition of FXa in the presence of calcium and phospholipids [42], implying an anticoagulant role. However, the exact role of PZ in hemostasis is not clear, since observations suggest contradictory effects. It has been proposed that PZ bound to phospholipids microparticles interacts with thrombin predisposing to thrombosis [43], but PZ levels in lower normal limits are not associated with bleeding tendency [44], that PZ is an independent prognostic factor for ischaemic stroke [45], acts as acute phase protein in ischaemic stroke or contributes to its pathogenesis [46]. Additionally, low PZ levels correlate with increased risk for thrombotic complications [47], although this is not confirmed by all studies [48]. Regarding inflammation, PZ was thought to be a "negative" acute phase reactant [49-51], i.e. had negative correlation with acute phase marker levels. However, experimental studies in murine models have demonstrated that PZPI, and not PZ, functions as acute phase protein and that the observed PZ increase in acute phase depends on PZPI, which prolongs PZ half-time by complex formation [52]. Similarly, the exact role of PZ in cancer remains obscure. PZ is expressed in breast cancer [53] and non-small-cell lung cancer, but less intensely compared to prothrombotic factors [54]. Its concentration in acute leukemia was found to be lower in comparison to controls [55].

PAI-1 is a fibrinolysis inhibitor that belongs to the family of serine protease inhibitors (SERPIN). High PAI-1 levels are associated with increased risk for thrombosis [56-60], while its deficiency has been correlated with bleeding tendency [61-65]. It is considered to be an acute phase protein [66,67] and its levels increase in conditions with acute phase response [67-71]. Furthermore, PAI-1 seems to promote tumor metastasis and angiogenesis [72] and its absence correlates with slow tumor proliferation rate in knock-out mice [73].

The subject of this work was to study plasma PZ and PAI-1 levels as hemostatic factors associated with thrombosis, inflammation and malignancy in patients with HL, a disease with intense inflammatory components. More specifically, the aim of this study was to compare plasma PZ and PAI-1 levels of newly diagnosed HL patients to age- and sex-matched controls, to correlate them with clinical and laboratory baseline characteristics, prognosis and outcome of patients and to evaluate their alterations during chemotherapy.

Methods

From January of 2013 to April of 2014, 16 patients diagnosed with HL and treated in the Department of Hematology of National and Kapodistrian University of Athens, as well as 16 age- and sex-matched healthy controls were enrolled prospectively in this study, which was approved by the Ethics Committee of the University. All patients and controls gave written informed consent. According to Departmental policy patients had undergone lymph node biopsy, baseline blood counts, biochemical and "inflammatory marker" profile, and clinical staging with physical examination, computed tomography (CT) of the neck, chest and abdomen and bone marrow biopsy. Patients were treated with 4-8 cycles of ABVD-based chemotherapy or combined modality therapy according to clinical stage and/or the German Hodgkin Study Group (GHSG) [74-77]. The standard ABVD regimen consisted of adriamycin 25 mg/m^2, bleomycin 10 IU/m^2, vinblastine 6 mg/m^2 and dacarbazine 375 mg/m^2, all on days 1 and 15 of 28-day cycles (day 1 part A and day 15 part B). Advanced-stage patients <60 years old received 2 ABVD cycles followed by interim PET. If interim PET was negative, they received 6 further ABVD cycles, but if positive they were treated with 6 cycles with BEACOPP-escalated (bleomycin, etoposide, adriamycin, cyclophosphamide, oncovin, procarbazine, prednisone) [78-80].

Four blood samples were collected using free flowing technique from each patient before ABVD cycles I_A, I_B, II_A and II_B and one sample from healthy controls (for each patient a control of the same gender and of similar age was chosen). Blood samples were centrifuged at 1500 rounds per min (RPM) for 15 min and plasma was collected in aliquots and stored at -20°C.

Enzyme-Linked Immunosorbent Assays (ELISA) were standardized for determination of PZ and PAI-1 plasma levels. These molecules were measured using commercially available ELISA kits according to manufacturer's instructions (HYPHEN Biomed, Paris, France).

Their levels before I_A ABVD (in fact at diagnosis) were compared to the corresponding ones of controls and were correlated between them and with clinical and laboratory characteristics, prognosis and outcome of patients. Furthermore, their variations between the 4 different time points were studied evaluating the effect of treatment.

Baseline PZ and PAI-1 levels of patients were compared to those of healthy controls.

Statistics

Normality was assessed using Kolmogorov-Smirnov test and the above comparisons were performed using independent-samples t-test or Mann-Whitney U test, as appropriate. Baseline PZ and PAI-1 plasma levels were also evaluated in relation with baseline characteristics of patients. Correlations between continuous variables were performed with Pearson's or Spearman's correlation test, as appropriate; between continuous and categorical variables correlations were assessed using independent-samples t-test and one-way ANOVA or Mann-Whitney U test and Kruskal-Wallis test, as appropriate. Subsequently, PZ and PAI-1 alterations during chemotherapy were evaluated: Comparison of values between 2 different time points was performed with paired-samples t-test or Wilcoxon test. P values <0.05 were considered statistically significant. Boxplots that define median values, 25% to 75% of values around median and outliers belonging to the 5% to 25% and 75% to 95% percentiles of the weighted distribution were used for graphic presentation of results. Statistical analyses were performed using SPSS-11.5 package for Windows.

Results

The median age of the 16 patients was 38.5 years (range 21-67) and 7/16 (44%) were male. Controls had similar median age (39 years, range 21-65) and 7/16 (44%) were male. Characteristics of patients and controls are summarized in Table 1. None of the controls was under anticoagulants or hormone therapy. All controls had free past medical history and were not receiving any systemic treatment, with the exception of a single individual that suffered from hypertension under treatment with angiotensin II antagonist. Most of them were smokers (10/16;63%). The International Normalized Ratio (INR) of controls, an indicator of biosynthetic capability of liver, ranged from 0.97 to 1.18 and the activated partial thromboplastin time (APTT) from 30.9 to 45.8 sec. Most patients had normal liver function tests and none of them had positive medical history for thrombosis or received anticoagulants or hormone therapy.

Comparing baseline PZ concentrations it was concluded that PZ levels were similar between patients and controls (mean values 1869.9 ng/ml vs

Table 1. Characteristics of patients and controls

Characteristics	*Patients / Controls*
Median age and range (years)	38.5 (21-67) / 39 (21-65)
Medical history, n (%)	
Positive	10/16 (62) / 1/16 (6)
Drugs, n (%)	
no	12/16 (75) / 15/16 (94)
yes	4/16 (25) / 1/16 (6)
Anticoagulants, n (%)	
no	16/16 (100) / 16/16 (100)
yes	0/16 (0) / 0/16 (0)
Hormone therapy, n (%)	
no	16/16 (100) / 16/16 (100)
yes	0/16 (0) / 0/16 (0)
Smoking, n (%)	
no	5/16 (31) / 10/16 (63)
yes	11/16 (69) / 6/16 (37)
PS, n (%)	
0	14/16 (88)
1	2/16 (12)
Histological subtype, n (%)	
NSCHL	9/16 (56)
LRCHL	2/16 (13)
MCCHL	5/16 (31)
Stage, n (%)	
I	3/16 (19)
II	6/16 (37)
III	2/16 (13)
IV	5/16 (31)
B symptoms, n (%)	
no	12/16 (75)
yes	4/16 (25)
IPS, n (%)	
1	3/16 (19)
2	3/16 (19)
3	3/16 (19)
4	6/16 (37)
Final PET, n (%	
negative	13/15 (87)
positive	2/15 (13)
PCT, n (%)	
normal	15/15 (100)
abnormal	0/15 (0)
Median CRP (mg/L)	18.3
Median ESR (mm/h)	39
Median Hb (g/dl)	12.3
Median WBCs (x 10^9/L)	8.6
Median Lymphs (x 10^9/L)	1.6
Median PLTs (x 10^9/L)	315
Median haptoglobins (mg/dl)	282.5
Median γ-globins (g/dl)	1.3
Median IgG (mg/dl)	1310
Median albumin (g/dl)	4.2
Median β_2-microglobulin (mg/L)	2.1
Liver function tests, n (%)	
normal	13/16 (81)
abnormal	3/16 (19)

PS: performance status, NSCHL: nodular sclerosis classical Hodgkin lymphoma, LRCHL: lymphocyte rich classical Hodgkin lymphoma, MCCHL: mixed cellularity classical Hodgkin lymphoma, IPS: international prognostic score, PET: positron emission tomography, PCT: procalcitonin, CRP: C-reactive protein, ESR: erythrocyte sedimentation rate, Hb: hemoglobin, WBCs: white blood cells, Lymphs: lymphocytes, PLTs: platelets

1721.1 ng/ml; p=0.546), as demonstrated in Figure 1. However, PAI-1 concentration in patients (before I_A ABVD) was higher than in controls (mean values 39 ng/ml vs 15.2 ng/ml; p=0.035), as shown in Figure 2.

When evaluation of PZ and PAI-1 was restricted to controls (as a representative sample of general population), it was observed that PZ had a wide range of "normal" values from 720.4 to 2598.7 ng/ml and that the range of PAI-1 was from 2.5 to 31.6 ng/ml. Neither PZ nor PAI-1 were correlated with age [correlation coefficient (cc)=-0.137, p=0.613, cc=-0.047, p=0.861 respectively)], gender (p=0.974 and p=0.902 for PZ and PAI-1 respectively vs gender) or smoking status (p=0.822 for PZ; p=0.828 for PAI-1).

Table 2 displays baseline PZ in relation with various clinical and laboratory characteristics, prognosis and patient outcome. It was observed that baseline PZ concentration had marginal negative correlation with β_2-microglobulin value (cc=-0.478, p=0.061). Moreover, patients with B symptoms at diagnosis or positive final PET had significantly or marginally lower PZ levels at diagnosis (p=0.029 and p=0.062 respectively), as shown in Figure 3.

Studying PZ levels in the 4 different time points, a declining trend was observed (Figure 4), but differences were not significant.

An issue that should be investigated was the possibility that PZ concentration before II_B ABVD is associated with the result of interim PET, i.e. the possibility that PZ concentration close to interim PET could "predict" its result. However, it was found that this was not true (p=0.906).

Table 3 shows baseline PAI-1 in relation with clinical and laboratory characteristics, prognosis and patient outcome. PAI-1 correlated significant-

Table 2. Correlation of Protein Z (PZ) with patient characteristics

Versus	*Correlation coefficient*	*p value*
Age	-0.064	0.813
PAI-1	0.014	0.958
CRP	-0.193	0.473
ESR	-0.098	0.717
Hb	-0.122	0.652
WBCs	0.265	0.321
Lymphs	-0.177	0.511
PLTs	-0.048	0.859
Haptoglobins	-0.204	0.449
γ-globulins	-0.142	0.599
IgG	-0.188	0.486
Albumin	0.249	0.352
β_2-microglobulin	-0.478	0.061
Gender		0.233
Medical history		0.972
Drugs		0.903
Smoking		0.467
Abnormal liver function tests		0.069
Histologic subtype		0.650
Stage		0.335
B symptoms		0.029
IPS		0.882
PS		0.751
Interim PET		0.670
Final PET		0.062

PZ: protein Z, PAI-1: plasminogen activator inhibitor-1, CRP: C-reactive protein, ESR erythrocyte sedimentation rate, Hb: hemoglobin, WBCs: white blood cells, Lymphs: lymphocytes, PLTs: platelets, IPS: international prognostic score, PS: performance status, PET: positron emission tomography

Table 3. Correlation of Plasminogen Activator Inhibitor-1 (PAI-1) with patient characteristics

Versus	*Correlation coefficient*	*p value*
Age	0.115	0.671
PZ	0.014	0.958
CRP	0.300	0.259
ESR	0.258	0.336
Hb	-0.165	0.542
WBCs	0.063	0.816
Lymphs	-0.293	0.270
PLTs	*0.522*	*0.038*
Haptoglobins	0.286	0.283
γ-globulins	0.027	0.920
IgG	0.142	0.600
Albumin	-0.271	0.309
β_2-microglobulin	0.319	0.229
Gender		0.199
Medical history		0.588
Drugs		0.115
Smoking		0.874
Liver tests		0.946
Histologic subtype		0.335
Stage		0.776
B symptoms		0.225
IPS		0.253
PS		0.153
Interim PET		0.162
Final PET		0.062

PAI-1: plasminogen activator inhibitor-1, PZ: Protein Z, CRP: C-reactive protein, ESR: erythrocyte sedimentation rate, Hb: hemoglobin, WBCs: white blood cells, Lymphs: lymphocytes, PLTs: platelets, IPS: international prognostic score, PS: performance status, PET: positron emission tomography

ly with the absolute number of platelets (cc=0.522, p=0.038). Furthermore, as in the case of PZ, it seems that PAI-1 levels at diagnosis were related with final PET. More specifically, PAI-1 levels in patients with positive final PET were marginally (p=0.062) higher in comparison to patients with negative final PET (Figure 5).

A slightly different pattern of variation in comparison to PZ was observed for PAI-1. Its levels showed a rising trend initially until I_B ABVD (not significant) and decline thereafter (p=0.020), as demonstrated in Figure 6.

Similarly to PZ, PAI-1 before II_B ABVD was not correlated with the result of interim PET (p=0.858).

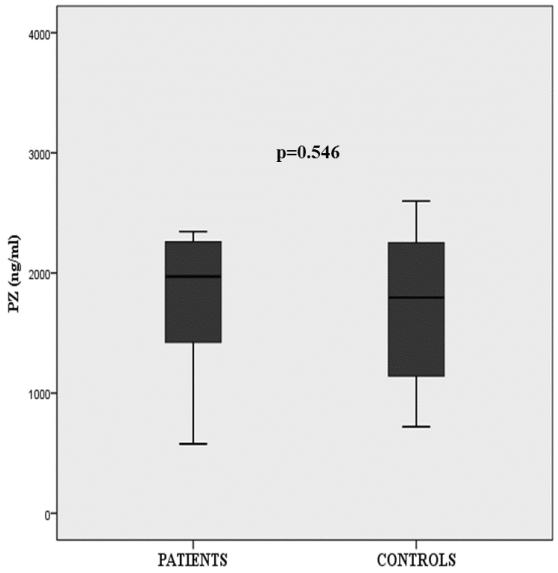


Figure 1. Comparison of Protein Z (PZ) levels between patients and controls.

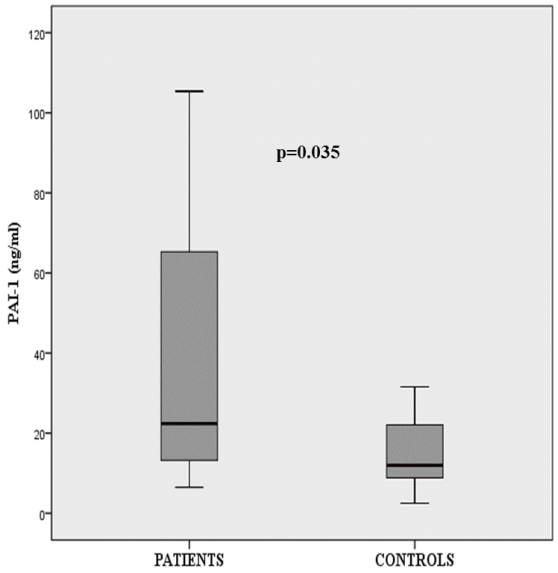
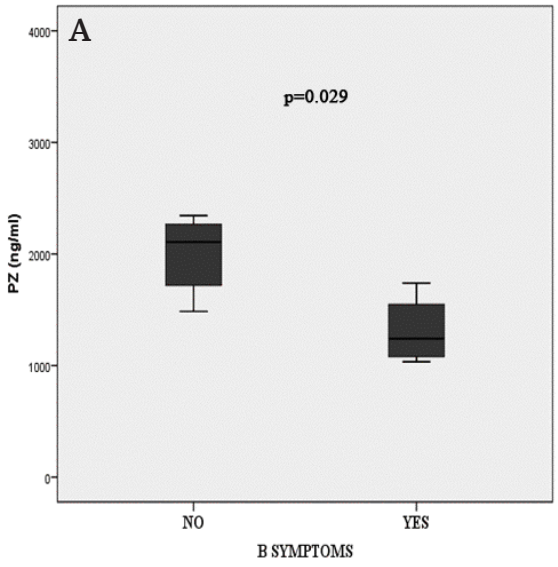


Figure 2. Comparison of Plasminogen Activator Inhibitor-1 (PAI-1) levels between patients and controls.



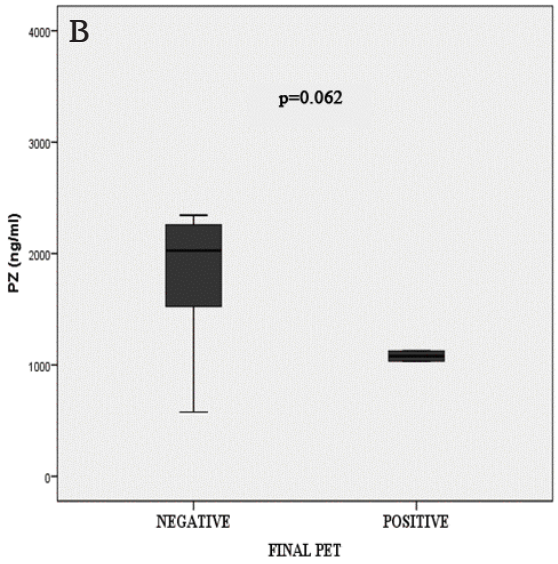


Figure 3. Protein Z (PZ) levels in relation with B symptoms **(A)** and final positron emission tomography (PET) **(B)**.

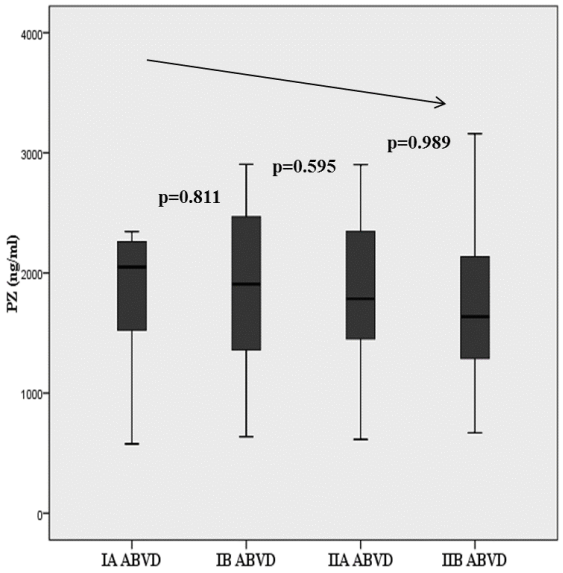


Figure 4. Variation of Protein Z (PZ) levels during treatment (*ABVD*: adriamycin, bleomycin, vinblastine, dacarbazine).

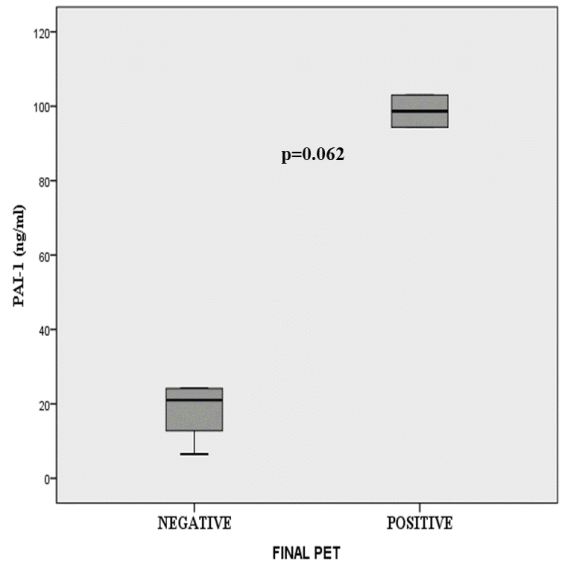


Figure 5. Plasminogen activator inhibitor-1 (PAI-1) levels in relation with final positron emission tomography (PET).

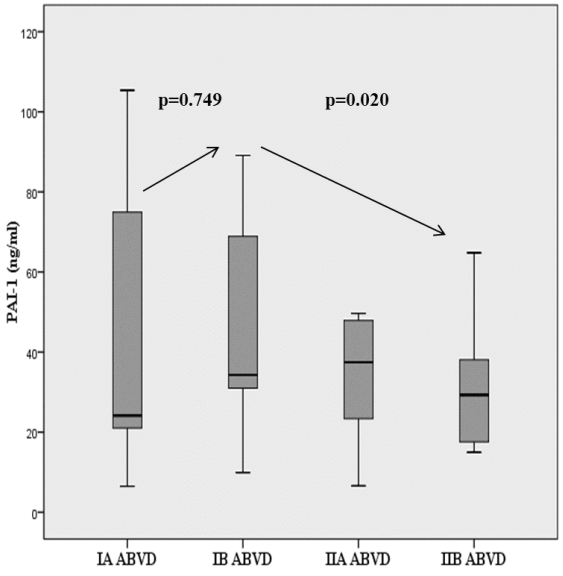


Figure 6. Variation of plasminogen activator inhibitor-1 (PAI-1) levels during treatment (*ABVD*: adriamycin, bleomycin, vinblastine, dacarbazine).

Discussion

In this work it has been attempted to study two components of hemostasis, PZ and PAI-1, which, according to the literature, are associated with thrombosis [43,46-48,56,59,60,81], inflammation [49-52,66-68,70] and malignancy [54,55,72,73], in patients with HL, an hematological malignancy with intense inflammatory background [35-38] and relatively low incidence of thrombotic complications [39]. For this purpose, patients with newly diagnosed HL and age- and sex-matched healthy individuals were studied prospectively.

The major findings are summarized as follows: 1) PZ was higher in patients compared to controls (not significantly), while PAI-1 was significantly higher in patients. 2) Both PZ and PAI-1 did not correlate with most of the patient characteristics. 3) Lower baseline PZ levels were associated with presence of B symptoms and positive final PET. Similarly, higher PAI-1 levels were related with positive final PET, too.

A limitation of the present study is that the small number of patients and controls could affect its power to detect potential underlying differences. However, significant differences emerging from this study were detected with non-parametric tests, that take into account the small size of the statistical sample and the possible deviation from normality, when appropriate.

All controls had normal INR, indicative of normal biosynthetic capability of liver (main site of PZ synthesis). Unfortunately, INR was not measured in patients. However, 13/16 (81%) had normal and only 3/16 abnormal liver tests restricted to serum alkaline phosphatase and γ-glutamyltransferase elevations, which are common in advanced HL due to cytokine effect [82]. Thus, we did not expect any impairement of liver synthetic capacity in this patient series. Additionally, no patient or control had positive history for thrombosis or was receiving anticoagulants or hormone therapy that could influence the levels of the two hemostatic factors (mainly PZ, a vitamin K-dependent protein). Furthermore, the use of free flowing technique for blood sample collection minimized activation of endothelium, the main site of PAI-1 release.

If controls are considered as a representative sample of the general population, it was observed that PZ had a wide range of "normal" values, a result in accordance with some reports [44,48]. Moreover, PZ and PAI-1 levels were not associated with gender, age or smoking in controls.

Baseline PZ levels were similar between patients and controls. Additionally, they were not correlated with most of patient characteristics associated with the inflammatory background of HL. However, a marginal negative correlation was observed between plasma PZ and serum β_2-microglobulin values. Serum β_2-microglobulin value is strongly correlated with other features of aggressive disease [83,84]; thus it could be assumed that lower PZ levels at diagnosis are associated with aggressive disease. The observation that low baseline PZ levels were associated with B symptoms is also suggestive of this hypothesis. Notably, low baseline PZ levels were associated with final PET positivity. Only 2/15 patients remained PET-positive and both of them progressed: these patients and a single additional advanced-

stage patient, who achieved PET-negative remission, had the lowest baseline plasma PZ levels.

The relationship of PZ with thrombosis could not be evaluated in this study, because no patient had positive medical history for thrombosis or presented with thrombosis during treatment or follow-up. Regarding the potential relationship of PZ with inflammation, it was not verified that PZ is a negative acute phase reactant, as proposed by various studies [49-51]. Its levels in HL patients do not differ from those in controls. The last observation goes with Cesari et al. [81] and Girard et al. [52]. The first found out similar patterns of variation, i.e. initial increase and subsequent decrease, between interleukin (IL)-6 and PZ in the acute phase of acute coronary syndrome, while the second argue that PZ increases in acute phase response, but it is not an acute phase protein, and, probably, the rising of PZ depends on PZPI. What is the relationship of PZ with malignancy? According to the results, lower PZ concentration at diagnosis seems to be associated with more aggressive disease (presence of B symptoms, positive final PET, elevated β2-microglobulin value). Finally, does PZ contribute to the expected prothrombotic state of HL? If the answer is positive, then the increased levels in comparison to controls mean that PZ promotes thrombosis [43-46] and the declining trend under treatment implies response of disease and progressive recession of the prothrombotic state. If the answer is negative [47,48], then higher PZ concentration in patients reflects a defence mechanism that tries to balance the prothrombotic state induced by inflammation and malignancy. In this case the declining trend of PZ levels during treatment could be attributed to recession of the prothrombotic state, due to response to treatment and consequently to recession of the need of the aforementioned defence mechanism. It should be emphasized that no patient had primary refractory disease. The only patient with positive interim PET is not considered as treatment failure, since he was about for treatment modification based on an unfavorable prognostic factor (positive interim PET).

Regarding PAI-1, patients had higher concentration compared to controls. Its concentration did not correlate with various characteristics of patients, except for platelet count. However, the small number of patients prevents extraction of definitive conclusions, since true existence of such relationships could not be reliably excluded. Notably, similarly to what was observed for low baseline PZ levels, higher baseline PAI-1 levels were marginally associated with positive final PET. Both patients who remained PET-positive and progressed and one more patient who remains in remission had the highest baseline plasma PAI-1 levels.

What is the relationship of PAI-1 with inflammation? In this study, it was confirmed that PAI-1 is an acute phase protein [66-70]. A reasonable question is why PAI-1 is not correlated with other inflammatory components of HL. Possibly, the small sample or a different pathogenetic mechanism did not allow such a difference to come out. What is the relationship of PAI-1 with malignancy? Similarly to PZ, higher PAI-1 concentration at diagnosis marks probably more aggressive disease biological behavior (positive final PET). Furthermore, it became clear that PAI-1 describes well the prothrombotic state that is expected to accompany HL. Initial increase of its levels might suggest a worsening of this potential prothrombotic state, possibly due to toxic effect of chemotherapy to endothelium (basic site of PAI-1 synthesis), while subsequent decrease could be attributed to recession of this prothrombotic state resulting from response to treatment.

Both PZ and PAI-1 at diagnosis and before II_B ABVD were not associated with interim PET result, whose predictive value is accepted, but there are cases in which exact prognosis needs more parameters. This observation should be verified in more patients, as only one patient had positive interim PET.

It is clear that studying of PZ and PAI-1 in larger series of patients and controls will strengthen the reliability of results. In addition, large prospective studies are needed in order to clarify the exact role of PZ in thrombosis, inflammation and malignancy, while studying of these markers in histological preparations is necessary, since it is possible that their plasma concentration does not reflect well the events that take place in tissue microenvironment. Finally, the relationship between lower PZ and higher PAI-1 levels at diagnosis with final PET positivity should be emphasized. Indeed, the importance of this finding is greater if it is counted that one of the two patients with positive final PET had positive interim PET too and his treatment was intensified to BEACOPP - escalated after interim PET (does hemostasis help to approach biology of HL better?). If this finding is confirmed in larger series either as an independent prognostic factor or in combination with other prognostic factors, it will be useful for prognostic stratification of patients and early treatment modification. Moreover, it strengthens the current opinion about the relationship of hemostasis with cancer and the effect of heparin in survival of patients with cancer [85].

To our knowledge, this work, besides the small number of patients and controls, is one of the first attempts to study PZ and PAI-1, two hemostatic factors, in patients with HL. The findings about the "behavior" of PZ in patients with HL are not clear, while PAI-1 seems to be an acute phase protein in this series of patients and to describe well the prothrombotic state that is expected in patients with HL. Additionally, the observation that lower PZ or higher PAI-1 levels at diagnosis may be associated with aggressive disease is considered remarkable. However, the last needs further verification in order to be incorporated in the prognostic stratification of patients with newly diagnosed HL.

Conflict of interests

The authors declare no confict of interests.

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