# Changes in oxidative stress haemostatic parameters during the thrombolytic treatment of pulmonary embolism

**PhD thesis**

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# 1. INTRODUCTION

Acute pulmonary embolism (PE) is the third most common cause of cardiovascular mortality with an incidence of 50-100/100000/inhabitants/year. Mortality of respiratory and circulatory compromised PE patients on hospital admission can be up to 95%. Hospital mortality is 80 % in patients requiring mechanical ventilation and 77% in those who need cardiopulmonary resuscitation in the first 24 hours. Only 29% of fatal PE cases (verified at hospital autopsies) were previously diagnosed clinically. Based on these facts, the primary goal in PE management is a rapid and definite diagnosis followed by the appropriate treatment. Thrombolysis (TL) is indicated urgently in PE before the occurrence of haemodynamic instability due to prolonged systemic arterial hypotension and multisystem organ failure. The mortality of PE following TL according to some studies can be reduced to 4.7 versus 11.4 % compared to heparin treatment. Based on the haemodynamic symptoms, PE either can be *massive*, characterised by systolic blood pressure lower than 90 mmHg or at least a decrease of 40 mmHg in systolic blood pressure or *not massive* which includes *submassive* state characterised by increased right ventricular pressure.

The importance of platelets in the pathophysiology of PE is debatable. It has been proved that increased platelet aggregation plays an important role in arterial occlusion syndromes but recent research shows that it is also plays a characteristic part in venous thromboembolism. It is well known that platelets release vasoactive substances which may cause diffuse vaso and bronchospasm in the affected regions. Considering these facts we found it interesting to observe the changes in platelet function during PE. No similar clinical trials have been conducted prior to our study. The aim of the present prospective randomised cohort study was to investigate the haemostatic alterations induced by TL in submassive PE. Therefore, we followed the changes in spontaneous and induced platelet aggregation and fibrinogen level for 30 days after submassive PE treated with two different TL regimes.

Acute PE is a life threatening disease that can be treated by TL leading to reperfusion of previously ischemic areas. This phenomenon is accompanied by oxidative stress (OS) and leukocyte activation. Experimental results have proven that hypoxia-reoxygenation and ischemia-reperfusion causes OS accompanied with production of oxygen free radicals exceeding the endogenous antioxidant capacity with simultaneously developing leukocyte activation and inflammatory reactions. The pathology of OS associated with PE have been studied mainly on animal models. Very few human models have been used to study the presence of OS in PE and deep vein thrombosis (DVT).

# 2. AIMS

1) Do patients suffering from massive or submassive PE show spontaneous platelet aggregation?

2) How does the time course of platelet aggregation change during and after thrombolytic therapy of massive or submassive PE?

3) Do elevated platelet aggregation levels lead to re-embolism following TL in massive or submassive PE?

4) Is there a difference in the effect of ultra high dose streptokinase (UH-SK) and alteplase (tPA) on platelet function during massive or submassive PE?

5) Is it possible to find an “easy to measure” laboratory parameter which dependably characterizes the efficacy of TL, without invasive examinations?

6) Can PE induced OS be detected on patients before the initiation of thromboltic treatment?

7) How does OS change during and after thrombolytic therapy of massive or submassive PE?

8) To what extent is OS changed following treatment with different thrombolytic drugs (streptokinase, alteplase)?

9) Can OS be used as a clinical marker following successful TL?

# 3. PATIENTS AND METHODS

The study protocol was carried out in accordance with the ethical guidelines of the 1975 Declaration of Helsinki and the permission of the Institutional Scientific and Human Research Ethics Committee of the University of Pécs (810/2001). Each patient provided written informed consent and was informed clearly about the details of study and blood sampling.

## 3.1 Study population

*3.1.1 Patients*

Between May 1, 2001 and May 1, 2005, we prospectively screened consecutive patients with definite massive or submassive PE referred to our Intensive Care Unit. Fifteen patients were divided into two therapeutic groups using block randomisation: 8 patients for ultra high dose (9 million units/6 hours) UH-SK and the other 7 patients for tPA 100 mg/2 hours as TL. We paid attention to homogenous medication during the supportive therapy. Upon suspicion of PE, heparin was administered according to the latest guidelines (5000 IU of unfractionated heparin intravenously or low molecular weight heparin adjusted to body weight subcutaneously as a single bolus). TL was begun after the verified diagnosis of PE.

*3.1.2 The inclusion criteria* were based on the haemodynamic state (massive or submassive PE) and on the extension of the unperfused area (>50%). These were verified with echocardiography, electrocardiography, spiral CT and perfusion lung scan. Inhalation lung scan was not carried out due to the progressive deterioration of the haemodynamic state of our patients.

*3.1.3 Exclusion criteria* defined patients who declined to give consent and absolute contraindication to TL.

- advanced malignant disease
- uncompressed punction of parenchymal organ within 24 hours (eg. kidney biopsy)
- active gastrointestinal bleeding
- encephalomalacia or cerebral haemorrhage in the recent past history (1 month)
- hypertensive crisis (not probable beside massive PE)
- major surgery in the past 1 week
- aneurysm with verified thrombus (eg. post infarct left ventricle, abdominal aorta)

Two patients declined to give consent. One patient suffered from haematological disease (lymphoma). Three patients had advanced stage malignant disease. Due to the above mentioned reasons, six patients weren't enrolled and therefore did not take part in the study.

*3.1.4 Control group:* Blood samples were taken nine healthy volunteers as controls for oxidative stress parameters.

A Heparin treated control group was not selected because withholding TL from patients suffering from haemodynamic instability due to massive and/or submassive PE would have been a violation against current guidelines.

*3.1.5 Discontinuation of examination:* Further examination was not performed if the patient countermanded the previously agreed informed consent in writing or in word or if the clinical end points mentioned below occurred.

*3.1.6 Clinical endpoints*

- Anaphylaxis caused by SK, by usage of other type of thrombolytic drugs (if introduction of other drug was clinically necessary).
- Death

## 3.2 Therapy

*3.2.1 Supportive therapy and diagnostic management before and during TL:* Anticoagulant therapy was initiated upon suspicion of PE, 5000 IU Na-heparin was administered as an i.v. bolus if the patient hadn't already received Low Molecular Weight Heparin (LMWH) previously. Besides stabilising venous security, the patients received immediate oxygen therapy with a 50% or 100% face mask. Morphine was given as i.v. bolus of 2mg for analgesia. For achieving optimal haemoreological parameters and a desirable volume state, aggressive fluid resuscitation was carried out intravenously in the acute phase (Sterofundin B 1.5-2 ml/kg/h). Supportive treatment was provided by homogenous medication and in case of airway difficulties, 200 mg i.v. theophyllin was administered. If required, noradrenaline and/or dobutamine was given as positive inotropic drugs. Using deep vein Doppler ultrasound, if re-embolisation was suspected, a temporary placement of vena-caval filter was considered before TL. A radial arterial line was introduced to monitor invasive blood pressure and to draw frequent blood samples upon the verification of massive or submassive PE. The treatment regimes of the two groups were therefore altered.

*3.2.2 UH-SK treament:* We initiated SK at 1.5 million IU/h in a perfusor which was continued for 6 hours. Anticoagulant therapy was started after TL with intravenous unfractionated heparin to maintain aPTT (checked every 4 hours) between 50-60 seconds for the first 48 hours and was continued with a therapeutic dose of LMWH. Using the "Heparin adjustment nomogram", the dose of Na-heparin was adjusted accordingly to reach a target aPTT of 60-70 sec. Heparin treatment was carried out until the next day's control measurement. The effect of the TL treatment was controlled between the $12^{th}$ and $24^{th}$ hour by a second look spiral CT or with a perfusion lung scan. If the clinical state did not improve or any of the examinations did not verify at least 30% decrease in the size of unperfused lung area after the first treatment, TL was repeated after 24 hours to a maximum of three thrombolytic cycles.

*3.2.3 Alteplase treatment:* In the tPA group unfractionated heparin was given parallel in the thrombolytic period, its dosage being alteplase 100 mg/2 h. The anticoagulant regiment which followed was similar to the UH-SK group. The effectiveness of thrombolytic treatment was controlled between $12^{th}$ h and $24^{th}$ h by a second look spiral computer tomography (CT) or perfusion lung scan. If any of the examinations did not verify at least 30% decrease in the size of unperfused lung area after the first treatment phase, thrombolysis was repeated after 24 hours elapsed. If the fibrinogen level had been lower than 2 g/l before second thrombolytic cycle, fresh frozen plasma was administered.

*3.2.4. Anticoagulant treatment after TL: A*nticoagulant therapy was provided with intravenous unfractionated heparin to maintain the actual partial thromboplastin time (aPTT) between 60-70 seconds for 48 hours. If TL was effective it was continued with a therapeutic dose of LMWH (dalteparine 2x 100 IU/kg/day). Following TL, long term anticoagulation (acenocoumarol) was started at 3-4 days.

*3.2.5 Follow up:* The follow up examination was carried out on the 30th day and physical examination, ECG and laboratory tests were performed. The blood sample of day 30 was taken at our outpatient ward and all patients were on anticoagulant therapy (acenocoumarol). The patients, were discharged home after the control examinations. We avoided arterial blood sampling due to possible bleeding complications. Furthermore, a pilot study showed that leukocyte of venous and arterial origin provided statistically not significant differences in platelets aggregation, cellular function tests and oxidative stress parameters. We referred patients below the age of 70 years to thrombophilia examination after 6 months following TL.

## 3.3 Measurement-techniques

*3.3.1 Frequency of blood collection*

Arterial blood samples were collected via a radial artery cannula prior to initiation of TL as baseline value and every 4 hours thereafter (4th hour (h), 8th h, 12th h, 16th h, 20th h and 24th h) to examine activated partial thromboplastin time (aPTT), prothrombin time, fibrinogen level, blood gas analysis and platelet aggregation. Blood gas analysis and platelet aggregation measurements were carried out in our department research laboratory. All other haematological measurements were carried out at the Institute of Laboratory Medicine, University of Pécs. Later, 8-hourly D-dimer levels and blood cell counts were measured. On the second day, we repeated the tests above twice a day and daily on 3rd, 4th and 5th day (d). Finally, venous samples were collected at 30th d. Arterial blood samples were collected to analyse OS markers prior to, and in the 8th and 24th hour following TL treatment, subsequently on the 3rd and 5th days. Venous samples were collected on the 30th day.

*3.3.2 Platelet aggregometry*

Carat TX4 type aggregometer based on Born's optical system was used [18]. Na-citrate anticoagulated blood samples were centrifuged with 600 rpm and platelet rich plasma was separated into 4 vials (450 µl). The remaining material was centrifuged for 10 minutes with 5000 rpm to obtain platelet poor plasma (PPP, 500 µl). Fifty µl of agonists were added separately to platelet rich plasma samples: 10 µM adenosine diphosphate (ADP), 2 µg/ml collagen, 10 µM adrenaline (Theracont TA-3 inductor kit, CARAT Diagnostica Ltd, Budapest, Hungary). We chose to use 10 µM ADP because we wanted to achieve a characteristic effect in platelet aggregation. To examine spontaneous platelet aggregation, 50 µl 0.9 % NaCl was used. We assumed spontaneous platelet aggregation to be higher than 10% and the inducible platelet aggregation value was considered normal if it was higher than 50%.

*3.3.3 Measurements of oxidative stress parameters and laboratory techniques*

*3.3.3.1 Measurement of malondialdehyde (MDA) with Ohakawa method*

The plasma MDA is one of the derivatives originating from oxidative damage of poly-unsaturated fatty acids, thus indirectly shows intensity of lipidperoxidation due to oxidative stress. We attained plasma from ethylene diamine tetraacetic acid (EDTA) anticoagulated blood - centrifuged at 4000 rpm for 10 minutes - and mixed with sodium-dodecyl sulphate, acid buffer and EDTA. Thiobarbiturate solution was added to the mixture and incubated for an hour at 90 $^{o}$C. After cooling, adding butanol and repeating centrifugation, the supernatant was measured with spectophotometry at 532 nm. We used tetrametoxipropane as a standard and MDA was expressed in µM/L.

*3.3.3.2 Measurement of reduced gluthation (GSH) in whole blood*

Reduced gluthation is a basic endogenous antioxidant, the level of which is reduced due

to oxidative stress of various origins. A sample of 0.2 ml EDTA anticoagulated blood, haemolysed with 0.8 ml of distilled water, was mixed with 4 ml trichlore acetic acid (TCA) of 10% concentration. After centrifugation the supernatant was mixed with 4 ml TRIS buffer of pH 8.7. A colour reaction was induced with 100 µl of 10 mM 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) followed by photometry at 412 nm. Using a standard GSH series for calibration, values were expressed in µM/L.

*3.3.3.3 Measurement of plasma protein sulfhydril groups (PSH) level with Ellman's reagen.*

Plasma SH groups originate predominantly from plasma proteins and participate in the defence against oxidative stress. To determine SH groups, 100 µl plasma, 100 µl Ellman's reagent (1 mM DTNB in methanol) and 800 µl EDTA containing TRIS buffer were mixed and photometry was performed at 412 nm. GSH standard series were used for calibration. The PSH amount was expressed in µM/L.

*3.3.3.4 Determination of superoxide dismutase (SOD) enzyme activity in whole blood*

Superoxide dismutase is an enzymatic endogenous antioxidant which catalyzes the dismutation of the superoxide free radical. To determine SOD activity, 100 µl of EDTA anticoagulated blood was haemolysed with 900 µl distilled water and a mixture of ethanol and chloroform (2:1) was used to remove haemoglobin. Determination of the enzyme activity was based on the inhibition of the spontaneous oxidation of adrenaline to adrenochrome. Spectrophotometric measurements were performed at 480 nm against sodium carbonate buffer (pH 10.2) blind. The values of SOD enzyme activity were given in IU/ml.

*3.3.3.5 Determination of reactive oxygen species (ROS) production in whole blood.*

Activated leukocytes, mainly neutrophils, are potential sources of reactive oxygen species during inflammation. Free radical generating capacity of circulating leukocytes was assessed by measuring the amount of reactive oxygen species in whole blood, with chemiluminescense (CL) method based upon the reaction of luminol with free radicals. To sum up, 20 µl EDTA anticoagulated blood was diluted in 1400 µl Dulbecco's modified Eagle's medium (DMEM) nutrient mixture of 37ºC. 30 µl of 3-aminophtalhidrazide was added and the cuvette was immediately placed to Chrono-Log Whole Blood Lumi-aggregometer. The mixture was stirred and incubated at 37 ºC during measurement. After determining the spontaneous radical production, 50 µl phorbol-12 myristate-13 acetate (PMA) was injected into the cuvette and the resulting light output was recorded on a chart recorder. The peak value of free radical production was calculated from the recorded curve, and the results were related to the white blood cell counts.

*3.3.3.6 Measurement of plasma myeloperoxidase (MPO) level.*

MPO is a lysosomal enzyme that is found in neutrophil granulocytes and its plasma level elevates during inflammation. Plasma MPO level was obtained by adding 200 µl of plasma to 1ml mixed solution (10.9 ml Na citrate, 100 µl o-Dianisidin, 1ml $H_2O_2$ and 5 µl of 0.05% Triton-x-100). Incubation followed at 37ºC for 5 minutes. After adding 1 ml of 35% perchloric acid to the solution, it was centrifuged for 10 minutes at 2500 rpm and was measured at 560 nm.

*3.3.3.7 Measurement of leukocyte CD11a, CD18, CD97 expression.*

Leukocyte surface adhesion molecule expression shows the activation state and emigration antigen expression. CD11a, CD18, CD97 (leukocyte surface antigens) were detected with direct immunofluorescence, evaluated by flow cytometry. For immunofluorescence staining 200 µl of the EDTA anticoagulated whole blood was mixed with 10 µl of fluorescence isothio

cyanate (FITC)-conjugated CD11a, CD18, CD97 mouse anti-human monoclonal antibody and incubated for 15 minutes in dark at room temperature. Erythrocytes were haemolysed with diluted Becton Dickinson fluorescence activated cell sorter (BD FACS) Lysing Solution for 12 minutes. The leukocytes were washed twice in phosphate buffer solution (PBS), and finally resuspended in CellFIX solution. Mouse $IgG_{1,}\kappa$ isotype control was used to determine the non-specific, background fluorescence. Cell immunofluorescence and light scatter data were acquired on a FACSCalibur flow cytometer and analysed by Cellquest software. Binding of CD11a, CD18, CD97 antibodies to leukocytes was quantified as the mean channel fluorescence in arbitrary unit that exceeded non-specific background fluorescence.

*3.3.3.8 Measurements of haemostatic parameters*

Haematological parameters like haemoglobin, platelet counts were tested by routine five part differential haematocytometers (CellDyn3700 – ABBOTT and XE2100 – Sysmex) using 3 ml of K3-EDTA anticoagulated Becton-Dickinson (BD) vacutainer tube samples. Measurements were carried out within 4 hours. Refering to the performance of coagulation tests (D-dimer – Helena, Trinity, immune turbidimetry test; fibrinogen, prothrombin time and aPTT – Stago) the test samples were 3 ml of 0.129 (3.8%) mmol/L citrated BD vacutainer tubes measured within 4 hours on CA1500 (Sysmex) coagulation automates utilyzing the optical (turbidimetric) coagulation measurement principles. Prothrombin time results were given in international normalised ratio. Daily internal and regular external quality controls are applied to both haematocytometers and coagulation automats of the laboratory.

### 3.4 Statistical analysis

Data of the figures are presented as means ± standard error of mean. Mean values were compared to baseline results within groups and to corresponding measurements between groups at same time-points. Data of the table are presented as means ± standard deviation. In case of normal distribution student's T test was applied and otherwise Wilcoxon Signed Rank Test was used for the analysis of non parametric data. The relations between the measured data were analysed using a correlation test (Spearman's Rho). The required number of patients was calculated by power analysis according to adrenaline and PSH results. Therefore, with type I alpha of 5% and type II (power) of 80%, we calculated we would need about 7 patients per group. Values of $p< 0.05$ were considered significant.

## 4. RESULTS

### 4.1 Patients

Fifteen patients were included in the study, aged between 21 and 84 years, mean of ages: 63 (±16) years. Table 1 shows the patinets' data: age, sex, New Simplified Acute Physiology Score (SAPS II) score of patients. The average pulmonary perfusion deficiency was 71 (±7.2) %, according to echocardiography findings, the calculated mean right ventricle pressure was 52.5 (±12.5) mmHg. Ten patients had D-sign (increased RV/LV (right ventricle/left ventricle) ratio caused by intraventricular septal bulging into the LV). Serious tricuspidal insufficiency was observed in 9 patients, 4 patients were in shock. All patients classified with massive or submassive PE. The average SAPS II score was 22.46 (±7.85). Eight patients (5 male, 3 female) received UH-SK and 7 patients (3 male, 4 female) underwent tPA treatment. The risk factors of developing PE were registered: deep vein thrombosis (8 patients), malignant disease (3 patients)

predisposition for thromboembolic history (7 patients). Table 1 compares baseline characteristics and therapeutic results of UH-SK and tPA groups.

Major bleeding complications (criteria: tachycardia, haemodynamic instability, mental status alteration caused by anaemia, and more than 20% drop in haemoglobin level) were not seen in any of the cases. Puncture site bleeding was experienced in 8 patients, and in one case haematuria was noticed. Six patients (UH-SK: 3, tPA: 3) received 2-3 units of cross matched blood between 2nd d and 5th d due to the slowly developing anaemia. None of the patients received platelet concentrates. Following complete therapy, the clinical status and lung perfusion improved significantly in every patient (Table I). Two patients did not attend the 30th d control examination. One of them (82 years old) died of cardiogenic shock following acute myocardial infarction at the coronary care unit on 8th d after successful TL. Another patient could not appear for the control examination because she underwent surgery due to a crural ulcer.

| | **UH-SK** | **tPA** |
|---|---|---|
| **Age (years)** | 62 ±18 | 63 ±15 |
| **Gender (Male/Female)** | 3/5 | ¾ |
| **Perfusion deficiency on admission (%)** | 72 ±9 | 71 ±6 |
| **Perfusion deficiency following TL (%)** | 21 ±8*** | 31 ±12*** |
| **Massive PE** | 4 | 4 |
| **Submassive PE** | 4 | 3 |
| **Right ventricular pressure (mmHg)** | 48 ±15 | 57 ±7 |
| **D-sign (n)** | 3 | 6 |
| **Circulatory shock (n)#** | 2 | 2 |
| **SAPS II** | 22 ±8 | 23 ±9 |
| **Deep vein thrombosis (n)** | 6 | 2 |
| **Hypertension (n)** | 6 | 6 |
| **Diabetes mellitus (n)** | 2 | 3 |
| **Arrhythmia (n)** | 2 | 3 |
| **Smoking (n)** | 1 | 1 |
| **Contraceptive (n)** | 1 | 1 |
| **Long term diuretics (n)** | 6 | 2 |
| **Lower limb varicosity (n)** | 4 | 4 |
| **Family drift (n)** | 4 | 3 |
| **Acetylsalicylate (n)** | 3 | 3 |
| **Chronic use of NSAID (n)** | 4 | 3 |

Table I. Comparison of patient data in two therapeutic groups. TL = thrombolysis, PE = Pulmonary Embolism. SAPS = Simplified Acute Physiology Scoring. # Circulatory shock was diagnosed when arterial blood pressure was lower than 100 mmHg for more than 15 minutes despite adequate intravenous fluid resuscitation. Data are expressed as mean± SD. *** $p<0.001$ vs. admission values

## 4.2 Platelet aggregation study

ADP induced platelet aggregation decreased at 4th h ($p<0.05$), then compared to this value, increased at 24th h ($p<0.02$) in UH-SK group. In tPA group we found similar but not significant changes (Figure 1A).

At 4th h there was a decreased adrenaline induced platelet aggregation, which was still present at 8th h ($p<0.03$) in the UH-SK group. In the tPA group, the changes were not significant. Comparing the two groups we found significant difference at 36th h and 3rd d ($p<0.03$) (Figure 1B).

In the UH-SK group the aggregation induced with collagen showed significant decrease at 4th h and 8th h ($p<0.05$). From 24th h the aggregation slowly increased finally reaching the normal level. In the tPA group a slight increase at 4th h and 8th h was followed by a decrease at 12th h and 24th h. After a remarkable increase at 36th h the aggregation stabilized within the normal range (Figure 1C). Spontaneous aggregation was considered significant if reached 10% or higher level. None of our patients had significant spontaneous aggregation (Figure 1D).

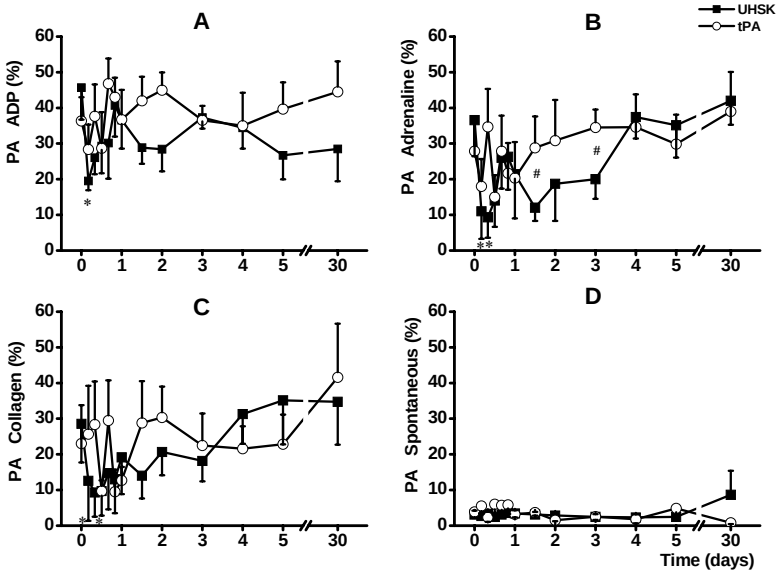


Figure 1

*Figure 1: Time course (in days) of platelet aggregation induced by adenosine-diphosphate (A), adrenaline (B), collagen (C) and 0.9% NaCl representing spontaneous aggregation (D) in ultra-high streptokinase (UH-SK, filled squares, n = 8) and alteplase (tPA, open circles, n = 7) treated groups. Data are presented as means ± standard error of mean, * $p < 0.05$ versus baseline value of the same group, # $p < 0.05$ versus corresponding value of the other group.*

## 4.3 Other haemostatic parameters

In UH-SK patients the platelet count showed an initial decrease compared to baseline ($p<0.05$) until 2nd d, then a slow increase lead to normalization on 5th d. In the tPA group a moderate initial decrease was followed by a slight increase. There was no significant difference between the two groups in this parameter (Figure 2A).

Fibrinogen levels were significantly lower at 4th h and on 4th d ($p<0.003$) in the UH-

SK group meanwhile in the case of tPA, the decrease was not statistically significant (Figure 2B). UH-SK group presented significantly lower fibrinogen levels than tPA group at all measuring points between 24$^{th}$ h and 5$^{th}$ d ($p<0.02$). Fibrinogen levels returned to the baseline in both groups by 30$^{th}$ d (Figure 2B).

Haemoglobin levels continuously decreased until 2$^{nd}$ d, especially in the UH-SK group ($p<0.02$). At 36$^{th}$ h and on 2$^{nd}$ d, there were significant differences between the two groups ($p<0.05$). From 4$^{th}$ d in both groups we found normal haemoglobin levels (Figure 2C).

Elevation of D-dimer levels showed an early peak at 8$^{th}$ h in the tPA group ($p=0.003$), the trend was slower in the UH-SK group and returned to the insignificant elevation at 36$^{th}$ h and on 2$^{nd}$ d in the UH-SK and tPA groups, respectively (Figure 2D).

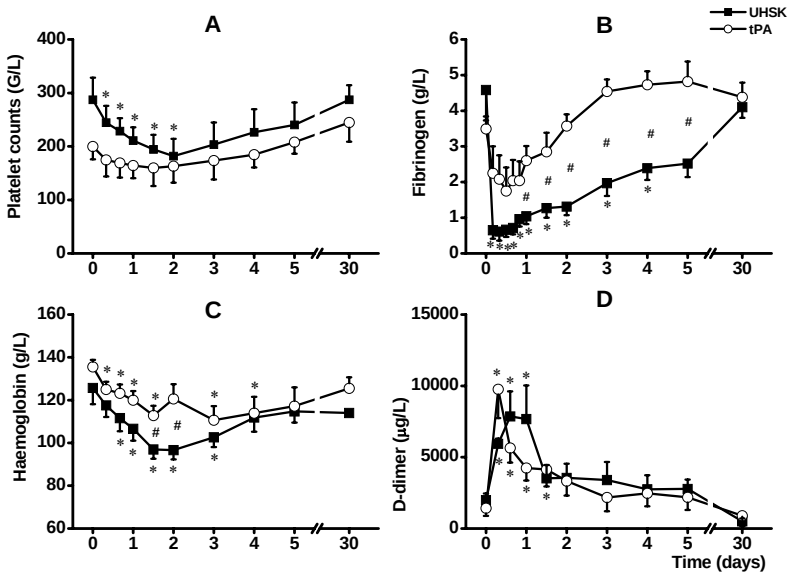


Figure 2

*Figure 2: Time course (in days) of platelet counts (A), fibrinogen (B), haemoglobin (C) and D-dimer (D) concentration in ultra-high streptokinase (UH-SK, filled squares, n = 8) and alteplase (tPA, open circles, n = 7) treated groups. Data are presented as means ± standard error of mean, * p < 0.05 versus baseline value of the same group, #p < 0.05 versus corresponding value of the other group.*

D-dimer concentrations showed negative correlation with fibrinogen levels ($r= - 0.462$, $p<0.004$). Fibrinogen levels and percentage of perfused pulmonary areas showed a strong significant positive correlation ($r=0,451$, $p<0,002$).

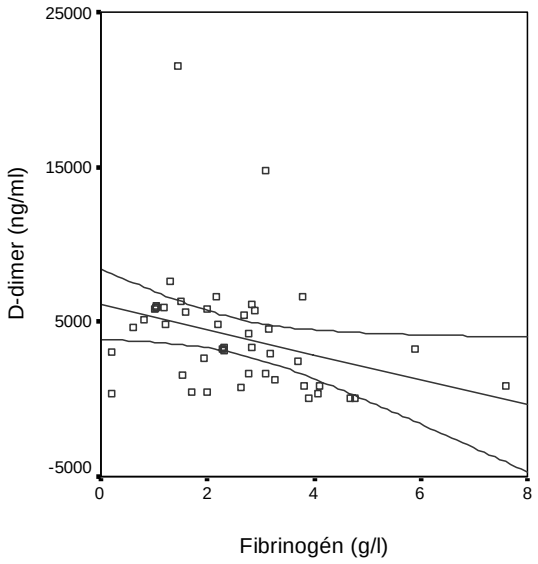


*Figure 3: Negative correlation between D-dimer and fibrinogen levels in all patients (n=15)*

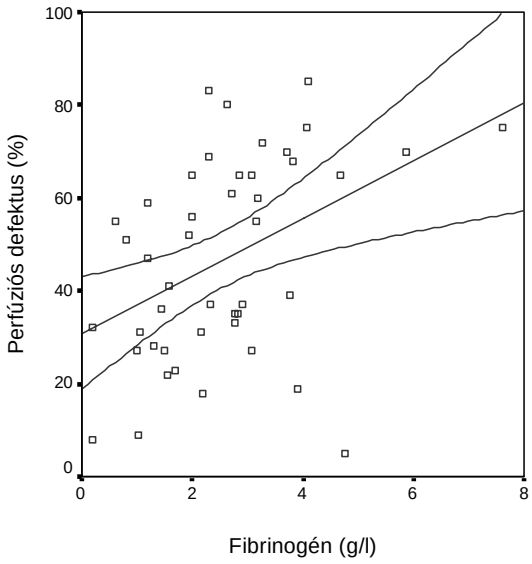


*Figure 4: Positive correlation between decreased perfusion defect and fibrinogen values in all patients (n=15)*

## 4.4 Oxidative stress study

### *4.4.1 Changes in leukocyte count*

Initially, the leukocyte count (normal value: 4.0-10.0 G/l) elevated slightly in the UH-SK treated patients following TL, then after the 3rd day that decreased gradually and reached the normal levels by the 30th day (Fig. 1). In the tPA treated patients, a progressive decrease in the leukocyte count turned to be significant ($p<0.05$) on the 3rd day.

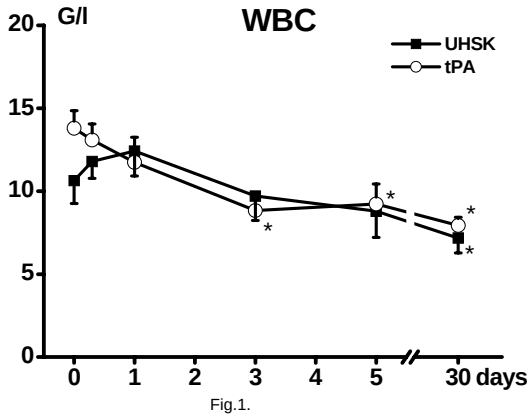


*Fig.1: Changes of the leukocyte count in UHSK (■; n=8) and tPA (○; n=6) treated patients(mean±SEM). * p<0.05 vs. 0*

*4.4.2 Plasma malondialdehide and reduced glutathion levels*

The plasma MDA level increased in both groups following thrombolysis (Fig. 2a). This increase was significant ($p<0.05$) in the 8th hour in the UH-SK treated group and on the first day ($p<0.05$) in tPA patients. The plasma MDA level remained high for 5 days in tPA treated patients. The GSH concentration in both groups decreased significantly ($p<0.05$) by the 8th hour, and stayed lower than the level prior to TL even on the 5th day (Fig. 2b).

*4.4.3 Plasma protein sulphhydril levels and superoxide dismutase enzyme activity*

The plasma protein sulphhydril (PSH) level decreased in both therapeutic groups following TL (Fig. 2c). This decrease was more marked ($p<0.001$) and lasted longer in the UH-SK treated group. The PSH level normalised by the 30th day. SOD activity decreased significantly ($p<0.05$) by the 3rd day in both groups (Fig.2d).

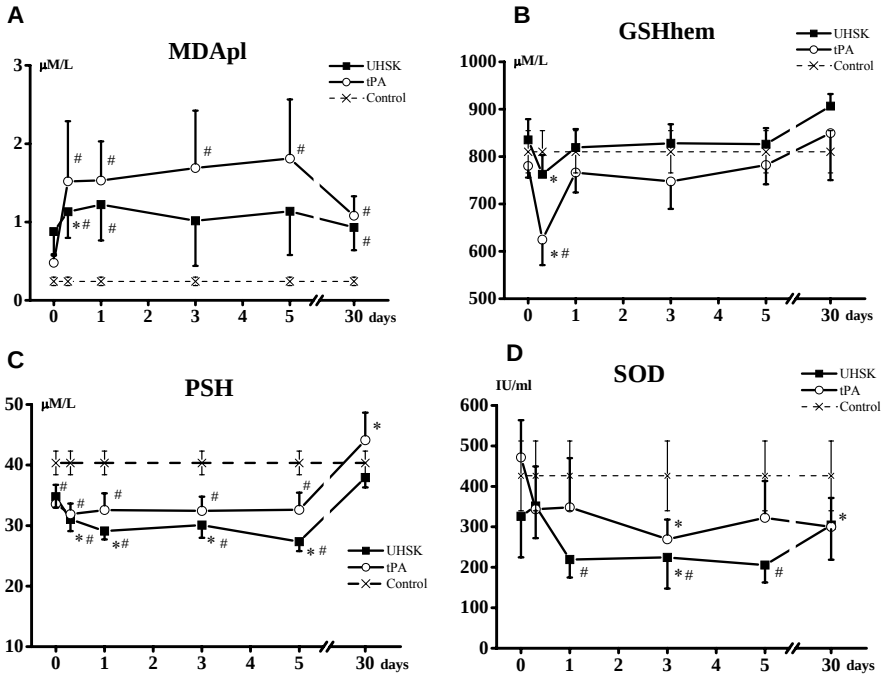


*Fig.2: Oxidative stress parameters. Plasma malondialdehyde (A), whole blood reduced glutathion (B), plasma sulphhydryl groups (C) and superoxide dismutase enzyme activity (D) changes in UHSK (■; n=8) and tPA (○; n=6) treated patients (mean±SEM). The broken line shows the normal values of healthy individuals. * p<0.05 vs. 0; # p<0.05 vs. control.*

*4.4.4 Granulocyte activation parameters*

The rate of production of PMA stimulated free radicals in the leukocytes was higher in the tPA patient group (Fig. 3a) and peaked 24 hours following TL. Afterwards a slow decrease started, which turned to be significant ($p<0.05$) by the 30th day. In the UH-SK treated group the level of free radical production peaked on the 3rd day and by the 30th day it decreased below the level prior to treatment. The mean plasma MPO enzyme activity was higher in UH-SK group (Fig. 3b) and gradually decreased from the first day of treatment. The MPO activity in tPA group increased in the acute phase and peaked on the first day ($p<0.05$) following the TL and then decreased.

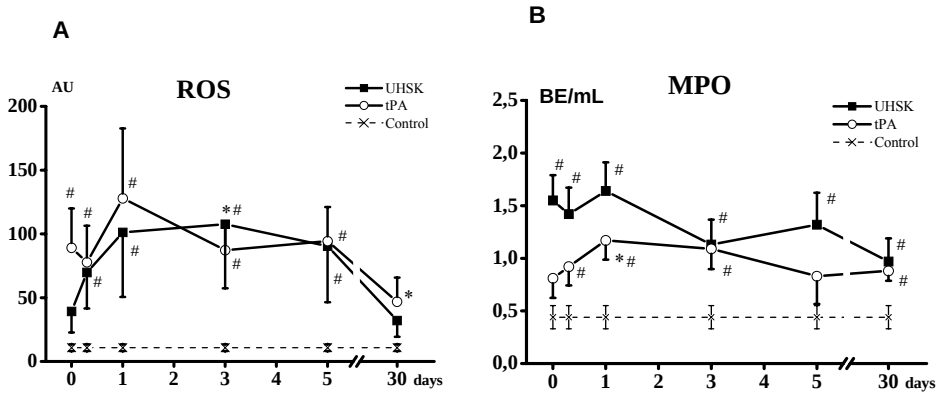


*Fig.3: Granulocyte activation parameters. The peak (A) value of the PMA stimulated free radical production and the changes in plasma MPO enzyme activity (B) in UHSK (■; n=8) and tPA (○; n=6) treated patients (mean±SEM). The broken line shows the normal values of healthy individuals. * p<0.05 vs. 0; # p<0.05 vs. control*

### *4.4.5 Adhesion molecule expression in circulating cells*

The expression of CD11a and CD18 adhesion molecules decreased on the cell surface of the circulating granulocytes and monocytes following TL (Fig. 4a, b, c, d). This change was significant (p<0.05) in the acute phase in UH-SK group. In tPA treated patients, the decrease in CD18 expression was even more prominent and lasted longer.

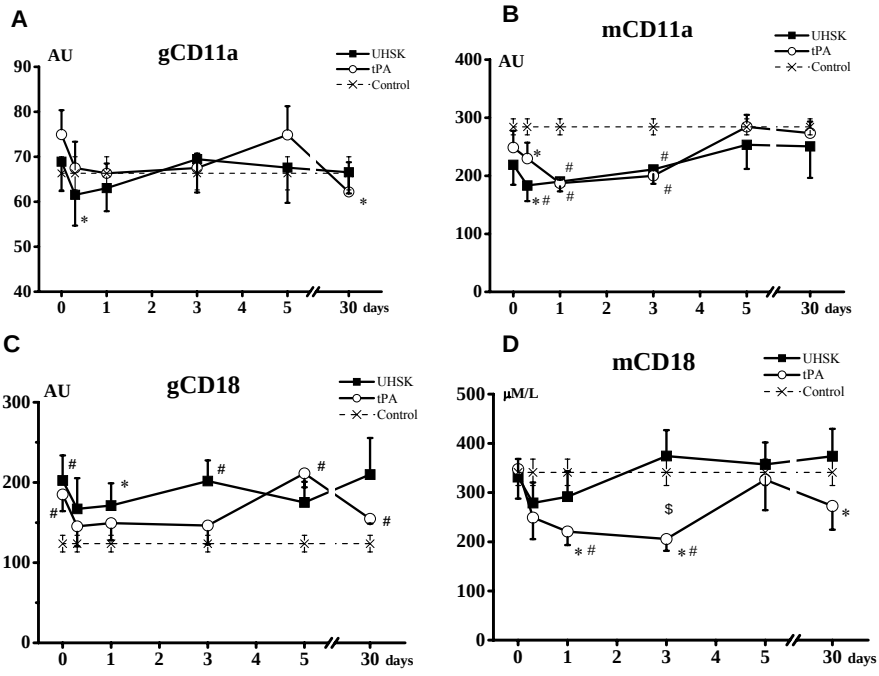


*Fig.4 : Adhesion molecule expression on circulating cells. Granulocyte surface expression of CD11a (A), CD18 (C) and monocyte surface expression of CD11a (B), CD18 (D) in UHSK (■; n=8) and tPA (○; n=6) treated patients (mean±SEM). The broken line shows the normal values of healthy individuals. * p<0.05 vs. 0; # p<0.05 vs. control; $ p<0.05 between two groups.*

*4.4.6 Inflammation marker expression*

Similar tendency could be seen in the expression of the inflammatory marker CD97 on the surface of granulcytes and monocytes (Fig. 5a, b), which decreased significantly in both groups at 8th hours following treatment. This decrease was more marked in the tPA group even on the 3rd day ($p<0.05$) following treatment.

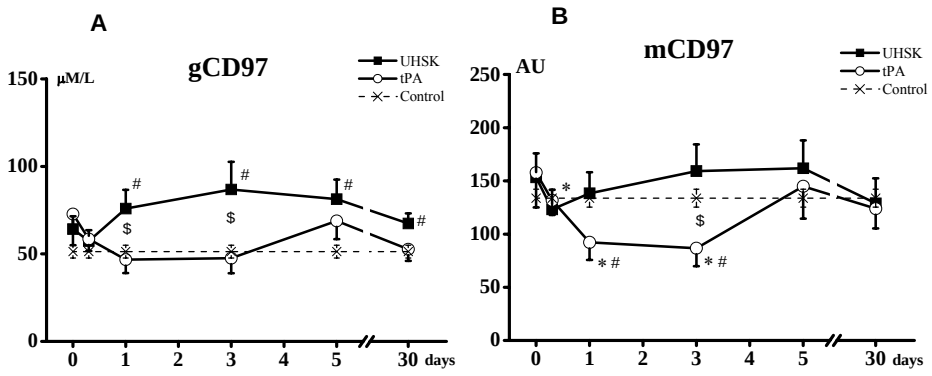


*Fig.5: Inflammation marker expression on circulating cells. Granulocyte (A) and monocyte (B) surfaceexpression of CD97 in UHSK (■; n=8) and tPA (○; n=6) treated patients (mean±SEM). The broken line shows the normal values of healthy individuals. * p<0.05 vs. 0; # p<0.05 vs. control; $ p<0.05 between two groups.*

# 5. DISCUSSION

*5.5.1 Platelet aggregation study*

In accordance with our previous reports, we observed that PE is not accompanied by spontaneous platelet aggregation. A decreased inducible platelet aggregation value was assessed before TL probably due to the heparin bolus given upon suspected diagnosis of PE. After TL, we observed a significant decrease in inducible platelet aggregation values compared to the above mentioned result in the UH-SK group, and it returned to the baseline at 36th h. We found a similar but insignificant decrease in platelet aggregation in the tPA group. This early restoration of inducible platelet aggregation explains that we noticed less bleeding complications than we expected according to previous publications on systemic TL. The significant decrease of haemoglobin levels was due to frequent blood sampling and aggressive fluid resuscitation in the acute phase of PE which caused haemodilution. After TL, all patients received intravenous 0.9% NaCl infusions at 1.5-2 ml/kg/hr. Blood samples were taken on the first day to measure platelet aggregation, aPTT and fibrinogen (16 ml every 4 hours). Blood gas, total blood count, D-dimer measurements (25 ml every 8 hours) were also carried out. From the second day onwards, we reduced the frequency of blood sampling but aPTT measurements remained unchanged until the patients received unfractionated heparin.

According to various investigations, the mortality of massive and submassive PE following TL is between 4.7 and 11.8 %. Analysis of 3-10 year survival shows a mortality of 18 to 33 % and a frequent cause of death is re-embolism. Despite previous reports on increased platelet aggregation and consequent early re-embolism, all of our patients were treated successfully and re-embolism was not detected in the follow-up period.

Recently many studies focused on platelet function in acute myocardial infarction (AMI) patients and in sepsis. Opinions are divided on the effects of thrombolytic agents on platelet aggregation. Some publications suggest inhibition, others are in favour of activation. According to our knowledge no such clinical trials has been conducted in PE. Moser and his co-workers studied platelet function of acute myocardial infarction patients after TL with reteplase or streptokinase, and reported a decreased platelet aggregation in the 1$^{st}$ h and 2$^{nd}$ h. Gurbel and co-workers studied platelet functions (aggregation and cell surface receptors) in myocardial infarction patients treated with alteplase or reteplase. According to their results during the first 4-6 hours there was a decrease in the surface receptor expression followed by progressive increase between the 12$^{th}$ h and 24$^{th}$ h. Our frequent measurements enabled us to detect similar findings to both authors, resolving the contradiction. Korbut et al. concluded that platelets play a vital role in the mediation of fibrinolytic activity of plasma. TL carried out with streptokinase or tPA leads to rapid platelet activation which can be responsible for early re-occlusion.

Our study revealed elevated platelet aggregation values compared to pre-thrombolytic values measured in the tPA group in the first 48 hours. These values measured at various time points were not linear and not even in one case exceeded the normal (>50%) range. Therefore, our experiments did not confirm the results of Korbut and re-embolism did not occur in our patients. Numerous work groups have confirmed that elevated platelet aggregation caused by TL can lead to early re-occlusion in AMI patients. Various studies have demonstrated that re-occlusion could be prevented by combining TL with anti-platelet therapy (ticlopidine and abciximab).

Topol analysed several international multicentric AMI trials and found that the dose of fibrinolytic therapy can be reduced with the combination of glucoprotein IIb/IIIa blocker plus salicylates which improves the effectiveness of TL, its speed and reduces bleeding complications, mortality and the frequency of re-occlusion. The pharmacological background of this effect is probably the prothrombotic tendencies of high dose fibrinolytic agents. It is important to note that the above mentioned studies were performed in AMI patients with arterial occlusion but in our current study we observed venous thromboembolism. We could not find higher platelet aggregation levels than normal in any of our patients in the PE groups, moreover consistently low levels were measured in the UH-SK group. Because of the above mentioned, we were not convinced about combining TL with platelet aggregation.

Agnelli and co-workers were seeking explanation for the phenomenon that continuous administration of tPA causes bleeding complications more often than single doses of the same drug. They concluded that single doses minimised the interaction with the haemostatic system, reduced the duration of systemic lytic state and decreased platelet function.

According to our results, we believe that both therapeutic regimens UH-SK and tPA are optimal thrombolytic agents in terms of effect in PE as a matter of fact we achieved successful TL in certain patients. According to the changes of D-dimer values, we can state that tPA secures a more rapid onset of TL. TPA reduces platelet aggregation moderately and does not reduce fibrinogen levels significantly due to lower duration of systemic lytic state in contrast to UH-SK treatment. Our opinion suggests tPA to be a more suitable drug but streptokinase is also effective because of its cost-benefit ratio.

The major limitation of our study is the small number of patients, but this is due to the low incidence of submassive and massive PE even in large centres. Clinical trials for management of critically ill PE patients have been dauntingly difficult to organise.

In the present study, UH-SK treatment decreased fibrinogen levels but tPA did not affect this parameter significantly. We followed platelet aggregation, the changes in fibrinogen levels and sought a quick laboratory parameter which well characterizes the efficacy of TL (>30% increase in pulmonary perfusion). In our results, fibrinogen levels

correlated with the efficacy of the TL ($r= 0.451$, $p<0.02$), therefore we concluded that it may be a useful marker of successful treatment.

*5.5.2 Oxidative stress study*

During the thrombolytic treatment of massive and submassive PE, leukocyte activation and particularly OS has not yet been elucidated clearly in human studies that is related to the ischaemia-reperfusion syndromes. During pulmonary embolism, the consequence of partial or complete block of pulmonary blood circulation is the decrease of left ventricle filling volume and the increase of right ventricle afterload. The progression of pulmonary hypertension and the the intrapulmonary shunt circulation induce an arterial hypoxia and the decrease of oxygen saturation. Acute massive and submassive pulmonary embolism without treatment can lead to fatal circulatory collapse. One of the modalities of the treatment of PE is TL improving the oxygen supply and restoring the perfusion of hypoxic and ischemic tissues.

It is widely known that hypoxia-reoxygenation and ischemia-reperfusion induces oxidative stress with a concomittant imbalance between the production of oxygen free radicals and endogenous available antioxidants. A cascade of production or consumption and activation of different cellular mediatiors, cytokines eventually increase the superoxide radicals and intracellular calcium level. The enhanced leukocyte activation and the increased aggregation of thrombocytes disturb microcirculation causing tissue and cellular damage.

We studied OS and accompanying leukocyte activation in patients suffering from massive and submassive PE and receiving either UH-SK or (8 patients) or tPA (7 patients) thrombolytic treatment.

The leukocyte count was elevated in both groups prior to TL as a result of systemic inflammatory reaction of PE that returned to the normal level within the next 30 day observational period.

Circulatory instability, hypoperfusion, hypoxia and ischemia were found at the time of admission due to the pulmonary circulatory failure caused by massive PE which could be aggravated by oxidative stress.

The MPO and SOD enzyme activity was elevated, the lipidperoxidation marker MDA was increased with a decreased level of endogenous antioxidant GSH and PSH groups. The free radical producing capacity of leukocytes measured during the first sampling was increased too. The effective TL opening the obstructed pulmonary arteries improved the pulmonary circulation, however, the reperfusion augumented the OS. The elevated MDA,

ROS and MPO and the decreased GSH and PSH levels were maintained in the first 5 days indicating the presence of oxidative stress. The cellular damage due to the hypoxia and reperfusion requires longer time for recovery after the restoration of pulmonary circulation. We could observe the improvement of the OS only 30 days after TL, however the clinical symptoms promptly showed an important and beneficial results of TL.

Following tPA treatment the OS markers showed more emphasized changes that was seen in the streptokinase treated patient. We assume that tPA has a more agressive and faster thrombolytic effect than streptokinase and the faster thrombolysis might induce a more intensive reperfusion cellular damage. Even if that is so, the early restoration of pulmonary circulation is lifesaving and a mandatory therapy of PE.

Another important finding of our study was that the expression of β2 integrins and CD97 inflammatory marker on the surface of the circulating granulocytes and monocytes temporarily decreased as a consequence of thrombolytic treatment. This effect was more marked on the monocytes CD18 and CD97 expression after use of tPA. These findings are concordant to the observation that the mature cells are sensitized following TL to adhere to the endothelium and they more actively migrate to the reperfused tissue. The extravasated

granulocytes and monocytes maintain the inflammatory reaction. The monocytes produce a large amount of pro and anti-inflammatory cytokines regulating the healing process.

Granulocytes release reactive oxygen radicals and tissue proteases. The activation and adherence of the granulocytes is especially high in the pulmonary capillaries. The increased MPO enzyme activity observed also supports this data. The tissue specific plasminogen activator - normally produced in the endothelium - inhibits the expression of the adhesion molecule on the surface of circulating cells. That can explain our findings together with the marked decrease noticed in in the tPA treatment group. The different effect of SK and tPA on the expression of the adhesion molecules was also verified in patients treated with thrombolysis after myocardial infaction.

Our clinical investigation revealed new data on PE and its thrombolytic treatment affecting leukocyte activation. It can be concluded that massive and submassive pulmonary embolism is accompanied by OS and leukocyte activation. Further increase was observed following thrombolysis. Different reactions were found in the tPA treated group assuming that the thrombolytic drugs might have different and direct effects on the cellular functions in the pulmonary tissue that requires further investigation.

## 6. CONCLUSIONS

1) We could not find spontaneous platelet aggregation in any of our patients suffering from severe PE. In concordance with previously published data, elevated platelet aggregation levels don't have a predictive role in critical venous thromboembolism either.

2) Following TL, on the first day there was a significant decrease in platelet aggregation in the UH-SK group. On the second day we saw an increase in platelet function which reached baseline values from the third day onwards.

3) We did not find a relation between the bleeding complications and decrease of platelet aggregation. In the case of all three inductors the platelet aggregation rapidly dropped in therapeutic groups and against this bleeding with haemodynamic relevance was not found.

4) As opposed to data in literature, we did not find elevated platelet aggregation in any of the treatment groups leading to re-embolism following TL in massive or submassive PE.

5) The changes in fibrinogen levels correlate significantly with the improvement of the pulmonary perfusion which shows the effect of TL. It is worth considering further studies to observe the connection between the changes of fibrinogen levels and the effectivity of TL.

6) A PE induced OS was detected on patients before the initiation of thrombolytic treatment as the result of submassive PE.

7) TL treatment of PE augmented the increase of OS response and leukocyte activation following reperfusion and these parameters normalised only on the $30^{th}$ day.

8) We noticed different effects of SK and tPA both over time and degree of change on the expression of leukocyte adhesion molecules and OS. This finding could reflect the effectivity of the administered thrombolytic drug.

9) OS and leukocyte activation markers can not be recommended for effective monitoring of TL in PE because their changes are prolonged beyond the $5^{th}$ day and reaches a normal range only by the $30^{th}$ day.