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The circadian rhythm of selected parameters of the hemostasis system in healthy people

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ABSTRACT

In this paper we tested a group of 66 healthy volunteers in terms of the influence of circadian rhythm on selected parameters of the coagulation system and fibrinolytic system. Blood was collected at 6-hour intervals, at 8 am, 2 pm, 8 pm and 2 am. Circadian variability was observed in the coagulation system parameters as well as in the fibrinolytic system. We observed increased platelet aggregation, APTT prolongation, along with increased levels of factors (fibrinogen, PAI-1) and PAP and TAT complexes that influence coagulation and fibrinolysis systems, in the blood samples collected in the morning (8 am). We also demonstrated a circadian rhythm in the number of circulating platelets (PLT), with a peak in the afternoon (2 pm) accompanied by increased concentrations of t-PA, D-dimers and PT prolongation. Based on the obtained results it was possible to conclude that circadian rhythm had an influence on the activation of coagulation processes in the morning, with a progressive activation of fibrinolysis up to the afternoon. Our results may be helpful in determining the transient risk of cardiovascular events, including myocardial infarction and ischemic stroke, and hence, can contribute to the effective prevention of such events. Such observations may also become a starting point of departure for further studies aimed at determining the circadian effect of secretion of parameters in the hemostasis system on the other systems and parameters in the human body.

1. Introduction

In recent years, researchers have paid a lot of attention to factors released into the blood at specific times of the day. Studies on circadian rhythms show that such variability can be observed with regard to many blood parameters, including parameters of hemostasis systems [1,2].

Over the last few decades, various authors have described the circadian variability of some components of the hemostasis system. The major finding was that circadian variability of blood vessel reactivity and activity of coagulation factors promotes morning hypercoagulability [3]. However, most of these works were written in the 1980s and 1990s, and so the methods used in our paper are quite different from those that were used in the past [4–15]. In addition, study groups were usually quite small in number and usually only two up to four factors in the coagulation system [4,5,11,13–16], or two fibrinolytic factors [7,9,17,18] were investigated. This paper presents a much more comprehensive view of circadian regulation in the hemostasis system, including, coagulation factors, fibrinolytic factors, the number of platelets (PLT) circulating in the peripheral blood and the process of their aggregation in the 24-hour cycle.

For a long time clinicians and scientists have noted that a variety of sex determining factors (e.g. sex hormones or differences in platelet biology) can contribute to the incidence of cardiovascular events or can influence the rates of bleeding following medical or invasive interventions [19]. These mechanisms are still not fully explained. In our work, we also paid attention to the potential occurrence of differences in the circadian rhythm in the context of sex.

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1.1. Circadian rhythm and the hemostasis system

The circadian rhythm of platelet count (one of the most important parameters of the coagulation system) is the subject of a few published studies. Haus et al. [4] observed that the number of platelets reached its peak in the afternoon, with the highest activity in the morning hours. Similar results were reported by Undar et al. [20]. In the case of studies on platelet aggregation induced by agonists such as adenosine diphosphate (ADP) and norepinephrine, the highest platelet response was observed late in the evening and early morning [4], which has been attributed to the morning activity and increased levels of catecholamines in the blood [21]. A circadian variability has also been observed in the case of platelet activation markers, i.e. beta-thromboglobulin (βTG) and platelet factor 4 (PF4). The highest concentration of these markers was demonstrated at 3 pm [5].

Circadian rhythms have also been investigated in terms of coagulation factors in the intrinsic and extrinsic pathways. In a study conducted by Kapiotis et al. [6] circadian variability was observed in the activity of factor VII, with the highest levels between 8 am and 12 am. Other researchers, in turn, found that the maximum activity of factors VIII and IX was at about 9 am [4–6]. Haus et al. [4] demonstrated circadian rhythm in routine tests concerning hemostasis, i.e. activated partial thromboplastin time (APTT) and prothrombin time (PT). Both tests indicated shortened coagulation times in the morning, and thus a possible tendency for blood hypercoagulability at this time. The difference between the longest and the shortest PT over 24 h was 0.95 s, and for APTT 3.27 s. The researchers concluded that these were small differences, not significant in routine diagnostics, but interesting from the point of view of pathophysiology [3].

Circadian rhythm has also been observed for F1 + F2 fragments, the markers of factor X activity (Xa), and D-dimers, the markers of fibrinolysis. The highest concentrations of these markers were observed in the morning [6,7,17]. Two teams of researchers, Rudnicka et al. [22] and von Känel et al. [23] observed the highest concentration of D-dimers in the afternoon, at about 2 pm, while Trifiletii et al. [24] did not report a circadian rhythm for D-dimers.

In studies on fibrinogen circadian variability, scientists have repeatedly pointed out the association between this protein and interleukin-6 (IL-6), the main stimulator of fibrinogen synthesis in the liver. A correlation between the circadian rhythms of these two factors was reported by Kanabrocki et al. [16], who found the highest IL-6 concentrations in the early night hours, while the highest fibrinogen levels in the morning. Rudnicka et al. [22] noted the highest concentrations of fibrinogen at midday and a progressive decline over the day.

Iversen et al. [17] observed a circadian variability in factor VIII activity, with the highest concentration occurring at around 8 am. Due to the fact that factor VIII is bound to its carrier protein, von Willebrand factor (vWF), it was speculated that protein may also exhibit circadian rhythm. One of the studies confirmed this hypothesis, demonstrating that the highest concentration of vWF factor occurred at 1 pm [22]. However, another study on a smaller group did not confirm a circadian rhythm for this factor [25].

A study on healthy men showed a circadian rhythm of endogenous coagulation inhibitors: protein C, protein S and antithrombin (AT) [25]. The highest concentrations of proteins C and S were recorded at 6 am and the lowest concentrations between midday and midnight. A different regularity was observed in the case of AT, with the highest values around 6 pm, the lowest around midday. In the study by Pinotti et al. [26], tissue factor pathway inhibitor (TFPI) had the highest levels in the morning, at the start of daily activities. Its circadian rhythm converged with the activity of factor VII, which indicated that their interaction is necessary for the equilibrium in the coagulation system.

Circadian variability in the fibrinolytic system was first described in the 1950s. At the beginning these were only clinical observations, which over time were confirmed by highly specific tests of the fibrinolytic system. Fearnley et al. [27] were the first to prove the occurrence of circadian rhythm in fibrinolytic activity, with the lowest at night and just before the morning.

Andreotti et al. [9] reported circadian variation in the activity and concentration of plasminogen activator inhibitor-1 (PAI-1) and tissue plasminogen activator (t-PA). They showed that the activities of both parameters had a high amplitude of circadian variability. They also found that t-PA activity rhythm was opposite to the PAI-1 activity rhythm. PAI-1 activity and concentration were the highest in the morning hours, thus determining the circadian rhythm of the fibrinolytic system, in which t-PA activity decreases in the morning, but its concentration increases, reaching a peak in the afternoon. Similar results on PAI-1 were obtained by Scheer et al. [18]. This large amplitude of circadian rhythm in the fibrinolytic system may lead to morning hypercoagulability, and, consequently, may increase the risk of hemorrhagic events in the late afternoon hours [3].

It is suggested that the interactions between the rhythms of individual elements of the hemostasis system increase the risk of cardiovascular events, i.e. acute myocardial infarction, pulmonary embolism, ischemic stroke or hemorrhage. These phenomena have a unique temporary pattern of occurrence. Numerous studies show that they occur with the greatest frequency at two time points during the day: in the morning, i.e. in the first hours of daily activity, and in the evening. It is postulated that they are a consequence of the existence of a circadian rhythm of the above-mentioned parameters of the hemostasis system and the concurrent environmental stimuli which strengthen this effect. The morning increase in blood viscosity, vasoconstriction, blood pressure, platelet activity and coagulation factors, with a simultaneous decrease in fibrinolytic activity promotes fibrin deposition and an increased risk of thrombus formation. A careful understanding of these unique oscillations can help predict the occurrence of these phenomena in people at risk and also introduce appropriate prevention [3]. In addition, more and more studies show that disorders in circadian rhythms in the hemostasis system can have an impact on many other systems, including the immune system. Factors such as plasmin, factor VIIa, Xa, XIa and thrombin appear to have the ability to activate the complement system by fragmenting its C3 and C5 components to their reactive forms [28]. Some studies indicate that these components also show a circadian rhythm, similar to the elements of the coagulation and fibrinolytic systems [29]. It is also suggested that there may be a close relationship between the pathways of the coagulation, fibrinolytic and complement systems, which may change the current understanding of the hemostasis system [28].

This paper presents a comprehensive look at circadian regulation in the hemostasis system. We examined many different factors in this system, including the number of platelets (PLT) circulating in the peripheral blood, their aggregation, and many parameters of the coagulation and fibrinolytic systems.

2. Materials and methods

2.1. Study group

66 healthy volunteers aged 20–50 years consciously signed a written consent for participation in the study. The participants were divided into two separate groups by sex; 33 women (31 ± 7 years) and 33 men (34 ± 10 years). Directly before the tests each participant filled in a detailed questionnaire on lifestyle, sleep duration, diet, health status, and intaken medicines and supplements. Exclusion criteria included pregnancy, intake of medicines to treat chronic diseases, contraceptives, and taking antibiotics and aspirin within 14 days prior to blood collection. The study group did not use any radical diets, led an active lifestyle (physical exercise minimum 2–3 times a week) and were not professional athletes. In addition, during blood donations, especially in the evening and night hours, it was ensured that the volunteers had a special room with a place to sleep, so that during the passing day the correct sleep–wake rhythm was preserved. The study was given the

approval of the Bioethical Commission at the Pomeranian Medical University in Szczecin (No.KB-0012/99/14).

2.2. Study material

Peripheral blood was collected 4 times from the basilic vein at equal 6-hour intervals (2 am, 8 am, 2 pm and 8 pm) by qualified medical personnel; into a tube as blood clot (4.9 ml), a tubes with hirudin (2.7 ml), K_2EDTA (2.7 ml), or sodium citrate (5 ml). In the whole blood collected in tubes with K_2EDTA a complete blood count (CBC) was performed (ABX Micros 60 analyzer), with particular emphasis on the PLT. The full blood collected in tubes with hirudin was subjected to two platelet aggregation tests: ASPI and ADP. Blood clot was centrifuged (1500*g*, 10 min, 20 °C). To confirm the health status in the group of subjects, additional determinations of basic biochemical parameters were performed. These included glucose, lipid panel [(total cholesterol, triglycerides, low-density lipoproteins (LDL), high-density lipoproteins (HDL)], total protein, albumin, creatinine, and uric acid. In the blood serum, the concentration of mineral components such as organic phosphorus, total magnesium and total calcium were determined. BioMaxima kits were used to make the determinations, and all analyses were carried out in accordance with the manufacturer's recommendations. The results of parameters which deviated from the established reference values disqualified volunteers from the group of subjects. The remaining serum was transferred to new tubes (Eppendorf type) and frozen at −20 °C until the assays were made. The blood collected in tubes with sodium citrate was centrifuged (1500*g*, 10 min, 20 °C), and the following parameters were determined: APTT, PT, fibrinogen, and D-dimers. The residual material was transferred to Eppendorf tubes and frozen at −20 °C until the analysis was performed. The stored material was used for the determination of t-PA, PAI-1 and Plasmin-Antiplasmin complexes (PAP) and Thrombin-Antithrombin complexes (TAT) concentrations

2.3. Laboratory tests

2.3.1. Determination of melatonin concentration

To confirm preservation of the normal rhythm of sleep and wake throughout the day, the concentration of melatonin was determined 4 times in blood serum. An ELISA reagent assay (Human Melatonin ELISA Kit, Cloud-Clone Corp.) was used for this analysis. All determinations were made in accordance with the guidelines provided by the manufacturer. The concentration of melatonin was calculated from a previously prepared standard curve based on serial dilutions of the standard solution included in the kit.

2.4. The influence of circadian rhythm on the coagulation system parameters

2.4.1. Determinations of APTT and PT times and fibrinogen concentration

Determination of APTT and PT times and fibrinogen concentration were measured using commercially available reagents (APTT: HEMOSIL® APTT-SP, Instrumentation Laboratory, Werfen Company, Bradford, USA; PT: RecombiPlasTin 2G, Instrumentation Laboratory, Werfen Company, Bradford, USA; Fibrinogen: Q.F.A. Thrombin (Bovine), Instrumentation Laboratory, Werfen Company, Bradford, USA) in the citrated plasma. In order to determine the APTT, PT and fibrinogen concentration, ACL ELITE PRO was used.

2.4.2. Determination of TAT complexes

For the determination of TAT complexes, commercial ELISA reagent kits were used (Human Thrombin/Antithrombin Complexes Kit, Cloud-Clone Corp.®). Principle of operation was based on the non-competitive "sandwich" method in which a plate coated with antibodies directed against thrombin and antibodies directed against antithrombin, that were also labeled with biotin. Determinations of the concentration of TAT complexes in citrated plasma were performed in accordance with the procedures attached to the kits. The absorbance was read at 450 nm using an EnVision microplate reader (PerkinElmer).

2.4.3. Determination of platelet aggregation(ADP and ASPI tests)

Determination of platelet aggregation was carried out by impedance using aggregometry (Multiplate aggregometry®; Dynabate Medical, Mannheim, Germany). The study was performed in whole blood mixed with hirudin. Two types of assay were performed: ASPI test and ADP test (Roche Diagnostics GmbH, Mannheim, Germany). The assessment of platelet aggregation was based on the measurement of electrical resistance, which increased as a result of the addition of platelet agonists: adenosine 5-diphosphate (ADP test) and arachidonic acid (ASPI test). The aggregation result was presented as the area under curve (AUC) expressed in AU*min.

2.5. The influence of circadian rhythm on the fibrinolysis system parameters

2.5.1. Determination of PAI-1 and t-PA concentrations

ELISA reagent kits (Human PAI-1 ELISA, BioVendor®, Human t-PA ELISA, BioVendor®) were used for testing PAI-1 and t-PA concentrations. In both cases, the determinations were based on the non-competitive "sandwich" method using a plate coated with antibodies directed against PAI-1/t-PA and anti-PAI-1 labeled with biotin, and anti-t-PA antibodies labeled with horseradish peroxidase (HRP). The determination of PAI-1 and t-PA concentrations in citrated plasma was performed in accordance with the procedures provided by the manufacturers of the kits. Absorbance was read at 450 nm using an EnVision microplate reader (PerkinElmer).

2.5.2. Determination of D-dimers

The concentration of D-dimers determined by Enzyme Linked Fluorescent Assay (ELFA) using a VIDAS PC apparatus. For this purpose, a BioMerieux D-dimers Exclusion II (DEX2) kit was used to perform the immunoenzymatic detection of fibrin degradation products in human citrated plasma. Fluorescence was read at 450 nm, and its intensity was directly proportional to the concentration of D-dimers found in the tested sample. After the analysis, the results obtained were automatically interpreted by the computer in relation to the calibration curve.

For the determination of PAP complexes, commercial ELISA reagent kits were used (Human Plasmin/Antiplasmin Kit, Cloud-Clone Corp.®). Principle of operation was based on the non-competitive "sandwich" method in which a plate coated with antibodies directed against the α2-antiplasmin molecule and antibodies directed against the anti-plasmin labeled with biotin. Determinations of the concentration of PAP complexes in citrated plasma were performed in accordance with the procedures attached to the kits. The absorbance was read at 450 nm using an EnVision microplate reader (PerkinElmer).

2.6. Statistical analysis

The obtained results were subjected to statistical analysis using Statistica PL 13 (StatSoft, Poland) and MS Excel 2013 software. Melatonin concentration and the hemostasis systems parameters were analyzed for all sampling times. In order to estimate differences between the sexes for all the analyzed parameters at subsequent time points, Mann–Whitney *U* tests were carried out. Non-parametric Friedman ANOVA test were used to assess whether the parameters showed a circadian rhythm. Finally, to directly assess the differences between the parameters at individual time points, allowing for the division of the participants by both sexes, a Wilcoxon signed-rank test was used. Results of all statistical tests were considered significant at $p \leq 0.05$

3. Results

The results of the statistical analysis are presented in the following 2 Tables and 3 Figures containing the results of the average concentration of each parameter and its standard deviation.

3.1. The influence of circadian rhythm on changes in melatonin concentration

Friedman's ANOVA confirmed the occurrence of circadian melatonin rhythm in both the women and the men ($p < 0.0001$). The Wilcoxon's signed rank tests confirmed the existence of statistically significant differences between all pairs of sampling times for both sexes (p < 0.0001). The average melatonin concentration (for both men and women) was the highest at 2 am [99.52 ± 11.25 pg/ml in women, 97.12 ± 11.73 pg/ml in men (NS)], and the lowest in the middle of the day, around 2 pm [8.70 ± 2.53 pg/ml in women, 7.97 ± 2.61 pg/ml in men (NS)]. In the other examined time points, the melatonin concentration was at 8 am [15,91 ± 3,78 pg/ml in women, 15.49 ± 4.42 pg/ml in men (NS)] and at 8 pm [12.94 ± 4.70 pg/ml in women, 12.09 ± 3.03 pg/ml in men (NS].

3.2. The influence of circadian rhythm on the coagulation system parameters

The results of the statistical analysis of differences between the sexes for the influence of circadian rhythm on the coagulation system parameters are presented in Table 1.

3.2.1. The influence of circadian rhythm on APTT time

Friedman's ANOVA confirmed the occurrence of a circadian rhythm of APTT in both women and men ($p < 0.0001$). The Wilcoxon signed-rank test confirmed the existence of statistically significant differences between each of the time points ($p < 0.0001$). The average APTT in both sexes was the longest at 8 am [29.1 ± 2.3 s in women, 29.3 ± 2.4 s in men (NS)], and the shortest in the middle of the day, around 2 pm [27.5 ± 2.1 women, 27.7 ± 2.3 men (NS)] (Table 1).

3.2.2. The influence of circadian rhythm on PTtime

Friedman's ANOVA confirmed the occurrence of circadian rhythm for PT in both women and men ($p < 0.0001$). The Wilcoxon signed-rank test confirmed the existence of statistically significant differences between most time points at p < 0.0001 [except between 8 am & 8 pm and 8 pm & 8 am, at $p = 0.0043$ in women, and between 8 am & 2 am and 2 am & 8 am, at $p = 0.0147$ in men]. The average duration of PT in both sexes was the longest at 2 pm [11.4 ± 0.5 women, 11.7 ± 0.7 s in men ($p = 0.0228$)], and the shortest at night, at approximately 2 am [10.8 ± 0.7 s in women, 11.1 ± 0.8 s in men (p = 0,0603)] (Table 1).

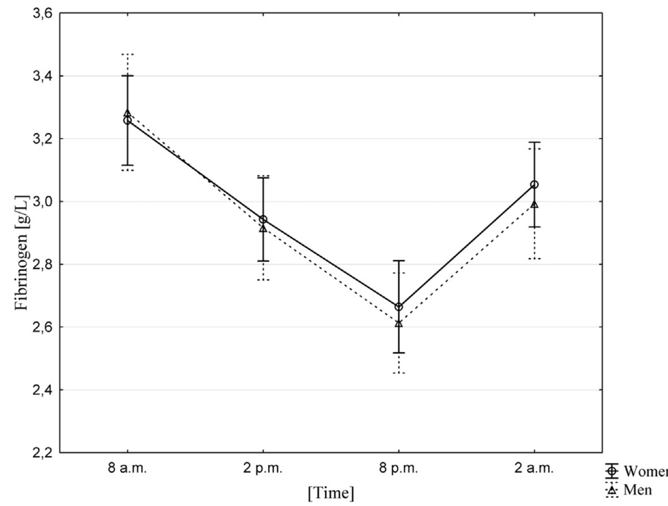

Fig. 1. The average fibrinogen concentration determined at four time points (8 am, 2 pm, 8 pm, 2 am) in the group of women ($n = 33$) and men (n = 33), data presented as means ± 95% confidence interval.

3.2.3. The influence of circadian rhythm on fibrinogen concentration

Friedman's ANOVA confirmed the occurrence of circadian fibrinogen rhythm in both women and men ($p < 0.0001$). The Wilcoxon signed-rank test confirmed the existence of statistically significant differences between all time points compared at p < 0.0001 (except between 2 pm & 2 am and between 2 am & 2 in men, where $p = 0.0068$). The average fibrinogen level in both sexes was the highest at 8 am [3.26 ± 0.40 g/l in women, 3.28 ± 0.52 g/l in men (NS)], and the lowest in the evening, around 8 pm [2.66 ± 41 g/l in women, 2.61 ± 45 g/l in men (NS)] (Table 1, Fig. 1).

3.2.4. The influence of circadian rhythm on TAT complexes concentration

Friedman's ANOVA confirmed the existence of circadian rhythms of TAT complexes in both women and men ($p < 0.0001$). The Wilcoxon's signed-rank test confirmed the existence of statistically significant

Table 1
Statistical analysis of differences between the sexes for parameters of the blood coagulation system in the subsequent time points of the day (8 am, 2 pm, 8 pm, 2 am). W – women, M – men; p – Mann–Whitney *U* test; NS – not significant.

Parameters	Times											
	8 am			2 pm			8 pm			2 am		
	W (x ± SD)	M (x ± SD)	P	W (x ± SD)	M (x ± SD)	p	W (x ± SD)	M (x ± SD)	p	W (x ± SD)	M (x ± SD)	p
APTT (s)	29.1 ± 2.3	29.3 ± 2.4	NS	27.5 ± 2.1	27.7 ± 2.3	NS	28.1 ± 2.1	28.1 ± 2.3	NS	28.5 ± 2.2	28.7 ± 2.3	NS
PT (s)	11.0 ± 0.6	11.3 ± 0.6	0.0968	11.4 ± 0.5	11.7 ± 0.7	0.0228	11.1 ± 0.6	11.5 ± 0.7	0.0148	10.8 ± 0.7	11.1 ± 0.8	0.0603
Fibrinogen (g/l)	3.26 ± 0.40	3.28 ± 0.52	NS	2.94 ± 0.37	2.92 ± 0.47	NS	2.66 ± 0.41	2.61 ± 0.45	NS	3.05 ± 0.38	2.99 ± 0.49	NS
TAT (ng/ml)	13.6 ± 4.2	14.7 ± 4.7	NS	7.6 ± 2.6	8.2 ± 2.4	NS	9.9 ± 2.9	10.1 ± 2.8	NS	11.4 ± 3.2	12.1 ± 3.8	NS
PLT (G/l)	252 ± 41	253 ± 40	NS	294 ± 44	282 ± 42	0.0917	279 ± 43	271 ± 41	NS	267 ± 42	263 ± 46	NS
ADP test (AUxmin)	926 ± 151	789 ± 132	0.0006	800 ± 132	686 ± 139	0.0027	715 ± 132	611 ± 134	0.0043	871 ± 147	720 ± 129	0.0001
ASPI test (AUxmin)	1007 ± 154	894 ± 123	0.0007	869 ± 160	780 ± 107	0.0027	778 ± 164	716 ± 114	0.0447	932 ± 168	820 ± 116	0.0010

differences between all time points compared with each other at $p < 0.0001$.The studies showed that the average concentration of TAT complexes in both sexes was the highest at 8 am [13.6 ± 4.2 ng/ml in women, 14.7 ± 4.7 ng/ml in men (NS)], and the lowest were observed in the afternoon, around 2 pm [7.6 ± 2.6 ng/ml, 8.2 ± 2.4 ng/ml in men (NS)] (Table 1).

3.2.5. The influence of circadian rhythms on the PLT

Friedman's ANOVA confirmed the occurrence of circadian rhythm in both women and men ($p < 0.0001$). The Wilcoxon signed-rank test confirmed the existence of differences at individual time points at $p < 0.0001$.The studies showed that the average number of platelets in both sexes was the highest at 2 pm [294 ± 44 G/l in women, 282 ± 42 G/l in men ($p = 0.0917$)], while the lowest were observed in the morning at approximately 8 am [252 ± 41 G/l in women, 253 ± 40 G/l in men (NS)] (Table 1).

3.2.6. Platelet aggregation test after ADP (ADP test)

Friedman's ANOVA confirmed the occurrence of circadian rhythms in the process of platelet aggregation in both men and women ($p < 0.0001$). The Wilcoxon's signed-rank test confirmed the existence of statistically significant differences between all time points at p < 0.0001.In the study it was observed that for both sexes, the platelet response to ADP was the strongest at 8 am [926 ± 151 AU*min in women, 789 ± 132 AU*min in men ($p = 0.0006$)], while the weakest at around 8 pm (715 ± 132 AU*min in women, 611 ± 134 AU*min in men ($p = 0.0043$)] (Table 1).

3.2.7. Platelet aggregation test after arachidonic acid (ASPI-test)

Friedman's ANOVA confirmed the occurrence of circadian rhythms in the process of platelet aggregation after arachidonic acid in both women and men ($p < 0.0001$). The Wilcoxon signed-rank test confirmed the existence of statistically significant differences between all time points compared with each other at the level of $p < 0.0001$. In both sexes the platelet response to arachidonic acid was the strongest at 8 am [1007 ± 154 AU*min in women, 894 ± 123 AU*in men ($p = 0.0007$)], and the weakest at around 8 pm [778 ± 164 AU*min in women, 716 ± 114 AU*min in men ($p = 0.0447$)] (Table 1, Fig. 2).

3.3. The influence of circadian rhythm on the fibrinolysis system parameters

The results of the statistical analysis of differences between the sexes for the influence of circadian rhythm on the fibrinolysis system parameters are presented in Table 2.

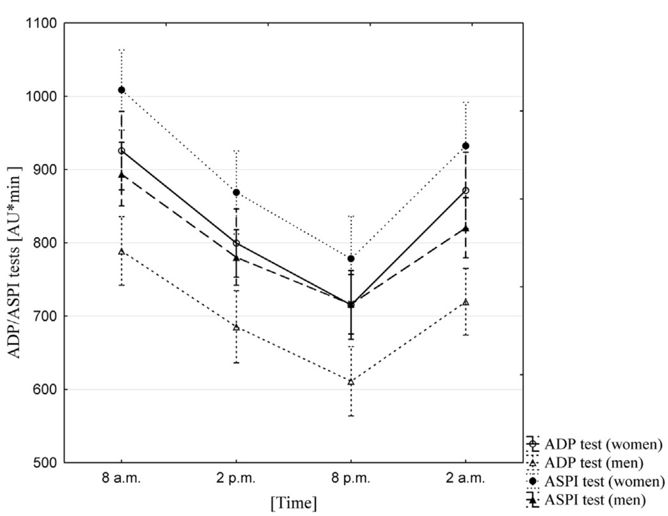

Fig. 2. The average platelet aggregation after ADP and arachidonic acid (ASPI) determined at four time points (8 am, 2 pm, 8 pm, 2 am) in the group of women ($n = 33$) and men ($n = 33$), data presented as means ± 95% Confidence Interval.

3.3.1. The influence of circadian rhythm on PAI-1 concentration

Friedman's ANOVA confirmed the occurrence of PAI-1 circadian rhythm in both women and men ($p < 0.0001$). The Wilcoxon's signed-rank test confirmed the existence of statistically significant differences between all time points $p < 0.0001$. The average PAI-1 concentration in both sexes was the highest at 8 am [20.5 ± 6.6 ng/ml in women, 24.2 ± 5.3 ng/ml in men ($p = 0.005$)], and the lowest at 2 pm [8.8 ± 2.4 ng/ml in women, 10.2 ± 2.1 ng/ml in men ($p = 0.0084$)] (Table 2, Fig. 3).

3.3.2. The influence of circadian rhythm on t-PA concentration

Friedman's ANOVA confirmed the occurrence of t-PA circadian rhythm both in women and in men ($p < 0.0001$). The Wilcoxon's signed-rank test confirmed the existence of statistically significant differences between all the time points of both sexes $p < 0.0001$.The average t-PA concentration in both sexes was the highest at 2 pm [7.14 ± 1.68 ng/ml in women, 8.54 ± 1.83 ng/ml in men ($p = 0.0028$) and the lowest were observed in the morning, around 8 am [5.16 ± 1.46 ng/ml in women, 5.61 ± 1.50 ng/ml in men (NS)] (Table 2, Fig. 3).

3.3.3. The influence of circadian rhythm on D-dimers concentration

Friedman's ANOVA confirmed the occurrence of circadian D-dimers rhythm in both women and men ($p < 0.0001$). The Wilcoxon signed-rank test confirmed the existence of statistically significant differences between all time points at $p < 0.0001$.The average concentration of D-dimers in both sexes was the highest at 2 pm [215 ± 60 ng/ml in women, 199 ± 57 ng/ml in men (NS)], and the lowest at 2 am [180 ± 55 ng/ml in women, 160 ± 49 ng/ml in men (NS)] (Table 2).

3.3.4. The influence of circadian rhythm on PAP complexes concentration

Friedman's ANOVA confirmed the occurrence of circadian rhythm of PAP complexes in both women and men ($p < 0.0001$). The Wilcoxon's signed-rank test confirmed the existence of statistically significant differences between all time points compared at $p < 0.0001$. The average concentration of PAP complexes in both sexes was the highest at 8 am [103.7 ± 31.7 ng/ml in women, 146.9 ± 58.0 ng/ml in men ($p = 0.0012$)], and the lowest in the evening, around 8 pm [40.2 ± 14.8 ng/ml in women, 52.3 ± 20.9 ng/ml in men ($p = 0.0012$)] (Table 2).

4. Discussion

This work is probably the first paper in which a large and homogeneous group of healthy participants was tested for circadian variation in as many as nine parameters of the hemostasis system (coagulation and fibrinolysis factors) and the effect of this rhythm on platelet aggregation in response to ADP and arachidonic acid. This work also includes information on the circadian regulation of the concentrations of individual parameters, including differentiation for the sex of the participants.

4.1. Circadian rhythm of melatonin

Melatonin is one of the best, if not the best, indicators of circadian rhythm. In this study the highest melatonin concentrations were observed at 2 am and the lowest at 2 pm. These results are in line with the results obtained by Fatima et al. [30] and many other researchers [8,29,31]. Therefore, in the present study we can confirm a normal 24-hour cycle sleep–wake rhythm of blood sampling.

Table 2
Statistical analysis of differences between the sexes for parameters of the blood fibrinolysis system in the subsequent time points of the day (8 am, 2 pm, 8 pm, 2 am). W – women, M – men; p – Mann–Whitney *U* test; NS – not significant.

Parameters	Times											
	8 am			2 pm			8 pm			2 am		
	W (x ± SD)	M (x ± SD)	p	W (x ± SD)	M (x ± SD)	p	W (x ± SD)	M (x ± SD)	p	W (x ± SD)	M (x ± SD)	p
PAI-1 (ng/ml)	20.5 ± 6.6	24.2 ± 5.3	0.005	8.8 ± 2.4	10.2 ± 2.1	0.0084	12.7 ± 4.4	16.7 ± 5.3	0.0027	15.9 ± 5.7	19.5 ± 5.0	0.0039
tPA (ng/ml)	5.16 ± 1.46	5.61 ± 1.50	NS	7.14 ± 1.68	8.54 ± 1.83	0.028	6.25 ± 1.57	7.40 ± 1.74	0.0131	5.90 ± 1.63	6.15 ± 1.49	NS
D-dimers (ng/ml)	197 ± 55	181 ± 53	NS	215 ± 60	199 ± 57	NS	188 ± 54	171 ± 48	NS	180 ± 55	160 ± 49	NS
PAP (ng/ml)	103.7 ± 31.7	146.9 ± 58.0	0.0012	56.2 ± 20.8	80.6 ± 32.1	0.0012	40.2 ± 14.8	52.3 ± 20.9	0.0012	80.1 ± 28.2	108.3 ± 51.3	0.0154

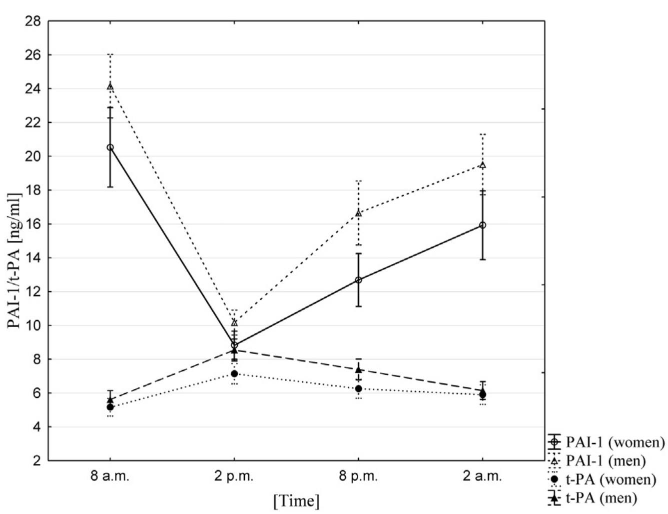

Fig. 3. The average PAI-1 and t-PA concentration determined at four time points (8 am, 2 pm, 8 pm, 2 am) in the group of women (n = 33) and men (n = 33), data presented as means ± 95% Confidence Interval.

4.2. The influence of circadian rhythm on the coagulation system parameters

4.2.1. APTT and PT

Both APTT and PT showed circadian variability. The longest APTT was determined at 8 am, and the shortest at 2 pm. Different results were obtained by Haus et al. [4] who observed shortening APTT during the day between 8 am and 4 pm, and prolongation at 8 pm. In the case of PT, our results were similar to those obtained by Haus et al. The shortest PT was recorded late at night and early in the morning hours, while the longest at noon. Differences between the longest and the shortest durations in our study (0.6 s for PT, 1.6 s for APTT) and in the study by Haus et al. (0.95 s for PT and 3.27 s for APTT) were so insignificant that they should not cause problems in routine diagnostics. The difference between our APTT results and those reported by Haus et al. [4] may be due to differences in the handling of the sampling material or the method of determination. In this work, clotting times were determined with an automated coagulatory apparatus, most likely characterized by a much higher sensitivity compared to the apparatus used in 1990 by Haus et al.

We did not find any literature data concerning differences in circadian rhythm for APTT and PT, depending on sex. However, our study at each of the time points confirmed the tendencies described in literature regarding the levels of these parameters depending on sex. Sivrikaya et al. [32] did not confirm statistically significant changes for APTT depending on sex in the 15–50 years age group, although it was noticed that this time was slightly longer in the men. The same trends, despite the lack of statistical significance, were observed in our research. In contrast to the APTT, Sivrikaya et al. [32] confirmed the occurrence of sex related statistical significance for PT as significantly higher in men compared to women, which we also confirmed in this research.

It is suspected that circadian variability of coagulation times (APTT and PT) may be the result of characteristic oscillations between the activity of particular coagulation factors involved in the extrinsic and intrinsic pathways of the blood coagulation activation [3]. We believe that the circadian changes of APTT and PT are unlikely to affect healthy people and they will not cause problems in routine diagnostics. However, these changes appear to be interesting from the pathophysiological point of view, e.g. in patients treated with warfarin, in which the PT in the morning was prolonged when compared to healthy individuals [33].

4.2.2. Fibrinogen

The highest fibrinogen concentrations were observed at 8 am, while the lowest at 8 pm. This is consistent with the results obtained by two other research teams who analyzed the circadian variability of fibrinogen concentration in a much smaller group of patients [4,16]. Rudnicka et al. [22] obtained a little different rhythm of fibrinogen concentration, with maximum at midday and the lowest levels at night. However, all the mentioned studies clearly indicate a tendency for higher fibrinogen levels in the first half of the day and low levels at night [4,16,22].

Rudnicka et al. [22] estimated that the circadian variability of fibrinogen is about 3%, stating that it should not affect the interpretation of test results in healthy people. It is suggested, however, that these observations may have a certain clinical value. There is a theory that people with cardiovascular diseases may not experience such circadian oscillations. Described in our study circadian-dependent rhythm of secretion of fibrinogen, with the peak in the morning, indicates that fibrinogen is rightly considered as a prognostic factor for the incidence of acute coronary and cerebral events [5].

We did not find any literature data concerning differences in circadian rhythm for fibrinogen concentration, depending on sex. Our research confirmed (regardless of the time of collection) the lack of a statistically significant difference in the concentration of fibrinogen depending on the sex, although our observations and other scientists indicate that the fibrinogen concentration is slightly higher in women [34,35].

4.2.3. TAT complexes

Our study demonstrated the circadian variability of TAT complexes, with the highest levels at 8 am, and the lowest at 8 pm. In available literature, we did not find any information on the occurrence of circadian rhythms of TAT complexes. Jafri et al. [5] examined 9 people aged 51 ± 10 years, from whom they took blood at six different time points. They did not observe any significant statistical differences between successive sampling times ($p = 0.36$). In turn, Deguchi et al. [11] studied a group of 10 healthy male volunteers aged between 23 and 30 years, whose blood was sampled at 9 am, 12 am and 3 pm. Researchers noticed a small variability of TAT complexes between specific time points, but without statistical significance. The lack of statistical significance in the results of both those study teams compared to our research, could be caused by a significant difference in the size of the groups studied by them. Jafri et al. [5] and Deguchi et al. [11] analyzed only 9 and 10 people, respectively. Moreover, the values of the TAT complexes obtained in our study in the group of men were significantly higher than those reported by Deguchi et al. [11]. The reason for this could be a different procedure for handling the sample, e.g. the time elapsed from collecting the material to determinations, age of the examined men (average age of men in the study was 51 ± 10 years, in our study it was 35 ± 10 years) and the use of ELISA manufactured by different companies.

We didn't find any literature data concerning differences in the concentration of TAT complexes, depending on sex, either in the circadian rhythm or at all. Our research indicates that such differences are not statistically significant, despite the slight tendency for higher levels of TAT complexes in men.

4.2.4. PLT and platelet aggregation

Our study showed that PLT was subject to circadian variation, with the highest at 2 pm and the lowest at 8 am. Statistically significant differences were also observed in platelet aggregation. The highest platelet response to the ADP agonist was in the morning hours, while the lowest was observed in the evening at 8 pm. Our results are in line with those observed by other researchers. For example, Toefler et al. [13] observed the largest platelet aggregation in the morning between 6 am and 9 am, in a group of 15 healthy men, non-smokers and non-smokers 2 weeks before an aspirin test. According to the researchers, this increase is associated with the start of the morning activity of the body and the increase in sympathetic nervous system activity. These conclusions were based on the parallel observation of circadian regulation of catecholamine concentrations. Those results were later confirmed by Brezinski et al. [14] who, additionally, noticed a relationship between the adoption of a vertical position just after waking up, and an increase in the aggregation of platelets and catecholamines in the blood. Similar results were obtained by Haus et al. [4] who observed the highest aggregation in the late-morning and early morning hours, and the lowest in the afternoon, around 4 pm. Similar to the results of our study, those researchers found a circadian rhythm of the platelet count, with the highest levels in the afternoon.

In 1992, a team of Japanese researchers showed increased aggregation of platelets under the influence of ADP, collagen, epinephrine and arachidonic acid in the morning and afternoon. However, the results of that study are quite unreliable because they were carried out on only a six-person group of healthy men [15]. Different results were obtained by Jafri et al. [5] who observed a low circadian variation in the response of the platelets to epinephrine, but this response did not show statistical significance ($p = 0.16$). Thus, the results presented in this paper, compared also with the results of other researchers, indicate the occurrence of the circadian rhythm in platelet aggregation, with a tendency to increase in the morning.

The results from the present study support previous findings that women have higher amounts of PLT [36–39] and physiologically increased platelet aggregation compared to men [37,40–42], which also does not change during antiplatelet therapy [43]. However, our study confirmed the existence of statistical differences between sexes for both aggregation tests, but not the existence of such significant differences in the case of PLT. Sex differences in ex vivo platelet function (i.e. higher in women) could be the result of the direct effects of estrogens, progesterone, orandrogens on platelets, or could be the indirect effect of sex hormones on the vasculature [44]. In the case of the aggregation tests, it is speculated that the cause of this situation is a higher hematocrit in men, with a relatively smaller amount of plasma and higher in vitro dilution (addition of anticoagulant solutions) which leads to a falsely lower amount of PLT in men compared to women [45].

Knowledge of possible circadian fluctuations in platelet aggregation seems to be of particular interest nowadays, as the implementation of ASPI and ADP tests for routine diagnostics is considered. We presume that the optimization of the time of performing these tests would be very important in the dose selection and could help in personalizing the antiplatelet therapy, what is especially important for patients undergoing cutaneous coronary intervention (PCI).

4.3. The influence of circadian rhythm on the fibrinolysis system parameters

4.3.1. PAI-1 and t-PA

We found a 24-hour variability of PAI-1, with its highest concentrations at 8 am and the lowest at 2 pm. Similar results were obtained by three other research teams. Andreotti et al. [9] observed that PAI-1 concentrations reached maximum in the morning, between 7 am and 9 am, and the lowest in the afternoon, between 3 pm and 6 pm. In a study by Sheer et al. [18] PAI-1 was characterized by high circadian variability, with the highest levels around 7 am and the lowest around 4 pm. In addition, PAI-1 circadian variation was 8-fold greater than the variability induced by exercise or other factors causing an increase in PAI-1. The results obtained by those scientists can therefore be considered very significant, as they show that PAI-1 daily variability is maintained regardless of lifestyle. Furthermore, the researchers suggest in their work that PAI-1 circadian rhythm may have appeared in the process of evolution, promoting coagulation system activity in the morning hours, a time of the day associated with an increased risk of blood loss due to higher blood pressure. A Japanese team of researchers observed the lowest of PAI-1 concentration at 4 pm, which after midnight began to increase significantly, reaching the maximum at 8 am [10]. Additionally, in 2009 von Känel et al. [23] conducted studies on differences in the occurrence of circadian PAI-1 in people with obstructive sleep apnea and healthy subjects (control group). The researchers observed the highest levels of PAI-1 in healthy persons in the early morning hours (5 am).

The results obtained in our research show that the concentration of t-PA reaches a maximum level at 2 pm, while the lowest at 8 am. The daily variability of t-PA was also observed by Andreotti et al. [9] who showed that the highest t-PA concentration occurred at 9 am, and the lowest at midnight. Similar results were obtained by Angelton et al. [7] who analyzed the range of t-PA variability at two time points; 8 am and 8 pm. Those researchers suggested that studies performed only at these two time points were aimed at avoiding the distortion of results caused by an increase in t-PA in the morning following endothelial damage during blood collection. Angelton et al. showed an increase in t-PA concentration in the morning hours compared to the evening hours [7]. Very similar results were obtained by the team of Rudnicka et al., [22] who noted the highest concentrations of this parameter at 10 am, and the lowest at around 7 pm. The results of the research of other aforementioned scientists, confirmed in our study, clearly indicate that the concentrations of PAI-1 and t-PA are subject to circadian variation.

The circadian rhythm of PAI-1 and t-PA activity is also well known in the literature. It directly translates into the efficiency of the fibrinolytic system. Therefore, in the case of PAI-1, its concentration and activity are likely to be characterized by the same pattern of variability over time, where the highest values of this parameter are observed in the morning hours and begin to decrease over the day. On the other hand, the circadian variability in t-PA activity shows a completely different rhythm than PAI-1, reaching its maximum values in the late afternoon hours, and minimum in the morning [7,9,10]. In turn, t-PA concentration is characterized by quite different behavior. In the morning, when PAI-1 concentration and activity are the highest, and t-PA concentration is the lowest [4,10,17]. In addition, the t-PA formed in the blood is very quickly inactivated by its inhibitor, therefore PAI-1 is the main regulator of fibrinolytic activity and determines the amount of plasmin produced [18,46]. This dependence is probably caused by the autoregulatory response from t-PA to the increase in PAI-1. The mutual relation between these two key parameters seems to be extremely important from the point of view of maintaining the proper performance of the fibrinolytic system [46]. Such a large amplitude of circadian rhythm in the fibrinolysis system may lead to hypercoagulability, and thus may have an impact on the increased risk of occurrence of hemorrhagic events in the late-afternoon hours [3].

We did not find any literature data concerning differences in circadian rhythm for PAI-1 and t-PA concentrations, depending on sex. Available literature reported that PAI-1 concentration is similar in healthy men and women [47–49]. However, there are also studies which show that men have statistically significantly higher mean PAI-1 antigen [50] and t-PA antigen values [47] than women. Our studies confirmed statistically significant differences depending on sex in PAI-1 concentration (higher in men compared to women), and slightly higher levels of t-PA in men compared to women, which, however, did not show such statistical significance.

4.3.2. D-dimers

The circadian variability of D-dimers has been demonstrated, with the highest values falling at 2 pm and the lowest at 2 am. Similar results were obtained by Rudnicka et al. [22] and Von Känel et al. [23]. Rudnicka et al. [22] found the highest concentration of D-dimers at 2 pm, then decreasing over the day. Von Känel et al. [23] demonstrated the existence of circadian rhythm in the concentration of D-dimers, with their highest levels in the afternoon (between 12 am and 2 pm) and the lowest at night. Different results were obtained by Trifiletti et al. [24] who did not find any circadian rhythm in the concentration of D-dimers. This discrepancy may be primarily due to the use of another D-dimers determination method and a small group size (only 10 people). The results obtained by us and other two teams of researchers confirm the participation of D-dimers in fibrinolysis (the commonly known increase in fibrinolytic activity in the afternoon is equivalent to an increase in the concentration of D-dimers at that time).

We did not find any literature data concerning differences in the concentration of D-dimers, depending on sex, either in the circadian rhythm or at all. Our research indicates that such differences are not statistically significant, despite the slight tendency for higher levels of D-dimers in women.

4.3.3. PAP complexes

In this study, we demonstrated the occurrence of the circadian rhythm of PAP complexes, with the highest levels at 8 am and the lowest at 8 pm. Different results were obtained by Kapiotis et al. [6], who in their experiments investigated the circadian variability of PAP complexes in a group of 10 people, between 8 am and 8 pm. They observed that higher concentrations of PAP complexes occur in the evening and lower concentrations in the morning ($p = 0.008$). Another team of researchers, Rosenfeld et al. [12] studied circadian variability of PAP complexes in a group of 12 healthy volunteers, at four time points: 8 am, 10 am, 4 pm and 8 pm. The study group was subjected to a 36-hour rest in a supine position. The aim of the researchers was to determine the effect of immobilization on the circadian rhythm of hemostasis parameters. PAP complexes did not show circadian rhythm, which could undoubtedly be attributed to a sudden change in lifestyle. Furthermore, the researchers did not observe the circadian variation in the levels of any of the studied factors (fibrinogen, plasminogen, α2-antiplasmin), with the exception of PAI-1. In turn, Akiyama et al. [10] examined the circadian variability of the hemostasis system in 16 healthy volunteers at 7 time points (first day at 4 pm, 8 pm, 12 pm and the second day at 8 am, 9 am, 12 am and 4 pm). One of the parameters analyzed by them was the concentration of PAP complexes whose concentrations did not show statistically significant differences between the designated hours. Therefore, in literature no confirmation was found for our results obtained in this research. This may be caused by the differences in the size of the analyzed groups. In our study, 66 healthy subjects participated the study, which significantly increases the reliability of the results obtained. However, it seems that our research may be helpful in assessing the risk of cardiovascular complications, as the measurement of concentration of formed PAP complexes allows the direct assessment of the amount of plasmin generated [51].

We did not find any literature data concerning differences in the concentration of PAP complexes, depending on sex, either in the circadian rhythm or at all. Our research indicates that the concentration of PAP complexes is statistically significantly higher in men than women.

4.4. Circadian rhythm and the hemostasis system- potential physiological or pathological significance of the found circadian changes

Circadian-dependent changes of many parameters of the hemostasis system in the physiology and pathophysiology of the coagulation and fibrinolysis systems seem to be a very interesting issue. It seems that the existing physiological range of circadian changes for described in our study parameters does not lead to diagnostic problems, however, our results may be helpful in determining the transient risk of thromboembolic events, including myocardial infarction and ischemic stroke.

Clinical observations indicate that the most frequent cardiovascular events occur in the morning due to the intensification of coagulation processes [52]. Our studies confirmed the existence of transient hypercoagulability in the morning hours, as evidenced by the highest morning concentrations of some of the tested hemostasis parameters, mainly fibrinogen, PAI-1 and TAT complexes, and the most intense platelet aggregation.

A better understanding of these temporal oscillations in hemostasis mechanisms may help in the optimization of appropriate treatment, mainly antiplatelet treatment (e.g., control of the dose of acetylsalicylic acid). This would be particularly important in the population of patients with coronary disease and concomitant metabolic disorders (e.g. diabetes and obesity), among which the phenomenon of aspirin resistance is the most common [53]. Therefore, knowledge of the regulation of circadian release of some parameters of the hemostasis system may also contribute to the effective prevention of cardiovascular events. In the future, our research will be extended with additional parameters of the hemostasis system.

The observed differences in the circadian circulation of hemostasis parameters may also become a point of departure for further studies aimed at determining the circadian effect of the secretion of parameters in the hemostasis system on the other systems and parameters in the human body. In our previous studies on mice, our team has observed the effect of hemostasis (both the coagulation and fibrinolysis systems) on complement activity [activation of C5b-9 (MAC)], which consequently leads to the release of many populations of stem cells from bone marrow niches into the peripheral blood during mobilization. Some experimental research showed that thrombin and plasminogen may possess C5 convertase-like activity, so our research hypothesized that also proteases generated in the activation of the coagulation and fibrinolysis systems play a huge role in the mechanism of compensation of this process [54]. Therefore, it would be interesting, to confirm such mechanisms in humans and to determine the impact of circadian regulation of secretion of these factors on stem cell mobilization.

5. Conclusions

In our study, we observed increased activity of the hemostasis processes in the morning, which is characterized by increased platelet aggregation, fibrinogen and PAI-1 concentrations, which is accompanied by increased in thrombin generation markers (TAT complexes).

We also observed fibrinolysis system activation progressing from the morning hours (increase PAP complexes) to the afternoon hours (2 p.m.) characterized by increase in the concentration of t-PA, D-dimers. We have also noticed a statistically significant difference between the sexes in the subsequent time points for a few parameters of hemostasis (ADP and ASPI tests – significantly higher in women, PAI-1 and PAP complexes – significantly higher in men). Such changes, both in platelet aggregation and fibrinolytic activity, may indicate the involvement of these processes in the pathogenesis of sex-dependent hypercoagulability.

6. Study limitations

The biggest limitation of our research was the too low number of volunteers who would meet the criteria of qualifying for research. This low number of volunteers resulted, among others, from the necessity of drawing blood 4 times in one day. It was not always possible or the participants resigned, despite the fact that they originally declared their willingness to participate in our study.

Declaration of competing interest

All the authors state that they have no conflicts of interest.

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