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ARAŞTIRMA MAKALESİ

THE LEVELS OF PLASMINOGEN ACTIVATOR INHIBITOR-1 (PAI-1), UROKINASE PLASMINOGEN ACTIVATOR (UPA), THROMBOMODULIN (TM) AND TISSUE FACTOR PATHWAY INHIBITOR (TFPI) IN ESSENTIAL THROMBOCYTHEMIA

ESANSIYEL TROMBOSITEMIDE PLAZMINOJEN AKTIVATÖR İNHIBITÖR-1 (PAI-1), ÜROKINAZ PLAZMINOJEN AKTIVATÖR (UPA), TROMBOMODULIN (TM), DOKU FAKTÖRÜ YOLAK İNHIBITÖRÜ (TFPI) DÜZEYLERI

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ABSTRACT

Introduction: Thromboembolic attacks are frequent in essential thrombocythemia (ET) patients, and the causes of thromboembolism include many acquired causes in addition to some genetic factors like JAK 2. This study is intended to evaluate the levels of some coagulation parameters such as PAI-1, uPA, TM, TFPI, and vWF (von Willebrant Factor) in thromboembolic attacks.
Materials and Methods: The study group consisted of 30 essential thrombocythemia patients with a mean age of 59.5±9.9 and 30 healthy controls with a mean age of 56.2±6.1. Plasma PAI, uPA, TM, TFPI, and vWF levels were determined by ELISA method according to manufacturer's instructions.
Results: There was a significant difference in PAI-1, uPA, TM, and TFPI levels between the patient and control groups ($p<0.05$). No statistically significant difference, however, was obtained in vWF levels between the two groups ($p>0.05$).
Conclusions: High levels of PAI-1, uPA, TM and TFPI indicate that some coagulation parameters are activated, and we believe that this may cause thromboembolic attacks regardless of genetic factors.

Keywords: essential thrombocythemia, plasminogen activator inhibitor-1, urokinase plasminogen activator, thrombomodulin, tissue factor pathway inhibitor.

ÖZET

Giriş: Tromboembolik ataklar esansiyel trombositemi (ET) hastalarında sık görülmekte olup tromboembolizmin nedenleri JAK 2 gibi genetik faktörlere ek olarak birçok edinsel nedeni de kapsar. Bu çalışma tromboembolik ataklarda PAI-1, uPA, TM, TFPI, ve vWF (von Willebrant Faktör) gibi koagülasyon parametrelerinin düzeylerini değerlendirmeyi amaçlamaktadır.
Materyal ve Metod: Çalışma grubunu ortalama yaşı 59.5±9.9 30 esansiyel trombositemi hastası ile ortalama yaşı 56.2±6.1 olan 30 sağlıklı kontrol oluşturmaktadır. Plazma PAI, uPA, TM, TFPI, and vWF düzeyleri üreticininin talimatları doğrultusundaki ELISA yöntemi ile belirlenmiştir.
Bulgular*: H*asta ve kontrol grubu arasında PAI-1, uPA, TM, ve TFPI düzeyleri bakımnıdan anlamlı bir farklılık mevcuttu ($p<0.05$). Bununla birlikte iki grup arasında vWF düzeylerinde istatistiksel oalrak anlamlı bir fark yoktu ($p>0.05$).
Sonuç: Yüksek PAI-1, uPA, TM ve TFPI düzeyleri bazı koagülasyon parametrelerinin aktive olduğunu gösterir ve biz inanıyoruz ki bu durum genetik faktörlerden bağımsız olarak tromboembolik ataklara yol açabilir.

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INTRODUCTION

Usually, ET is a myeloproliferative disorder characterized by persistent peripheral thrombo cytosis and abnormal megakaryocytic proliferation in the bone marrow (1). ET is a rare cause of thrombocytosis and may be associated with both venous and arterial thrombosis in 10-75% of cases, depending largely on the study and whether vasomotor symptoms are included in thrombotic manifestations (2–4). Thrombotic events may be observed in both microvascular and macrovascular arterial circulation in patients with ET (3).

In general, risk factors for thrombosis in patients with ET include: increased age, a prior thrombotic episode, and the presence of risk factors for atherosclerotic heart disease (5). Several authors agree that there does not appear to be a linear relationship between platelet counts and thrombosis (2,6), but others attribute both the development of thrombosis and hemorrhage to an increasing platelet count (1). Furthermore, there is no consistent evidence that the in vitro assessment of platelet aggregation will identify patients at risk for thrombosis (3). It is therefore not surprising that the task of appropriately adding those patients with ET who are at increased risk for thrombotic events poses both a clinical and laboratory challenge. As a result, a detailed understanding of the thrombotic mechanisms becomes difficult because of the possible multifactorial nature of thrombosis in these patients.

It is well recognized that plasminogen activator inhibitor (PAI-1) acts as the primary physiological inhibitor of plasminogen activation in vivo, and is associated with a variety of thrombotic conditions when elevated, including myocardial infarction and deep venous thrombosis. Elevated PAI-1 concentration was found to be a reliable predictor of coronary events in patients with angina pectoris or previous myocardial infarctions (7). Moreover, PAI-1 appears to be upregulated in obesity, atherosclerosis, hypertension, glucose intolerance, insulin resistance, and malignancy (10). Although not often reported, an association between PAI-1 elevations and thrombogenesis has also been a feature in patients with ET (6).

PAI-1 is the central physiologic inhibitor of the plasminogen activator tPA in blood. PAI-1 ia an important inhibitor of uPA. TFPI, is a major anticoagulant present in plasma and associated with vascular endothelium. TFPI binds to and neutralizes factor Xa to inhibit coagulation. TM functions as a cofactor for α-thrombin in the activation of protein C and Thrombin-Activatable Fibrinolysis Inhibitor. One of the major activators of coagulation in blood is uPA. vWF is a multifunctional protein with several key roles in coagulation.

We believe that activation of coagulation system contributes to thromboembolic events in patients with ET. Therefore, we found it appropriate to choose the coagulation parameters including PAI-1, uPA, TM, TFPI, and vWF.

MATERIAL AND METHODS

Patients

A total of 30 patients diagnosed with ET and 30 healthy persons as control were enrolled in this study. The ET diagnoses were made as per the diagnostic criteria suggested by the World Health Organization [36]. Age averages of both groups were similar (59.5±9.9 vs. 56.2±6.1, p:0.121) (Table 1). No symptoms of acute infection or inflammatory disease were seen in the ET cases and healthy control group. None of the ET patients nor healthy controls was using oral contraceptives or taking hormone replacement therapy at the time of the study. All research was approved by the local ethics committee. The procedure used was in compliance with the 1975 Helsinki Declaration as revised in 2000, and their blood samples were collected after their informed consents were taken. A total of sixteen patients with ET were JAK 2 positive while 14 were JAK 2 negative.

Laboratory analyses

Venous fasting blood samples were collected from an antecubital vein in evacuated tubes with 3.8% trisodium citrate as anticoagulant (Vacuette, Greiner Bio-One, Austria). After centrifugation, the plasma samples were aliquated, frozen and stored at -80°C for PAI-1, uPA, thrombomodulin (TM), TFPI and vWF analysis. Repeated freezing and thawing processes were avoided. Plasma PAI (Bender MedSystems Inc, Vienna, Austria), uPA, TM, TFPI and vWF (American Diagnostica Inc., Stamford, CT, USA) levels were determined by ELISA method according to the manufacturer's instructions.

TFPI

The necessary number of precoated microwells were removed from the foil pouch and they were placed in the plate holder. Hundred µL of TFPI Standard, TFPI Reference Plasma, diluted samples (1:40) were added to the microwells, covered with an acetate sheet and incubated 16-20 hours at 2°-8°C. The following day, the contents of the microwells were emptied and washed 4 times with Wash Buffer. The droplets were removed by tapping the plate 4-5 times face down against absorbing material. Hundred µL of Detection Antibody was added to each microwell, covered with the acetate sheet and incubated for 1 hour at room temperature. After the incubation, the contents of the microwells were emptied and washed 4 times with Wash Buffer. The droplets were removed by tapping the plate 4-5 times face down against absorbing material. Hundred µL of diluted enzyme conjugate was added to each microwell, covered with the acetate sheet and incubated for 1 hour at room temperature. After the incubation, the contents of the microwells were emptied and washed 4 times with Wash Buffer. The droplets were removed by tapping the plate 4-5 times face down against absorbing material. Hundred µL of Substrate solution was added to each microwell, covered with the acetate sheet and incubated for 15-20 minutes at room temperature.

A blue color developed. The enzymatic reaction was stopped by adding 50 µL of Stop solution to each microwell. The sides of the microwell frames was tapped to ensure even distribution. The solution color turned yellow. The absorbances were read on a microwell plate reader at a wavelength of 450 nm within 10 minutes. The background average of the blanks was deducted from the standards and sample readings. The standard curve was constructed by plotting the mean absorbance value for each TFPI standard versus its corresponding concentration in ng/mL. The TFPI concentrations for diluted samples were Interpolated directly from the standard curve by multiplying by the dilution factor of the sample.

Thrombomodulin

The necessary number of precoated microwells were removed from the foil pouch and they were placed in the plate holder. Two hundred µL of standard, diluted plasma sample (1:4) were added to the microwells, covered with the lid and incubated for 1 hour at room temperature. The contents of the microwells were emptied and washed 4 times with Wash Buffer. The droplets were removed by tapping the plate 4-5 times face down against absorbing material). Two hundred µL of Detection Antibody was added to each microwell, covered with the acetate sheet and incubated for 30 minutes at room temperature. The contents of the microwells were emptied and washed 4 times with Wash Buffer. The droplets were removed by tapping the plate 4-5 times face down against absorbing material). Two hundred µL of Substrate solution was added to each microwell, covered with the acetate sheet and incubated for 20 minutes at room temperature. A blue color developed. The enzymatic reaction was stopped by adding 100 µL of 0.5M H_2SO_4 to each microwell. The solution color turned yellow. The absorbances were read on a microwell plate reader at a wavelength of 450 nm within 30 minutes. The background average of the blanks was deducted from the standards and sample readings. The standard curve is constructed by plotting the mean absorbance value for each thrombomodulin standard versus the corresponding concentration of thrombomodulin in ng/mL. Thrombomodulin concentrations were obtained for each test sample, as interpolated from the standard curve. This concentration was multiplied by the dilution factor of the sample to calculate thrombomodulin concentration of original sample.

uPA

The necessary number of precoated microwells were removed from the foil pouch and they were placed in the plate holder. Hundred µL of uPA Standard and diluted sample (1:20) were added to the microwells, covered with an acetate sheet and incubated 16-20 hours at 4°C. The following day, the contents of the microwells were emptied and washed 4 times with Wash Buffer. The droplets were removed by tapping the plate 4-5 times face down against absorbing material). Hundred µL of Detection Antibody was added to each microwell, covered with the acetate sheet and incubated for 1 hour at room temperature. After the incubation, the contents of the microwells were emptied and washed 4 times with Wash Buffer. The droplets were removed by tapping the plate 4-5 times face down against absorbing material. Diluted enzyme conjugate was prerared by adding 12 µL of Enzyme Conjugate to 12 mL of Enzyme Conjugate Diluent. Hundred µL of diluted enzyme conjugate was added to each microwell, covered and incubated for 1 hour at room temperature. After the incubation, the contents of the microwells were emptied and washed 4 times as described above. Hundred µL of Substrate solution was added to each microwell, covered with the acetate sheet and incubated for 20 minutes at room temperature. A blue color developed. The enzymatic reaction was stopped by adding 0.5N H2SO4 to each microwell. . The sides of the microwell frames was tapped to ensure even distribution of H_2SO_4. The solution color turned yellow. The absorbances were read on a microwell plate reader at a wavelength of 450 nm within 10 minutes. The standard curve was constructed by plotting the mean absorbance value for each uPA standard versus its corresponding concentration in ng/mL. uPA concentrations were obtained for each test sample, as interpolated from the standard curve. This concentration was multiplied by the dilution factor of the sample to calculate uPA concentration of original sample.

vWF

The necessary number of precoated microwells were removed from the foil pouch and they were placed in the plate holder. Hundred µL of standard, diluted plasma sample (1:100) were added to the microwells, covered with the acetate sheet and incubated for 1 hour at room temperature. The contents of the microwells were emptied and washed 4 times with Wash Buffer. Working strength Detection Antibody was prepared by diluting the concentrate 1:100 as follows: 20 µL of Detection Antibody was added to 2 mL of Detection Antibody Diluent. Then 100 µL of working strength Detection Antibody was added to each microwell, covered with the acetate sheet and incubated for 1 hour at room temperature. The contents of the microwells were emptied and washed 4 times with Wash Buffer. Hundred µL of Substrate solution was added to each microwell, covered with the acetate sheet and incubated for 20 minutes at room temperature. A blue color developed. The enzy matic reaction was stopped by adding 50 µL of 0.5N H_2SO_4 to each microwell. . The sides of the microwell frames was tapped to ensure even distribution of H_2SO_4. The solution color turned yellow. The absorbances were read on a microwell plate reader at a wavelength of 450 nm immediately. A standard curve was constructed by plotting the mean absorbance value for each vWF standard versus its corresponding concentration. The mean absorbance value for each diluted sample was used to interpolate its vWF concentration from the standard curve. The concentration was determined from the standard curve by multiplying with the dilution factor to obtain the vWF concentration in the original plasma sample.

PAI-1

The samples were prediluted 1:50 with Assay Buffer (1x) before starting with the test procedure. The necessary number of microwells were removed from the foil pouch and they were placed in the plate holder. Microwell strips were washed twice with approximately 400 µl Wash buffer per well with thorough aspiration of microwell contents between washes. Hundred µl of Assay Buffer was added to the blank wells, 50 µl of Assay Buffer was added to the sample wells. Fifty µl of prediluted sample was added to the sample wells. Biotin-Conjugate was prepared and 50 µl of Biotin-

Conjugate was added to all wells. The plate was covered with an adhesive film and incubated at room temperature for 2 hours on a microplate shaker set at 400 rpm. Streptavidin-HRP was prepared. After incubation, microwell strips were washed 3 times and proceeded immediately to the next step. Hundred µl of diluted Streptavidin-HRP was added to all wells, including the blank wells and covered with an adhesive film and incubate at room temperature for 1 hour on a microplate shaker set at 400 rpm. The adhesive film was removed and the wells were washed 3 times and proceed immediately to the next step. Hundred µl of TMB Substrate Solution to all wells was added and incubated at room temperature for about 10 min. The enzyme reaction was stopped by pipetting 100 µl of Stop Solution into each well and the results were read immediately after the Stop Solution was added on a spectro-photometer using 450 nm as the primary wave length. The concentration was determined from the standard curve by multiplying with the dilution factor to obtain the PAI-1concentration in the original plasma sample.

Statistical analyses

All statistical analyses were performed using the Statistical Package for Social Sciences (SPSS, version 11.0 for Windows, Chicago, Ill, USA). Data were expressed as mean±standard deviation (SD). A "p" value <0.05 was accepted as significant. A Kolmogorov-Smirnov test was performed to assess the normality of the variables. T-test and Mann-Whitney U tests were used to compare the variables. According to the Kolmogorov-Smirnov test, the distribution of TFPI and vWF were normal (p>0.05) whereas the distribution of PAI, uPA and TM were not normal. Normally distributed variables were compared with the t-test whereas other parameters were compared with the Mann-Whitney U test.

Results

The study group consisted of 30 ET patients with a mean age of 59.5±9.9 and 30 healthy controls with a mean age of 56.2±6.1 (Table). Mean PAI-1, uPA, TM, TFPI and vWF levels of groups are presented in Table 1. There was a significant difference in PAI-1 (Fig. 6), TM (Fig. 7), TFPI (Fig. 5), and uPA (Fig. 8) levels between patient and control groups (p<0.05). However, no statistically significant difference was obtained in vWF levels between two groups (p>0.05).

Pearson's correlation analysis revealed that platelet count was correlated with thrombomodulin (Fig. 1; r=0.30, p=0.020), TFPI (Fig. 2; r=0.30, p=0.019), uPA (Fig. 3; r= 0.27, p<0.001) and with PAI-1 (Fig. 4; r=0.144, p=0.273) in the whole study group.

No difference was observed between JAK 2 positive and negative patients regarding these parameters.

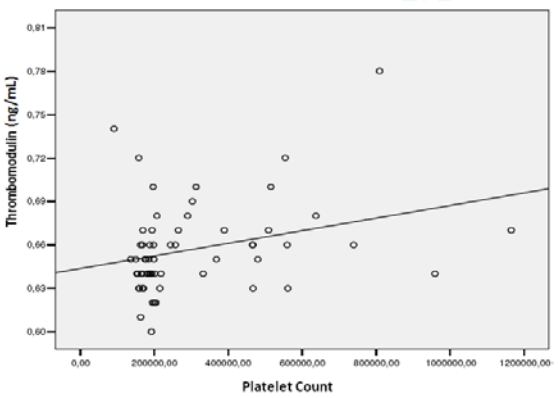


Figure 1: Positive correlation between platelet count and thrombomodulin level in the study group.

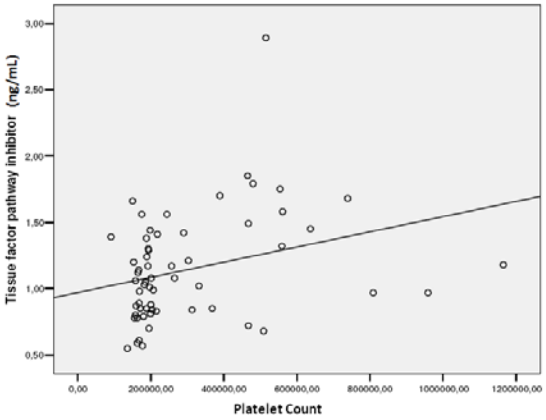


Figure 2: Platelet count was correlated with Tissue Factor Pathway Inhibitor in the study group.

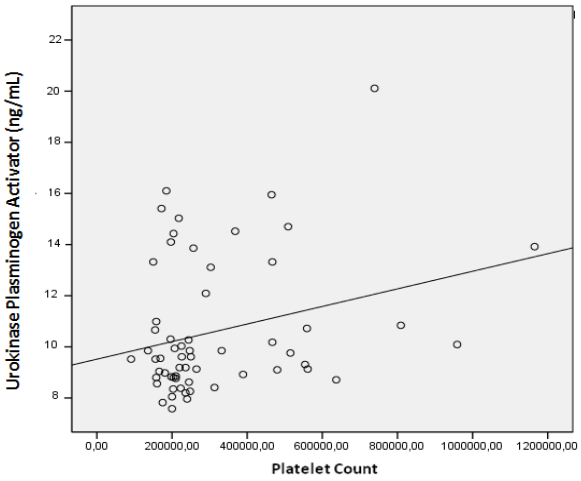


Figure 3: Platelet count was correlated with Urokinase Plasminogen Activator in the study group.

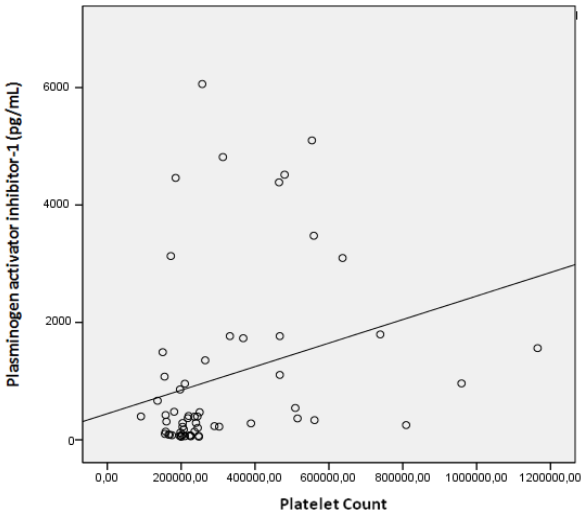


Figure 4: Platelet count was weakly correlated with Plasminogen Activator Inhibitor-1 in the study group. (r=0.144, p=0.273).

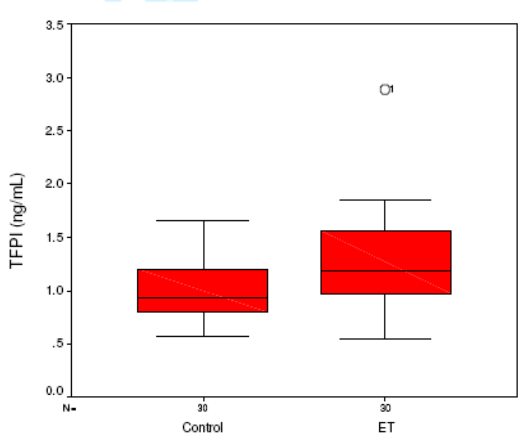


Figure 5: The boxplot graph of TFPI in the control and patient group.

	Patient group	*Control group*	*p*
n	30	30	
Age (years)	59.5±9.9	56.2±6.1	0.121
TFPI (ng/mL)	1.28±0.46	1.01±0.29	0.008
vWF (mU/mL)	11.34±6.67	8.69±4.54	0.070
PAI (pg/mL)	1643.2±1740.2	510.3±971.4	<0.001
TM (ng/mL)	0.67±0.03	0.64±0.003	<0.001
uPA (ng/mL)	11.66±2.79	9.54±2.01	<0.001

Table : Comparison of parameters in patients and controls (mean±SD).

DISCUSSION

Plasminogen activator inhibitor-1 (PAI-1), a member of the serine proteinase inhibitor (serpin) family, is produced in the liver and endothelial cells and is expressed in thrombocytes, liver cells,monocytes,and smooth muscle cells(11).
Most active proportion of PAI-1 is found primarily in the liver and secondarily in the spleen (11).
A small portion of PAI-1 is found in the brain and myocardium (12). Normal human blood contains independent pools of plasma and platelet PAI-1 as well, in varying amounts and activity (13). Although found in small quantities (7%), it is the plasma PAI-1 that shows the highest activity. Platelets, in contrast, account for the remaining 93% of blood PAI-1, with only 3–5% activity (14). PAI-1 is affected by gender, age, obesity, plasma concentrations of tumor necrosis factor (TNF-alpha), interleukin-1(IL-1), tumor growth factor (TGF)-β, lipopolysaccharides, phorbol esters, glucocorticoid hormones, VLDL, and insulin (15). Possessing a circadian rhythm, PAI-1 shares similarities with hormones and other cytokines associated with thromboembolic events (including myocardial infarctions), with peak activity in the early morning followed by rapid tapering (16). Among other features, PAI-1 is known to be an acute phase reactant. Increased plasma PAI-1 activity is found during acute events such as myocardial infarction, postoperative period, endotoxemia, and septicemia (12). In fact, PAI-1 levels have been shown to be a good prognostic factor for fatal outcomes in septicemia because PAI-1 is increased up to five-fold in response to endotoxin, lipopolysaccharides, and IL-1 elevations (12).
The PAI-1 molecule reacts rapidly with tissue plasminogen activator (tPA) and uPA, forming very stable, stoichiometric 1:1 complexes, as is typical for serpins (16). PAI-1, however, lacks cysteines (and hence disulfide bonds) unlike other serpins, but contains multiple methionines. The detection of PAI-1 mRNA and PAI-1 antigen in megakaryocytes suggests that PAI-1 may be deposited into alpha
granules during the maturation of these cells. Studies have shown that approximately 4000 molecules of PAI-1 per platelet are localized in the alpha granules (19). On activation, platelets will secrete both PAI-1 and alpha 2-antiplasmin, inhibiting plasmin formation and activity, respectively (20). Most of the platelet PAI-1 appears to be in the latent form, a phenomenon that may be associated with the stabilizing effects of vitronection (17). It has been suggested that the latent form of PAI-1 may be responsible for the resistance to thrombolytic therapy by platelet-rich thrombi. The presence of a large storage pool of latent PAI-1 in platelets, however, also raises the possibility that mechanisms may exist for the activation of this form of PAI-1 in vivo.
Although found in low concentrations in the blood, PAI-1, is responsible primarily for in vivo inhibition of both tissue type (tPA) and urokinase-type (uPA) plasminogen activators. Under the influence of both genetic and environmental factors, PAI-1 elevation has been associated with the promotion of both venous and arterial thrombus formation in a multitude of medical conditions (24). PAI-1 has been correlated positively with severe hypertension, myocardial disease, deep venous thrombosis, malignancies, including myeloproliferative diseases, disorders of glucose and lipid metabolism, type II diabetes mellitus,

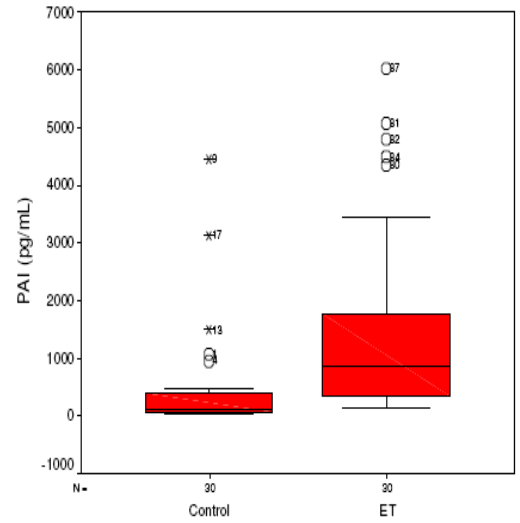


Figure 6: The boxplot graph of PAI in the control and patient groups.

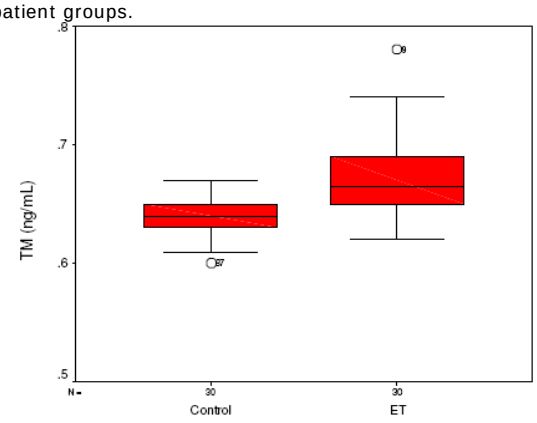
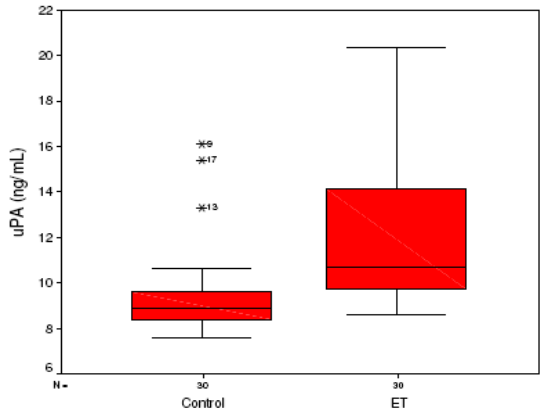


Figure 7-8: The boxplot graph of TM in the control and patient groups.(up)The boxplot graph of uPA in the control and patient groups.(down)



polycystic ovarian syndrome, and is influenced by both alcohol consumption and acute infection (23,24,25). Although the association between arterial thrombosis and myeloproliferative disorders has been established previously, the relation between ET, PAI-1 elevations, and arterial thrombogenesis only has been sporadically mentioned in the literature.

PAI-1 is expressed within invasive foci of multiple types of human cancer (8). It was reported that PAI-1 is not produced by epithelial cancer cells but by the stromal cells contained within various tumors. High levels of PAI-1 have been found within the endothelial cells of small vessels in breast, gastric, liver, pancreas, lung, ovary, skin, brain, and colon carcinomas. High PAI-1 levels in certain tumors may predict poorer outcome. Additionally, a strong correlation between the number of tumor-positive axillary lymph nodes, particularly in breast cancer, and increased levels of PAI-1, has been made (8). This association may have significant prognostic and therapeutic implications and may serve as supportive evidence for PAI-1 surveillance in cancer patients at risk for thromboembolic events.

Tissue factor pathway inhibitor (TFPI) is an endothelial membrane-associated protein (26). TFPI co-localizes with tissue factor and macrophages in atherosclerotic plaques, where it may reduce thrombotic and inflammatory potential (27). In vascular injury models, TFPI infusion or local gene delivery had favorable effects on thrombosis and restenosis (28), while TFPI deficiency in atherosclerosis-prone mice was associated with more atherosclerosis, more plaque tissue factor activity, and decreased time to occlusive thrombosis with vascular injury (29). TFPI circulates bound to lipoproteins primarily with a smaller free component; both can be measured with commercial assays (30). Both free and total TFPI concentration may be higher with acute or chronic vascular disease (31). Not all studies agree, however (32). Epidemiologic studies have reported conflicting results concerning correlation of TFPI with vascular risk factors (33). TFPI, however, is essentially the stoichiometric inhibitor of extrinsic pathway (FVIIa-tissue factor) of coagulation. The complex that it forms through binding with FXa rapidly inactivates the FVIIa-tissue factor. It should be considered that this increase seen in the TFPI level is caused by the co-activation of the tissue factor with FVIIa.

As we know, Thrombomodulin (TM) binds with α-thrombin, and neutralizes its procoagulant activity; and plays a cofactor role of α-thrombin in the activation of Protein C and TAFI (Thrombin Activatable Fibrinolysis Inhibitor). It also has anticoagulant and antifibrolinytic function. Its increase in ET indirectly shows that α-thrombin is activated in this disease group.

Increase in the PAI-1 and uPA levels in ET patients indicates that coagulation is activated in addition to vascular injury. Although genetic factors such as JAK2 are shown to cause thromboembolism in ET, vascular injury as well as activation of coagulation may also cause this. uPA is one of the major extrinsic activators in the blood, and is effective in both vascular tissue and extravascular tissue. uPA is reported to have a significant role in the activation of matrix metalloproteinase, as well as degradation of extracellular matrix through its plasminogen activation (34,35). In particular, it has an amplifying effect on the formation speed of plasmin secondary to fibrin in the fibrinolytic process as does t-PA on vascular tissue. Although it is believed that uPA primarily is effective in extracellular matrix degradation, as well as tissue repair and remodeling, the existence of its

profibrinolytic features should not be ignored. Since it has shown negative correlation with the number of thrombocytes in our study, we consider that fibrinolysis occurs after coagulation in these cases, and the number of thrombocytes decrease after occurrence of this process. The parameters (TM, TFPI, uPA, and PAI-1) studied show a positive correlation with platelet counts, suggesting that coagulation activation is likely to be a part of the nature of ET. (Figures 1-4)

As we know, elevation of both TFPI and TM act as a compensatory mechanism to protect from thrombosis by neutralizing TF and thrombin respectively, secondary to coagulation activation. Hence, elevation of these parameters also suggests an activation of coagulation.

Consequently, due to increase in the PAI-1, uPA, TM and TFPI values in ET, we believe that some of the coagulation parameters activated in this disease should be included in the factors that cause thromboembolism.

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