Hemorheological and hemostatic changes in acute and chronic vascular diseases

PhD Thesis Booklet

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The investigation of pathological conditions of the circulation in vivo surgical models and patients

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List of abbreviations

ACC: American College of Cardiology
ACE2: angiotensin converting enzyme 2
ACS: acute coronary syndrome
ADP: adenosine-diphosphate
AHA: American Heart Association
ANOVA: analysis of variance
APS: antiphospholipid syndrome
aPTT: activated partial thromboplastin time
AUC: area under the curve
CAHA: COVID-19-associated hemostatic abnormalities
CD: cluster of differentiation
CFT: clot formation time
COVID-19: coronavirus disease 2019
CT: computer tomography
DIC: disseminated intravascular coagulopathy
e.g.: exempli gratia, meaning for example
ECA: ecarin-based assay
ECG: electrocardiography
ECLIA: electrochemiluminescence immunoassay
EDTA: ethylenediamine tetra-acetic acid
ESR: erythrocyte sedimentation rate
etc: et cetera, meaning and so on
ETT: endotracheal tube
FDP: fibrin degradation product
FSC: forward scattered light
G: gauge
g: g-force (exerted by a centrifuge while spinning a sample)
GP: glycoprotein
H-IPF: high-immature platelet fraction
HPS: hemophagocytosis syndrome
HRPR: high-residual platelet reactivity
hs-CRP: (high-sensitivity) C-reactive protein
HTPR: high on treatment residual platelet reactivity
ICU: intensive care unit
IL: interleukin
INR: international normalized ratio
IPF: immature platelet fraction
IQR: interquartile ratio
LAR: leukocyte antisedimentation rate
LCR: larce cell ratio
LE: level of evidence

LMWH: low-molecular-weight heparin
LT: lysis time
MCF: maximal clot firmness
mPFT: modified platelet function test
MV: microvesicles
NAR: neutrophil antisedimentation rate
NLR: neutrophil-to-lymphocyte ratio
NSAID: nonsteroidal anti-inflammatory drug
OR: odds ratio
PAC-1: procaspase activating compound-1
PAI-1: plasminogen activator inhibitor-1
PAR: platelet antisedimentation rate
PAR1: protease-activated receptor 1
PDE: phosphodiesterase
PE: phycoerythrin
PE: pulmonary embolism
PECAM-1: platelet endothelial cell adhesion molecule-1
PGI2: prostaglandin I2
PLT: platelet
PMV: platelet-derived microvesicles
RECOVERY: Randomised Evaluation of COVID-19 Therapy
ROC: receiver operating characteristic
rtPA: recombinant tissue plasminogen activator
RT-PCR: (real-time) polymerase chain reaction
SARS-CoV-2: severe acute respiratory syndrome – coronavirus 2
SD: standard deviation
SEM: standard error of meaning
SFL: fluorescent light
SIC: sepsis-induced coagulopathy
SPSS: Statistical Package for the Social Sciences
SSC: side scattered light
TEG: thromboelastography
TIA: transient ischemic attack
TMA: thrombotic microangiopathy
TNFα: tumor necrosis factor α
tPA: tissue plasminogen activator
TRAP: thrombin receptor activating peptide-6
vWF: vonWillebrand factor
vWF:Ag: vonWillebrand factor antigen
vWF:Rco: vonWillebrand factor ristocetin cofactor activity
YKL-40: mammalian chitinase matrix protein of specific granules in human neutrophils

1. Prologue

Biomarkers are extensively used for diagnosis, prognosis, and treatment monitoring in patients with cardio- and cerebrovascular diseases. The ideal biomarker possesses high sensitivity, allows early recognition, and sufficiently high specificity for a given disease outcome. It is fortunate if it can be measured easily, noninvasively, and inexpensively to produce rapid, reproducible results. Biomarker research should help to better understand underlying pathophysiological processes in special medical conditions, which can lead to new therapeutic perspectives potentially improving the outcomes.

My PhD thesis is focused on two main types of vascular diseases: post-stroke research is referred as a „chronic" vascular event, meanwhile COVID-19-associated thromboembolic complication investigation is referred as „acute disease"-associated alterations in hemorheology and hemostasis.

CHRONIC VASCULAR DISEASE: POST-STROKE HEMORHEOLOGICAL AND HEMOSTATIC RESEARCH

2. Introduction

A stroke is a medical emergency requiring prompt diagnosis and treatment to reduce brain damage. Diagnosis is upon clinical symptoms and radiological imaging. The primary aim of the treatment is to restore cerebral blood flow as soon as possible to improve or resolve neurological deficits (with intravenous thrombolysis or mechanical thrombectomy). Up to the recent stroke statistics for the year 2022, the crude annual incidence of stroke ranges between 41-297/100000/year in different countries. The incidence seems to decrease in high-income countries and increase in low- and middle income countries. The 28-30-day case mortality ranged between 10-40%. An increase in stroke survivors contributes to a rise in recurrent ischemic events. To reduce the burden of recurrent ischemic stroke episodes, antiplatelet therapy is a critical component of secondary prevention (with I.A evidence). The most commonly discussed antiplatelets in practice guidelines are aspirin, aspirin-dipyridamole, clopidogrel, and ticagrelor. Drug-therapy should be started as soon as possible after brain imaging excludes hemorrhage within 24 hours of symptoms onset (with I.B evidence). The physiology of platelets is relevant to understand stroke better. Thrombogenesis is initiated by endothelial damage; exposure of the vascular subendothelium causes platelet activation, aggregation, and fibrin generation via the coagulation cascade. Many studies suggested that

platelets can be excessively activated in the acute convalescent phase of cerebral ischemia.

2.1. High - on treatment - residual platelet reactivity (HTPR or HRPR)

High residual platelet reactivity (HRPR) is defined as the high level of platelet reactivity present after receiving a loading dose of an antiplatelet agent. It should be based on laboratory techniques that can quantify the activity of the target receptor before and after the administration of the drug. The HTPR value - determined by ROC analyses - is >468 arbitrary aggregation units/min in response to ADP measured by Multiplate® analyzer. Previous meta-analyses declared an independent correlation between HRPR and recurrent ischemia; the higher the residual platelet reactivity, the higher risk of cardio-cerebrovascular adverse events.

2.2. Prediction of stroke reccurrence with biomarkers

We can shortly divide the recently used biomarker into two main subtypes: classical- and novel biomarkers. Classical biomarkers are used in wide spectrum, they are easy to measure and they can be measured easily on a daily basis, these biomarkers are: C-reactive protein, fibrinogen and D-dimer level. Novel biomarkers are mainly used during clinical researches and in academic hospitals: neutrophil lymphocyte ratio, genetic testing, interleukin-6, YKL-40 protein were independently associated with recurrent stroke and poor functional outcome.

2.3. Antisedimentation of differenct cellular components

Leukocyte flotation during gravity sedimentation of the whole blood was described by Bogár et al. in 2000. They noticed that the upward motion of leukocytes could predict bacteremia in critically ill patients, later it was established that leukocyte antisedimentation rate could help in the early recognition of post-stroke infection, and postoperative complications. A modified version of the leukocyte antisedimentation rate (LAR) was developed by our research team. We examined the motions of platelets; platelet antisedimentation rate (PAR) reflects the percentage crossing the midline of the blood column upwards during one-hour gravity sedimentation. Activation of neutrophil granulocytes is reflected by the neutrophil antisedimentation rate (NAR).

2.4. Platelets-derived microvesicles

Peripherally circulating microvesicles (MVs) are small cell membrane-derived particles (diameter: 0.1-1.0 micrometer); they can be found in liquor, tear, saliva, urine, breastmilk, and

bronchoalveolar lavage as well. Circulating platelet-associated MVs (PMVs) are the most copious type of MVs found in human circulation, and they express several platelet surface markers, for example, CD42a (glycoprotein IX, GPIX) and CD62P (P-selectin). The CD42a expression is restricted to platelets and megakaryocytes. The GPIb-IX-V complex primarily functions as the platelet receptor for the von Willebrand factor. Microvesicles have numerous biological functions; they play a significant role in antigen presentation and different immune reactions. They enable intercellular communication by delivering lipids, proteins, and genetic material to cells nearby or distant places and modulation the function of these targets. PMVs might be essential biomarkers for identifying various recurrent cardio- and cerebrovascular diseases.

3. Hypothesis and objectives

The aims or our research project on post stroke patients were:

- to observe peripheral blood cell characteristics as predictors of recurrent ischemic episodes
- to find contributing factors to the long-term outcome of stroke patients
- to establish a predictive value of a modified platelet function test compared to conventional platelet impedance aggregometry during follow-up of the patients
- to explore differences between circulating microvesicles from a different origin comparing convalescent ischemic stroke patient with healthy controls
- to observe the correlation between platelet function and microvesicles in patients on antiplatelet therapy
- to detect association with high-on treatment residual platelet reactivity and peripherally circulating microvesicles

4. Methods

4.1. Study design and subjects

We performed a prospective observational pilot study. The University of Pécs Clinical Centre Regional and Institutional Research Ethics Committee approved the study protocol; reference number: 6735. Clinical Trial Identification Number of the research: NTC03679858. All procedures were performed by the ethical guidelines of the 1975 Declaration of Helsinki. Written informed consent was obtained from patients and healthy controls as well. A total of 52 patients (age: 66 ± 8 years, male: 31) taking antiplatelet medication (clopidogrel 75mg/day)

due to secondary stroke prevention were prospectively recruited into the study to detect the effectiveness and utility of the modified platelet function test to provide early recognition of high-risk population for recurrent ischemia. On the other hand, 18 patients (age: 66 ± 8 years, male: 12) from the same group were enrolled in the microvesicle examination study. All selected patients suffered large artery atherothrombosis and were on regular medical follow-up at the Outpatient Ambulance of the Neurology Clinic, Clinical Centre, University of Pécs.

4.2. Sample collection

Patients were instructed to take their daily medication at least two hours before blood sampling. Fasting blood samples were taken via a 21G peripheral venous cannula from each patient and healthy subjects after short strangulation from the antecubital vein into a closed blood collection system tubes. Blood samples were transported immediately for laboratory measurements and were processed within one hour.

4.3. Platelet and neutrophil antisedimentation rate

A modified whole blood gravity sedimentation technique was developed for studying platelet and neutrophil sedimentation properties. After one-hour gravity sedimentation, the upper and lower half of the venous blood column were removed separately from the EDTA and hirudin tubes and transferred into another EDTA and hirudin tube for further analysis. It is presented in ***Figure 1.*** Total blood cell count, platelet, and neutrophil (%) count were measured from the whole blood after the gravity sedimentation from the upper and lower fraction of the blood on Sysmex XN 9000 integrated hematology analyzer. Next, the platelet antisedimentation rate (PAR; %), leukocyte antisedimentation rate (LAR; %), and neutrophil antisedimantation rate (NAR; %) were – respectively – calculated based on this equation:

$$\frac{\text{sejtszám (felső frakció)} - \text{sejtszám (alsó frakció}}{\text{sejtszám (felső frakció)} + \text{sejtszám(alsó frakció)}} \text{ x } 100$$

4.4. Platelet function test

Platelet function test was performed in the whole blood after one-hour sedimentation from the upper and lower fraction of the hirudin anticoagulated blood with Multiplate® analyzer. Platelet aggregometry was uniformly carried out sixty minutes after blood sampling using adenosine-diphosphate (ADP; 6.5M) as an agonist. The aggregation level was expressed as the area under the curve (AUC). AUC was calculated by the machine software using the

product aggregation (defined in aggregation unit; AU) × time (minutes). After ADP stimulation, the normal aggregation range was expected as AUC: 53-220. Based on the whole blood AUC, patients on clopidogrel were categorized as „responders" with AUC <53, and „low-responders" (obtaining the high residual platelet reactivity on clopidogrel status) with AUC ≥53.

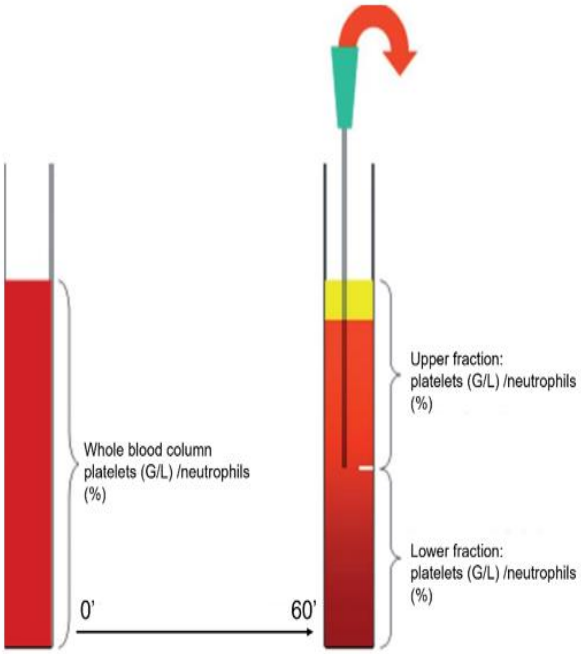

Figure 1. *Sample preparation, separation of the upper and lower sample fractions after one-hour gravity sedimentation. This figure was designed by D. Schrick.*

4.5. Sample preparation and measurement of microvesicles

After one-hour gravity sedimentation, the upper and lower part of the citrated blood was centrifuged at 2500×g for 20 minutes at room temperature. The supernatant was transferred into a new test tube and centrifuged at 2500×g for further 20 minutes to obtain cell-free plasma. The top of the cell-free plasma was transferred into an Eppendorf tube, and it was immediately frozen on liquid nitrogen and stored at –80°C until further measurements. The selected CD markers and their cellular origin, the fluorescent dye used for labeling, and the manufacturer specification for our MV measurements are summarized in ***Table 1.***

Flow cytometric measurements and data analysis were performed on Beckman-Coulter FC-511 cytometer with CXP software. The MV's reference gate was defined with Biocytex Megamix beads. Side scatter, forward scatter, and fluorescence channels were set on a logarithmic scale. MV size gate was determined between 0.5 μm size range. Events in the MV gate were further discriminated by labeling with annexin. MVs were defined as annexin V positive events in the MV gate with fluorescence intensity above the isotype control. To

determine the MV number, known concentration (1×10^6/mL) of 3μm diameter microbeads were used to determine the optimal labeling concentrations.

CD marker	Cellular origin	Fluorescent dye	Manufacturer
CD62P (P selectin)	Platelet	PE	Beckman-Coulter
CD41 (GPIIb/IIIa)		Cy5	Beckman-Coulter
CD42a (GPIb/V/IX)		FITC	Becton-Dickinson
PAC1 (GPIIb/IIIa, near fibrinogen binding site)		FITC	Becton-Dickinson
CD31 (PECAM-1)	Endothelial cell	PE	Becton-Dickinson
Annexin V	Phosphatidyl-serine	FITC, Cy5	Becton-Dickinson
Mouse IgG1	Isotype control	FITC, PE, Cy5	Becton-Dickinson

Table 1. *The selected CD markers for MV measurement, cellular origin, fluorescent dye, and manufacturer specification. Abbreviations: Cy5, Cychrome5; FITC, fluorescein isothiocyanate; PE, phycoerythrin; Ig, immunoglobulin*

4.6. Data collection and statistical analysis

Comorbidities, medications, and smoking status were also recorded. The incidence of vascular events (acute coronary syndrome, recurrent ischemic stroke, transient ischemic attack) in the total study population was evaluated in a 36-month follow-up. Acute coronary syndrome (ACS) was defined by the most up to date guidelines (based on the clinical presentation of symptoms, ECG results, level of cardiac necroenzymes, and stress testing results). Each recurrent cerebrovascular ischemia was confirmed by neuroimaging.

Data were evaluated by the SPSS software package (IBM SPSS Statistics® 27.0). We presented medians and interquartile ranges or means and standard deviations for continuous variables and frequencies and percentages for categorical variables. Differences between the groups were explored using the Student-t test, Mann-Whitney U test, one-way ANOVA and Kruskal-Wallis test for continuous variables, and Fisher exact test or $\chi2$ test for categorical variables, where appropriate. Correlation analysis was performed by calculating Spearman's correlation coefficient (rho). Binary logistic regression was used to explore the independent predictor of high residual platelet reactivity. A p-value<0.05 was considered statistically significant

5. Results

A total of 52 convalescent stroke patients were prospectively recruited into the study. All patients suffered large vessel occlusion, diagnosed by neuroimaging. Eleven vascular events occurred (stroke n=5; ACS n=6) during the 36-month follow-up. Out of the antisedimentation properties, only NAR showed a significant difference between the „uneventful" and „vascular events" subgroups. No difference was observed in the baseline blood count parameters, while a trend-like difference was detected in ESR.

The AUC_{upper} was significantly higher in patients with recurrent stroke compared to those with uneventful follow-up (p=0.003), shown in ***Table 2***.

	Total population n=52	Uneventful n=41	Stroke + ACS n=11	p-value
AUC	40.5 (27.53.5)	40 (27-54)	42 (32.5-44)	0.866
AUC_{upper}	56 (22.5-76.5)	51.5 (19.5-77.5)	65 (42-75.5)	0.247
AUC_{lower}	18 (13.5-22)	28 (14-34)	17 (13-20)	0.567
	Total population n=52	Uneventful n=41	Recurrent stroke n=5	p-value
AUC	40.5 (27-53.5)	39 (27-53)	43 (42-44)	0.347
AUC_{upper}	56 (22.5-76.5)	49 (21-74)	77 (71-92)	0.020
AUC_{lower}	18 (13.5-22)	18 (14-44)	17 (11-19)	0.763

Table 2. *The area under the curve (AUC) in the whole blood and AUC in the upper and lower samples after one-hour gravity sedimentation in the total population and comparison between uneventful and stroke+ACS subgroups, as well as uneventful and recurrent stroke subgroups. Abbreviations: AUC, area under the curve*

The ROC curves of variables predicting the recurrence of vascular events during 36 month follow-up period are shown in ***Figure 2.*** In this cohort, NAR with cut-off ≥-0.431 independently predicted the recurrence of total vascular events (stroke + ACS, n=11) with a sensitivity of 82%, and specificity of 88% during 36-month follow-up (area: 0.847, p=0.002, 95%CI: 0.703-0.992), shown in ***Figure 2A***. Besides, ROC of platelet function test based on impedance aggregometry in the upper blood sample after one-hour gravity sedimentation revealed that AUC_{upper} with a cut-off ≥70 predicts recurrent ischemic stroke with a sensitivity of 80%, and specificity of 74% during 36-month follow-up (area: 0.813, p=0.023, 95%CI: 0.689-0.937), shown in ***Figure 2B***. A more precise model was created when a ROC analysis

was performed with the predicted probability of the combination of NAR and AUC_{upper} (area: 0.881, p=0.001, 95%CI: 0.754-1.0), shown in ***Figure 2C***.

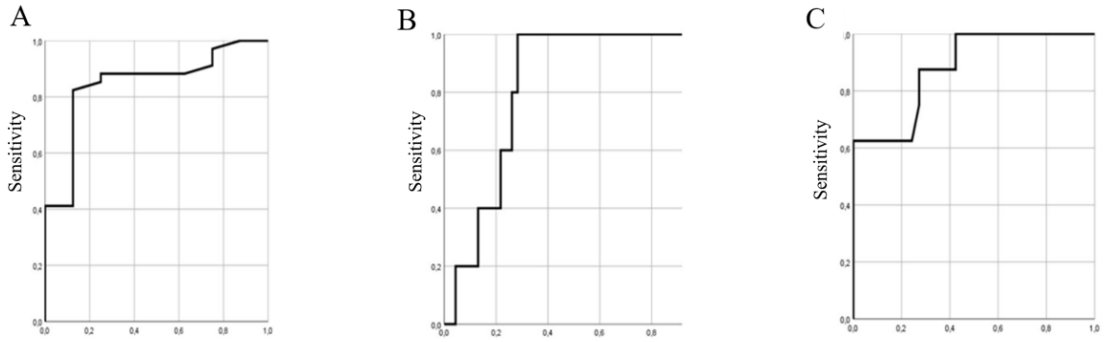

Figure 2. *ROC of variables predicting the vascular event recurrence during 36-month follow-up.* ***A****: ROC of neutrophil antisedimentation rate (NAR) with the area=0.847, p=0.002, 95%CI 0.703-0.992;* ***B****: ROC of* AUC_{upper} *with the area=0.813, p=0.023, 95%CI 0.689-0.937;* ***C****: ROC of predicted probability of the combination of NAR and* AUC_{upper} *with area:0.881, p=0.001, 95%CI 0.754-1.0*

From the same group of patients, 18 patients' blood were furtherly analyzed in comparison with 20 healthy controls. Demography and baseline microvesicle parameters of patients and their healthy controls are summarized in ***Table 3.*** All of the considerable MV data from *Table 3.* are presented in ***Figure 3.***

AUC and velocity in the whole blood sample showed a negative correlation with the total number of MVs ($x10^5$/ml) in the lower blood sample after one-hour gravity sedimentation. Notably, a significant negative correlation was observed for the velocity (r=-0.801, p=0.005) but not for the AUC in responders (n=11).

$PAC\text{-}1^+$ microvesicles in the lower blood sample showed a significantly positive correlation with the percentage of neutrophil granulocytes in the lower blood sample after one-hour gravity sedimentation (r=0.634, p=0.008). On the contrary, this positive correlation dissipated when whole blood indices were analyzed. Additionally, $CD42a^+$ MVs measured in the upper blood fraction showed a significant correlation with the percentage of neutrophils in the lower blood fraction (r=0.652, p=0.006). Though, no significant correlation was found in the whole blood samples.

	Post-stroke patients n=18			Healthy controls n=20	p-value
Age	65 (60-70)			57 (49-63)	0.078
Male/female	12/6			10/10	0.298
MVs ($x10^5$/ml)	whole blood	upper sample	lower sample	whole blood	
Total MVs	3.43 (2.34-4.70)	1.79 (0.37-2.82)	1.53 (0.96-1.89)	0.22 (0.13-0.37)	<0.001
CD31+	0.43 (0.12-0.55)	0.25 (0.10-0.46)	0.07 (1.13-1.255)	0.08 (0.04-0.16)	0.016
CD42a+	0.21 (0.09-0.48)	0.13 (0.07-0.32)	0.05 (0.02-0.10)	0.02 (0.01-0.03)	<0.001
CD41+	0.25 (0.13-0.56)	0.15 (0.10-0.53)	0.04 (0.03-0.24)	0.15 (0.09-0.25)	0.251
CD62P+	0.48 (0.09-0.85)	0.26 (0.07-0.59)	0.15 (0.03-0.24)	0.17 (0.09-0.33)	0.105
PAC-1+	0.009 (0.009-0.03)	0.008 (0.007-0.02)	0.003 (0.002-0.007)	0.01 (0.009-0.02)	0.515

Table 3. *Demography and baseline microvesicle parameters of patients and healthy controls. Abbreviations: MV, microvesicles; CD, cluster of differetiation; PAC, procaspase activating compound*

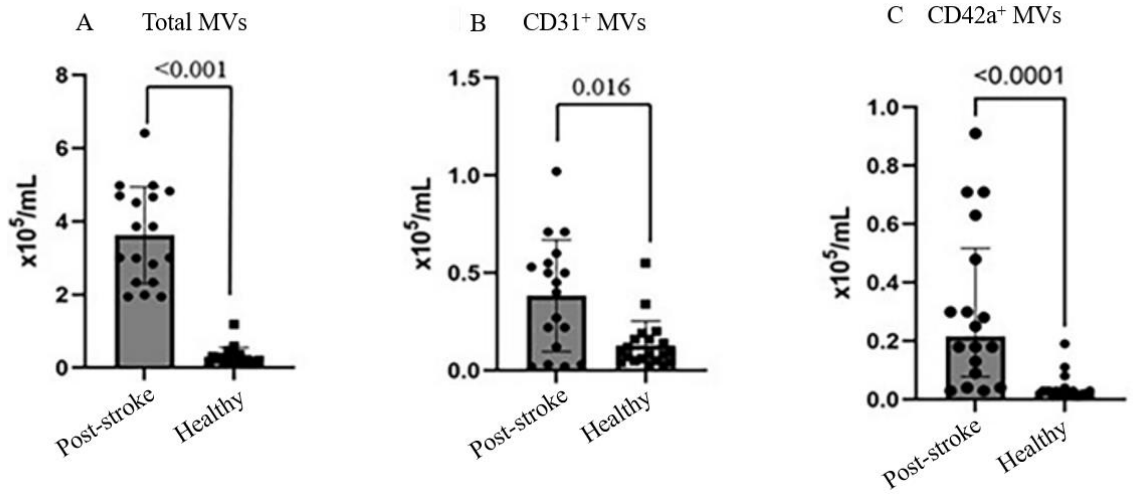

Figure 3. A*: Comparison of the total MVs count;* ***B****: CD31+ MVs;* ***C****: CD42a+ MVs in the whole blood of post-stroke patients and healthy subjects (Mann-Whitney U test)*

6. Discussion

Neither LAR nor PAR was found to be predictive for recurrent vascular events in convalescent stroke patients. This exploration suggests that platelets and leukocytes exert their actions mainly in the acute phase of the disease, just as previously described in post-stroke acute infections and burn patients. Our finding suggests neutrophils are important markers for stroke outcomes, as their predictive role was previously shown in patients with acute coronary syndrome. Both animal and human clinical data support the pivotal role of activated peripheral blood cells in neuroinflammation after ischemic stroke. The sustained detrimental effects of activated white blood cells in the systemic circulation carry a constant risk in patients with chronic inflammatory states (subclinical vascular diseases with endothelial dysfunction). Neutrophil activation (reflected by NAR) seems to be the most sensitive marker of recurrence of ischemic cerebral attacks in post-stoke patients on clopidogrel. Interestingly, the downward motion of neutrophil granulocytes during one-hour gravity sedimentation expressed by the negative value of NAR was observed in those patients who suffered composite vascular events during 36-month follow-up.

Numerous data highlight that many patients with cardiovascular disease have ex vivo high residual platelet reactivity despite compliantly taking an antiplatelet regimen. Although several studies showed an increase in the rate of recurrence of cerebral ischemia in patients presenting high residual platelet reactivity, the routine diagnostic for the state has been unsolved so far by neurologists. So, there is a need for more large, randomized controlled trials that account for potential confounders such as ischemic stroke subtypes, technical variations in the testing protocols, pharmacogenetic differences, patient behavior, and adherence to therapy. Risk stratification and individually tailored antiplatelet therapy based on platelet function testing may lower the rate of ischemia recurrence.

In our study, the low response to clopidogrel based on Multiplate® Analyzer from the whole blood could not predict recurrent stroke. However, a higher AUC (≥70 as a cut-off value) from the separated upper blood sample after one-hour gravity sedimentation emerged as a novel independent predictor of future stroke episodes. Our observation suggests that the upward motion of the platelets might be associated with increased thrombotic tendency due to increased platelet activity. When the combination of NAR and PFT_{upper} was used in the statistical model, the predicted probability of a recurrence of the future vascular event was even more accurate.

Wang et al. observed that pooled concentration of total microvesicles; endothelial-, platelet-, leukocyte-, and monocyte-derived microvesicles were significantly increased in post-

stroke patients compared to noncerebrovascular controls. Comparably with this recently published metaanalysis, we found that the total number of peripherally circulating microvesicles, endothelial-derived ($CD31^+$) and platelet-derived ($CD42a^+$) microvesicles were significantly higher in convalescent post-stroke patients compared to age-matched healthy controls. We presumed that the origin and number of circulating microvesicles might affect the response to clopidogrel in post-stroke patients. Although we did not observe any correlation between the platelet function test and the total number of microvesicles in the whole blood, we discovered a negative correlation between $AUC_{\text{whole blood}}$ and the total number of microvesicles in the lower blood sample after one-hour gravity sedimentation. Supporting our hypothesis, Kafian et al. already described elevated levels of platelet-derived microvesicles in patients acquiring high residual platelet reactivity during clopidogrel treatment, thus indicating ongoing platelet activation, despite the antiplatelet medication. In contrast, Rosinska et al. revealed no correlation between peripherally circulating microvesicles and platelet aggregation in post-stroke patients taking aspirin, suggesting that residual platelet reactivity is not affected by microvesicles in the presence of aspirin. Nevertheless, elevated concentrations of PAC-1^+/$CD61^+$, $CD62P^+$/$CD61^+$, and $CD31^+$/$CD61^+$ microvesicles were found in acute stroke patients indicating antiplatelet treatment failure. Moreover, in recent research, high levels of MVs with different origins were found predictive for estimating stroke severity and prognosis. We observed a negative correlation between the velocity of platelet aggregation and total MV count measured in the lower blood sample after one-hour gravity sedimentation, suggesting that this sample separation technique could be suitable for discrimination of clopidogrel responders from low-responders with residual platelet reactivity.

Another vital aspect is emerging evidence of platelets and PMVs and their crucial role in immune processes. Michelson et al. supported our results, who identified platelet-neutrophil complexes as markers for platelet activation. An increasing number of animal and human studies recognize that neutrophils and platelets together exhibit a diverse biological repertoire of function in thromboinflammatory conditions contributing to „immunothrombosis", which can occur in the venous or arterial system, such as stroke.

7. Limitations of the studies

When interpreting the study results, it is crucial to consider the potential limitations because of

- small sample size
- acute stroke patients were not investigated

- only focused primarily on recurrent coronary and cerebral ischemic episodes, which required somehow hospitalization, however small silent ischemic lesion recurrence on MRI was not detected
- variance in time elapsed between the index event and blood sampling
- only patients taking clopidogrel were investigated, but other antiplatelet agents would be worth screening in the future

ACUTE VASCULAR DISEASE: COVID-19 AND COAGULATION RESEARCH

8. Introduction

On the 1st of December 2019 in Wuhan of Hubel Province in the People's Republic of China, a highly contagious respiratory disease emerged caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), and several severe pneumonia cases of unknown cause were reported. On the 11th of march 2020, WHO declared the novel coronavirus outbreak (COVID-19) a global pandemic. Up to the latest data (end of 2022) more than 0.6 billion cases and > 6 million death happened due to the infection globally. Gladly, > 13 billion preventive vaccines were administered to people worldwide.

The incubation time of COVID-19 is 5-6 days up to 14 days; during this asymptomatic stage, the infected individuals can be contagious and transmit viruses to healthy people. The pathophysiology of the disease is presented in ***Figure 4.*** Diagnosis is made upon clinical, blood tests, and radiological findings. Infection is detected by real-time polymerase chain reaction (RT-PCR) from nasopharyngeal swabs or bronchoalveolar lavage and conserves portions of the SARS-CoV-2 genetic code identified in the amplified genetic material. Furthermore, the COVID-19 antigen presentation technique could also be helpful for a prompt diagnosis.

It is widely known, that the virus infects the pulmonary epithelial cells causing pneumonia, but extrapulmonary manifestation of the disease must not be forgotten. The most common extrapulmonary manifestations of the disease are summarized in ***Figure 5.***

Immunothrombotic dysregulation might explain the multiorgan failure and the systemic hypercoagulability in severely ill patients with SARS-CoV-2 infection. In summary, COVID-19 promotes a hypercoagulable state, termed COVID-19-associated hemostasis abnormalities (CAHA). CAHA has no consensus definition yet. ***Figure 6.*** presents a schematic view of the overlap-like syndromes of different coagulation abnormalities presented in COVID-19 patients.

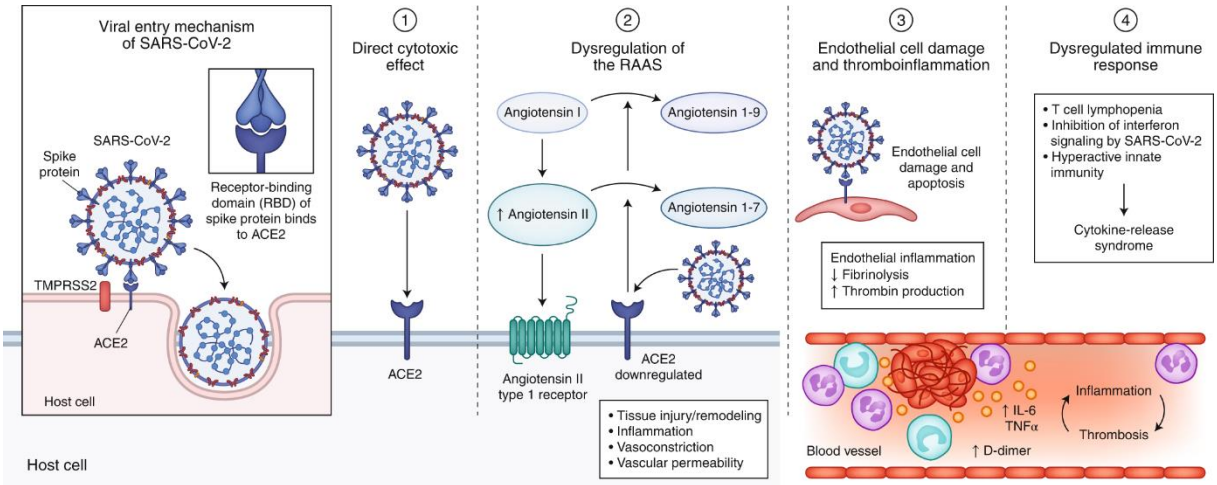

Figure 4. *Pathophysiology of COVID-19. (1) direct virus-mediated cell damage via ACE2 receptors on host cells (2) dysregulation of the renin-angiotensin-aldosterone system (RAAS) (3) endothelial damage and thromboinflammation (4) dysregulation of the immune response causing T cell lymphodepletion, and there is an increased production of proinflammatory cytokines, particularly IL-6 and TNFα. This image was initially published by Gupta et al. increased production of proinflammatory cytokines, particularly IL-6 and TNFα. This image was initially published by Gupta et al.*

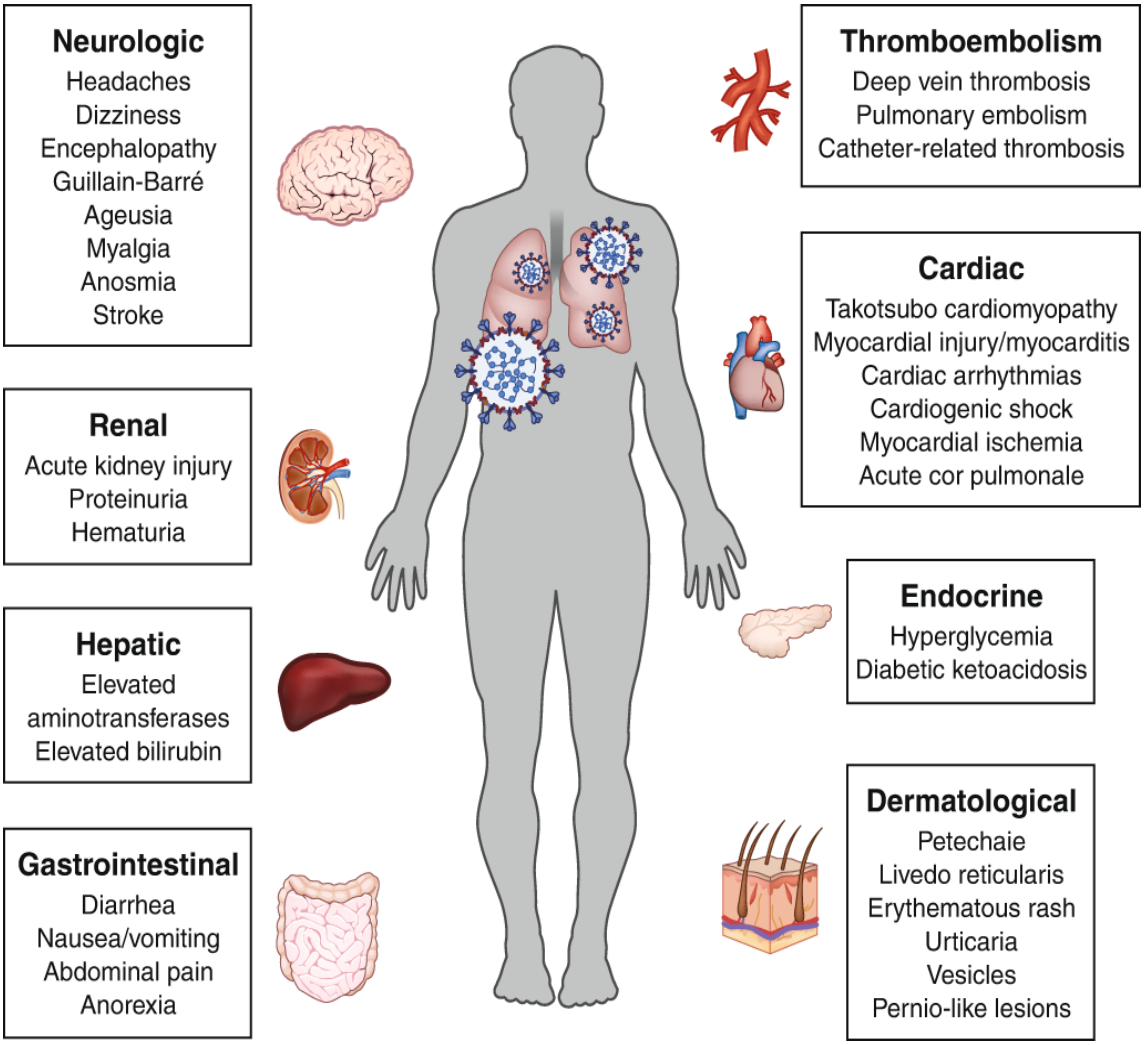

Figure 5. *Extrapulmonary manifestations of COVID-19. This image was initially published by Gupta et al.*

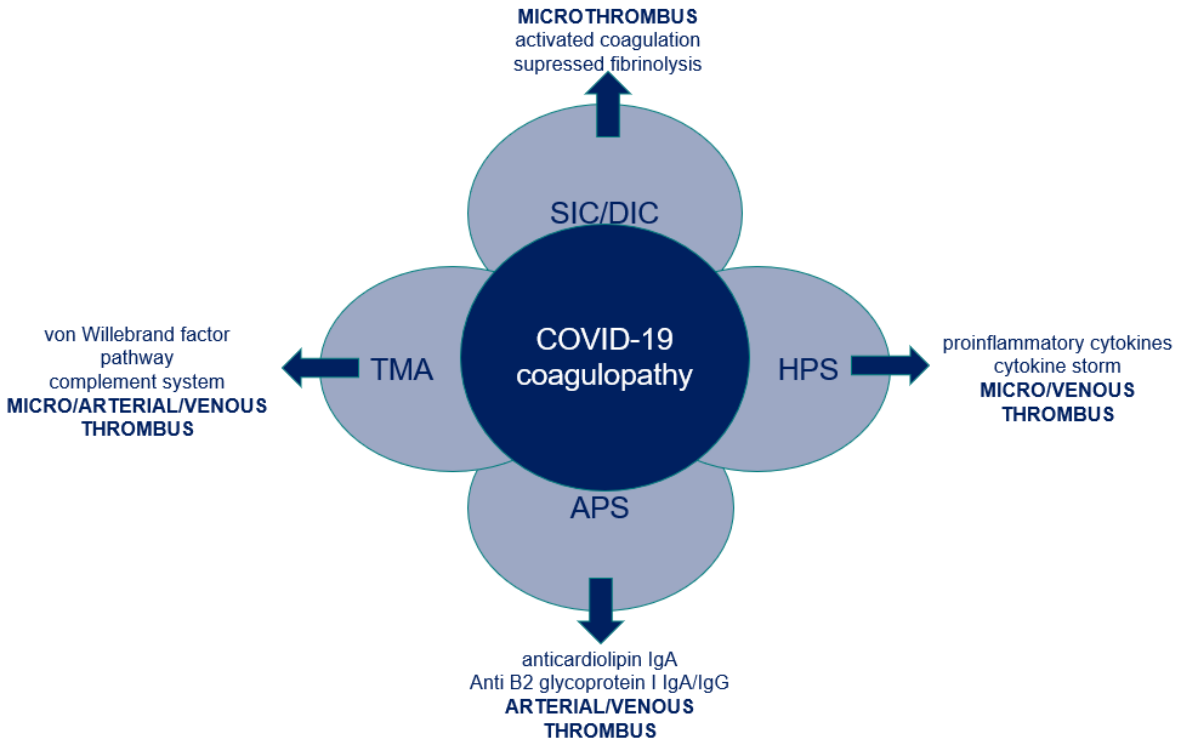

Figure 6. *COVID-19-associated coagulopathy. Special clinical features partially overlap with haemophagocytosis syndrome (HPS), antiphospholipid syndrome (APS), thrombotic microangiopathy (TMA), and sepsis-induces coagulopathy (SIC) / disseminated intravascular coagulation (DIC), however, it is not perfectly matched to any of the coagulopathies. The figure is made by D. Schrick. based on the unique characteristics of COVID-19 coagulopathy written by Toshiaki et al.*

9. Hypothesis and objectives

As written above, COVID-19 has several extrapulmonary manifestations and thromboembolic complications. Hemorhelogical and coagulation disturbances were the focus of our interest. The aims of our research were:

- to establish special features of hemostatic disturbances
- to identify the associations between alterations in conventional hemorheological and hemostasis parameters with mortality and thrombotic complications in severe COVID-19
- to reveal changes in platelet reactivity and microthrombi formation
- to detect altered fibrinolytic response contributing to the etiology of an increased thrombotic risk associated with COVID-19
- to explore personalized antithrombotic strategies

10. Methods

10.1. Subjects

The Hungarian Medical Research Council approved our prospective pilot study (ETT – TUKEB; 20783- 5/2020/EÜIG). The research was planned as multicentric, collaborative research with Semmelweis University, but our research unit in Pécs provided the data on which this thesis is based. All procedures were performed by the ethical guidelines of the 1975 Declaration of Helsinki. 21 patients with severe SARS-CoV-2 infection were retrospectively analyzed from a prospective database. Patients only with SARS-CoV-2 RT-PCR positivity, the requirement of O_2 (at least nasal-high flow oxygen therapy and/or need for respiratory support - noninvasive ventilation, or invasive ventilation with analgosedation and intubation - were enrolled into our research after signed informed consent (by the patient or relative). Patients were hospitalized at the ICU, Coronavirus Crisis Centre, Clinical Centre, University of Pécs, Pécs, Hungary. All the patients were on 100mg/day aspirin and got prophylactic anticoagulation with enoxaparin uniformly (1x/day, exact dosage based on their weight), based on our local therapeutic protocol. Patients who were enrolled in this study did not receive regular NSAIDs; only paracetamol was given occasionally (e.g., in case of fever), and basic analgosedation was conducted with propofol (±midazolam) and opioid (sufentanil uniformly). 21 age-matched, healthy, SARS-CoV-2 RT PCR negative healthcare workers served as controls.

10.2. Whole blood count, immature platelet fraction (IPF) measurement, eryhtrocyte sedimentation rate (ESR) and hs-CRP, ferritin, IL-6 measurements

The total blood cell count from the whole blood and absolute neutrophil count were measured on Sysmex XN 9000 integrated automated hematology analyzer. The platelet number was calculated on the fluorescent platelet channel of the analyzer. In this channel, the platelets are specifically stained intracellularly with a fluorescent dye and measured on the principle of flow cytometry, analysing the forward scattered light (FSC), side scatters light (SSC), and fluorescent light (SFL). Platelets are counted, and the plots with high fluorescence intensities are separated as the immature platelet fraction and the research parameter high immature platelet fraction (H-IPF). mnHigh sensitivity C-reactive protein (hs-CRP) was measured from patients' serum on Roche Cobas 6000 fully automated chemistry analyzer. For the measurement of ferritin and IL-6, we used Roche Elecsys® reagents on Cobas e801 fully automated analyzer.

Both tests use the electrochemiluminescence immunoassay (ECLIA) technique which allows the in vitro qualitative detection of analytes in the sample.

10.3. Measurement of hemostasis parameters

10.3.1. Routine and special hemostasis measurements

Activated partial thromboplastin time (aPTT), D-dimer, and fibrinogen were measured as part of the routine hemostasis parameters on ACL-TOP-750 analyzer, respectively. The special hemostasis tests were measured on an ACL-TOP-500 analyzer. The quantitative determination of von Willebrand factor antigen (vWF:Ag) and von Willebrand factor ristocetin cofactor activity (vWF:Rco) as essential markers of endothelial damage were performed with an automated gated enhanced immunoassay. For quantitative measurement of plasminogen (critical factor of fibrinolysis) and alpha$_2$-antiplasmin (an essential regulator of the fibrinolytic system), we used an automated chromogenic assay.

10.3.2. Platelet function tests

To monitor aspirin therapy, we performed a platelet function test from hirudin anticoagulated whole blood within one hour after collection on Multiplate® using ASPI test (which uses arachidonic acid as an activator of clotting). For other platelet function measurements, used ADP-test (ADP as an agonist), TRAP-test (thrombin receptor activating peptide-6 as an activator), and RISTO-test (ristocetin as an agonist). Given the lack of universal cut-off values, the normal aggregation range for ASPI-test was expected as AUC: 71-115U; AUC: 53-122U for ADP-test, AUC: 94-156U for TRAP-test and AUC*:* 90-201U for RISTO-test, respectively, according to the manufacturer (laboratory cut off value). However, previous studies suggest that patients were considered as „responders" with an AUC<40; and „low-responders" with an AUC≥40 to aspirin therapy.

10.3.3. Viscoelastic tests

Viscoelastometric testing was carried out by ClotPro®. It uses active pipette technology, which means the pipettes are prefilled with starting reagents and 340µl of citrated whole blood for initiating measurements. For measurement, it uses a stationary pin placed in a moving cup, from which the reduction of movement is detected and charted as the amplitude, resulting in thromboelastometry curves. We uniformly performed several tests as standard tests' in COVID-19 patients and control people. EX-test (tissue factor-activated assay with polybrene), IN-test

(ellagic acid-activated assay), FIB-test (tissue factor activated assay, without functional platelet), ECA-test (ecarin-based assay), and tPA-test (recombinant tPA within an extrinsic pathway-based assay) were performed in everyone, who were enrolled into this research. In each test, we recorded the following parameters which characterize the whole course of coagulation: clotting time (CT), clot formation time (CFT), α angle („amplitude of the clot" at a given time x (A(x)), maximum clot firmness (MCF), maximum lysis (ML) and lysis time (LT). The critically ill COVID-19 patients were divided into two groups based on their fibrinolytic response. A decreased fibrinolytic response was defined as LT >393s, determined upon previous literature.

10.4. Data collection and statistical analysis

Comorbidities, medication history, actual medication, full laboratory parameters, and requirement of organ replacement therapies were recorded. The incidence of vascular events (acute coronary syndrome, ischemic stroke, transient ischemic attack, pulmonary embolism, deep vein thrombosis) in the patient population was evaluated only during hospitalization (due to high mortality). Acute coronary syndrome (ACS) was defined by ACC/AHA guidelines (based on the clinical presentation of symptoms, ECG results, level of cardiac necroenzymes, and stress testing results). Each recurrent cerebrovascular ischemia was confirmed by neuroimaging (CT or MRI). All of the venous thromboembolisms were established upon clinical presentation of symptoms, laboratory parameters, and radiologically as well.

Data were statistically evaluated by IBM SPSS Statistics® 27.0. The chi-square test was used for categorical data to detect demographic and clinical factors. Comparisons of continuous non-normally distributed data between patients and the control group were conducted using the Mann–Whitney U-test. In contrast, patients and controls with or without ASA subgroups were tested using a one-way ANOVA. Kolmogorov–Smirnov test was applied to test for the normality of continuous variables distribution. Student's t-test was used to analyze normally distributed continuous data. Continuous variables are reported as median and interquartile range or mean and standard error of the mean (SEM). Correlation analysis was performed calculating Spearman's correlation coefficient (rho). Correlations between variables were analyzed with univariate and multivariate linear regression with corresponding beta values and 95% confidence intervals. Multivariable logistic regression was used to identify factors independently associated with decreased fibrinolytic response, defined as hypofibrinolysis. P-value <0.05 was considered statistically significant.

11. Results

21 COVID-19 patients (median age was 69 years, IQR: 52–71; male: 12) and 21 age-matched (67 years; IQR 63–69, male: 11) SARS-CoV-2 PCR negative healthy controls were enrolled into our prospective observational pilot study. All patients had positive SARS-CoV-2 PCR results, and they required intensive care with some level of oxygen support (with (NIV, IV via ETT) or without ($NHFO_2$) ventilatory support). Not surprisingly, significantly higher erythrocyte sedimentation rate, D-dimer level, von Willebrand factor antigen, and von Willebrand factor ristocetin cofactor activity were observed in patients on admission to the ICU. Serum levels of IL-6 and ferritin also exceeded the normal reference range.

Sadly, only three patients were discharged from the hospital alive, while a total of 18 patients died despite intensive care and organ support. H-IPF (%) showed a considerable difference when these two subgroups (survivor vs. non-survivor) were compared to each other (2.5, 1.0–4.2 vs. 0.5, 0.45–0.55; p = 0.011), presented on ***Figure 7.***

A significant negative correlation was seen between H-IPF (%) and plasma plasminogen (%) among non-survivors (r=-0.572, p=0.002), shown in ***Figure 8.***

Despite aspirin alone or combined with LMWH (enoxaparin uniformly), eight patients suffered symptomatic thromboembolic events during their ICU-stay. We divided patients into two subgroups based on their ex vivo platelet reactivity measured by Multiplate® analyzer. High-on aspirin platelet reactivity (HAPR) was found in eight COVID-19 patients using the cut-off for AUC >40 by the ASPI test. Then, patients were dichotomized based on their fibrinolytic response; the AUC measured by ASPI-, Risto-, TRAP-, and ADP-tests showed significant differences when ASA responders and low-responders were compared (all p=0.024, respectively). Data are presented in ***Figure 9.*** below.

Maximal clot firmness (MCF) was significantly higher measured by ECA-test (p=0.016) in patients with high-on aspirin platelet reactivity (n=8) compared to the responder subgroup (n=13), suggesting larger and more solid clots despite antiplatelet-therapy, presented in ***Figure 10.***

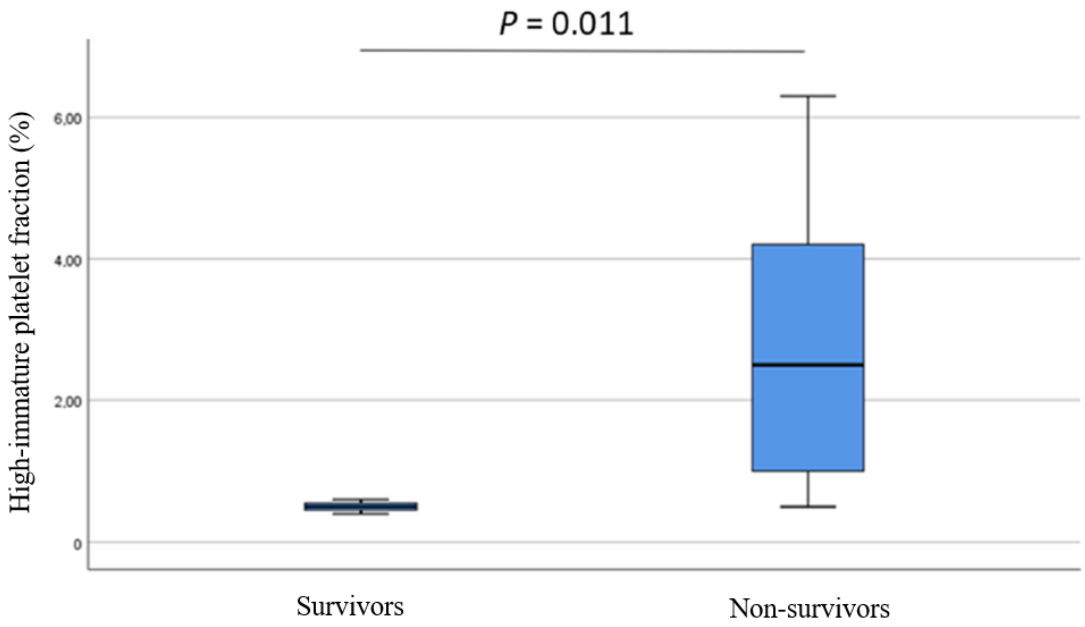

Figure 7*. High-immature platelet fraction (%) in survivors and non-survivors*

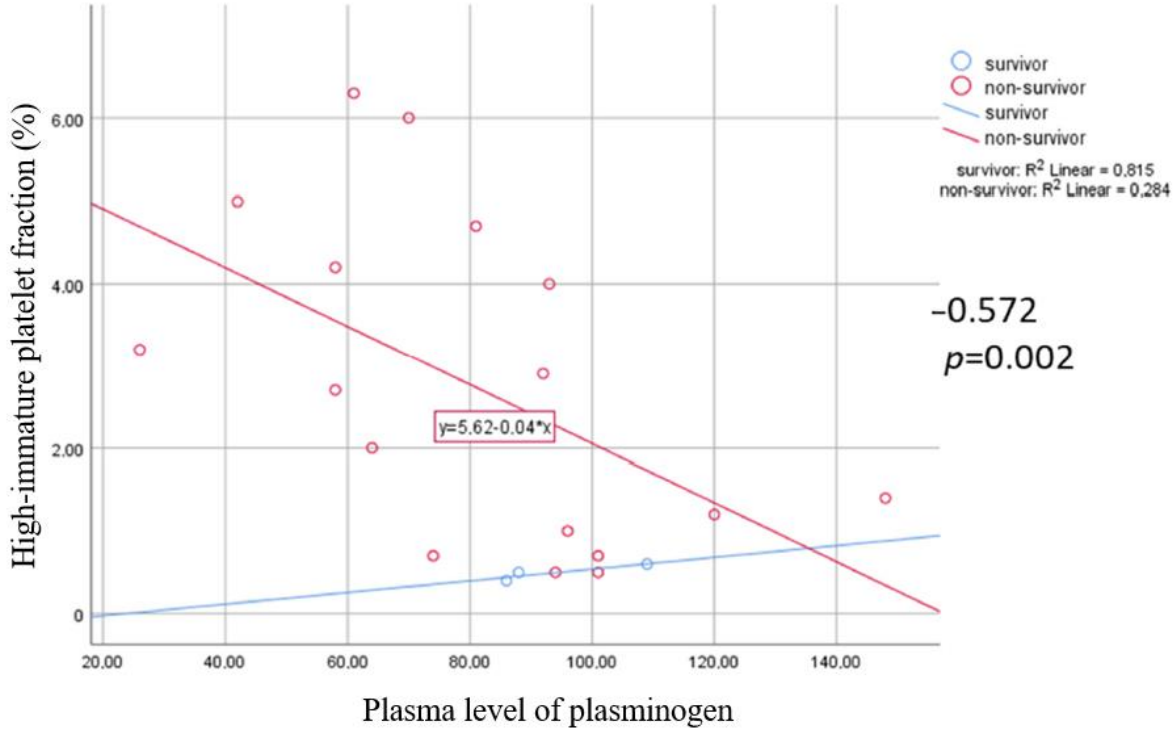

Figure 8. *Correlation of high-immature platelet fraction (H-IPF; %) and plasma plasminogen level in survivors (blue line) and non-survivors (red line). Rho and p indicate a negative correlation among the nonsurvival subgroup*

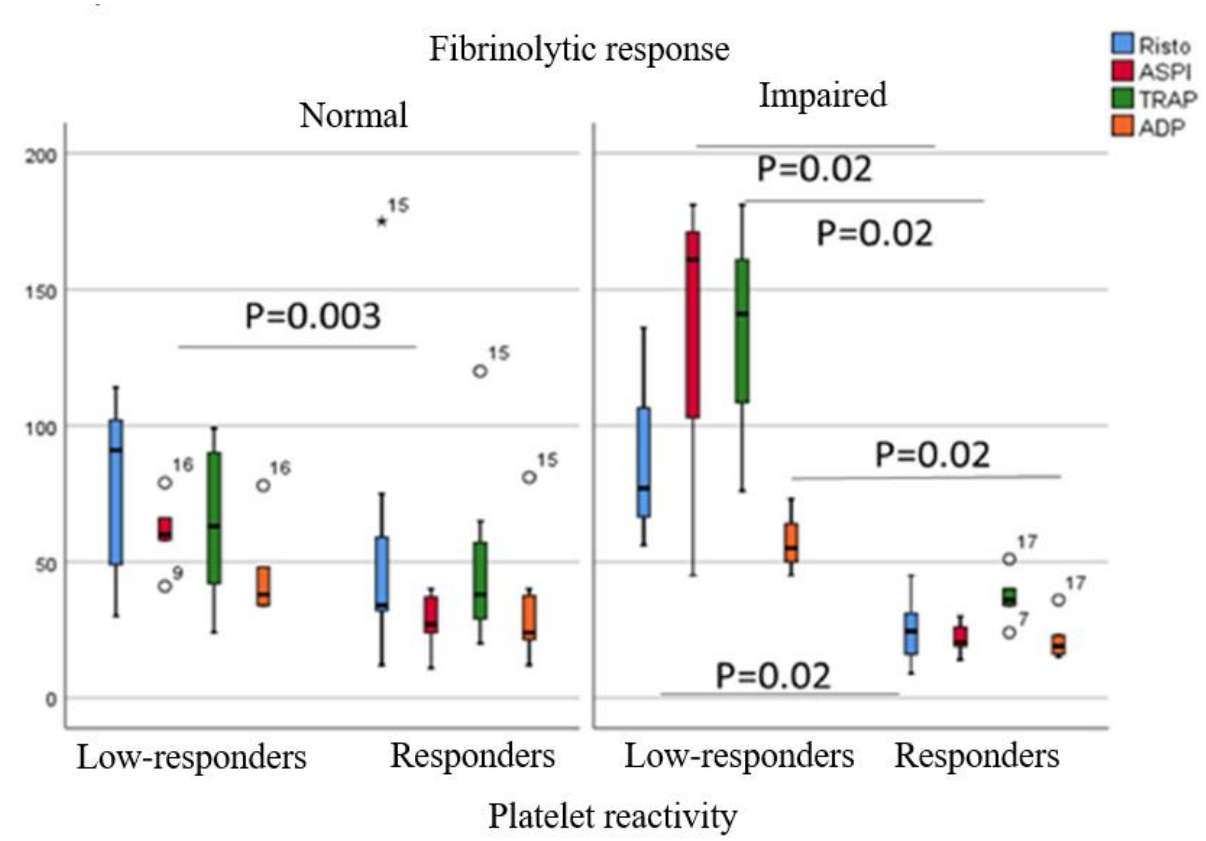

Figure 9. *Comparison of Risto-, ASPI-, TRAP, and ADP-tests (AUC) in patients with normal and impaired fibrinolytic response, dichotomized based on their responsiveness to aspirin (low-responders and responders). Performed by Mann-Whitney test (Asterix and white circles indicate extreme values)*

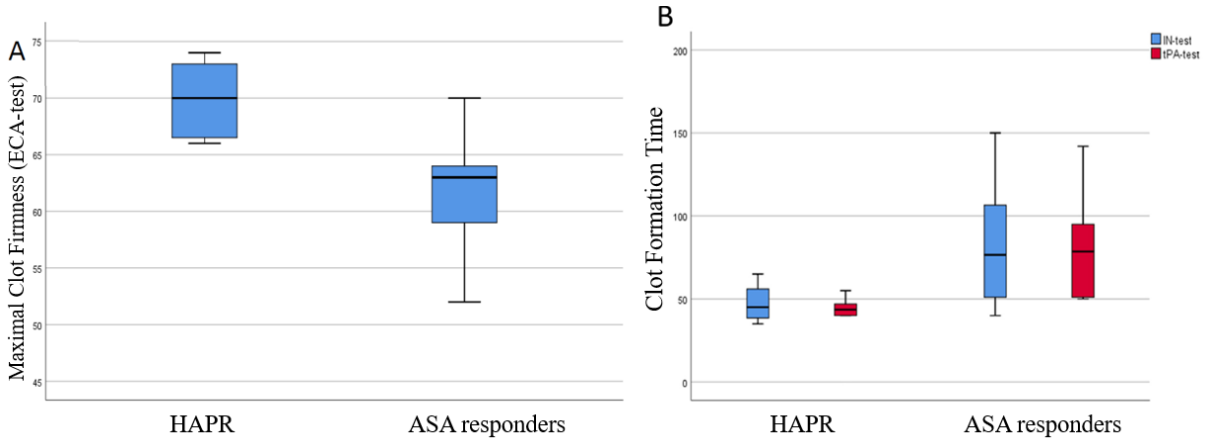

Figure 10. A*: Maximal clot firmness (MCF) measured by ECA-test in patients with high-on aspirin platelet reactivity (HAPR) and aspirin (ASA) responders (Mann-Whitney test, p=0.016);* ***B****: clot formation time (CFT) measured by IN- and tPA-test in patients with high-on aspirin platelet reactivity (HAPR) and aspirin (ASA) responders (Mann-Whitney test, p=0.039 and p<0.001, respectively)*

The ROC analysis of plasma fibrinogen level as a predictor of hypofibrinolysis revealed a cut-off value of 3.86 g/l (AUC of 0.800; 95% CI: 0.623-0.976; p=0.006) with the sensitivity of 78% and the specificity of 73% - presented in ***Figure 11.***

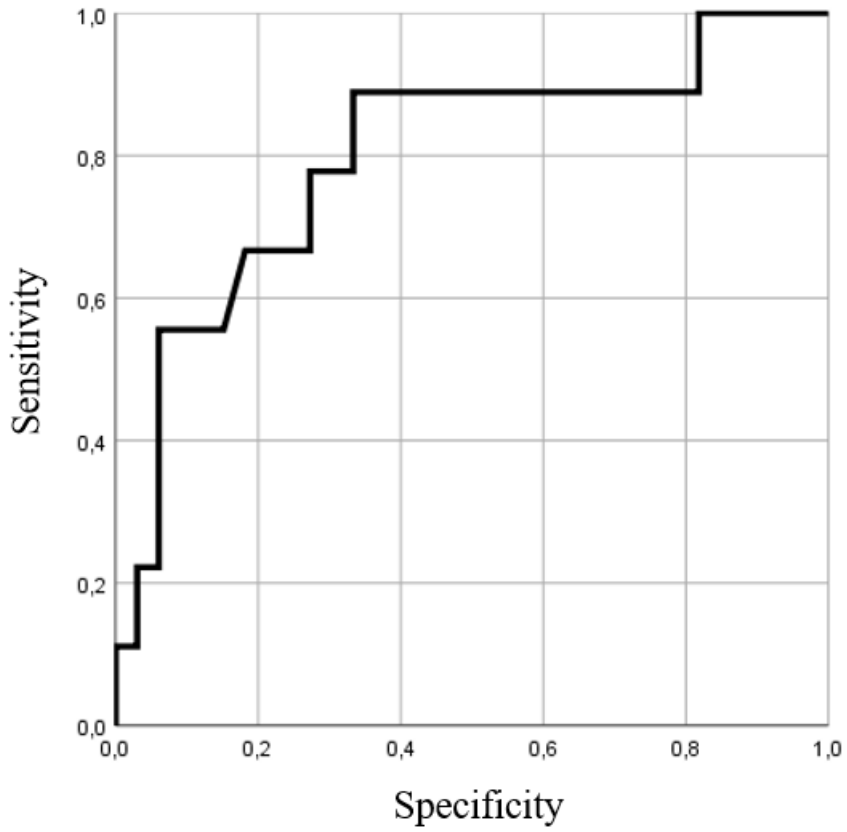

Figure 11. *Plasma fibrinogen level predict hypofibrinolysis with a sensitivity of 78% and a specificity of 73%*

12. Discussion

Regardless of the small number of enrolled patients, an elevated H-IPF (%) level was found to predict fatal outcomes in this research. Welder et al. found previously that a high percentage of IPF on admission to a hospital was predictive for the length of hospitalization, need for intensive care, and mechanical ventilation. Detecting the immature platelet fraction would be helpful for the optimal allocation of the available capacities. Importantly, elevated H-IPF was associated with lower plasminogen levels in cases of nonsurvival. Our finding was indirectly supported by Betolin et al., who disclosed increased plasminogen activator inhibitor (PAI-1) activity in COVID-19 patients, which may be due to the increased consumption of plasminogen in association with hypercoagulable state.

Collectively, endothelial dysfunction (represented by elevated vWF level), with the release of fibrinolysis inhibitor PAI-1, and young, active platelets (higher level of H-IPF) with

the dysregulated immune response (increased ferritin, interleukin-6, CRP, ESR) seem to be eloquent contributors to thrombogenesis in COVID-19.

Increased level of vWF – as we also observed in our small cohort – implies activated or damaged endothelium. It would be anticipated that damaged endothelium would result in the release of ultra-large vWF multimers, which are capable of interacting with platelets and leukocytes as well, leading to platelet activation, platelet-leukocyte aggregation formation, microthrombi production, and platelet consumption. Previous studies showed that patients with COVID-19 have significantly higher levels of vWF:Ag and vWF:Rco, likely contributing to an increased risk of thrombogenesis. Accordingly, we also found a positive correlation between H-IPF (%), vWF antigen, and activity among patients with high-on aspirin platelet reactivity.

Notably, in our cohort study, the aspirin low-responder patient subgroup presented with higher platelet count and increased platelet reactivity based on either ASPI-, ADP-, Risto- or TRAP test in the hypofibrinolytic (LT >393s, measured by ClotPro®) group of patients. Manne et al. explored that it can be due to increased P-selectin expression basally and upon activation, which may lead to faster and increased spreading of thrombus formation on both fibrinogen and collagen. The presumed concept of platelet activation and aggregation was supported by the results of Bachler et al. as well. To oppose, Bertolin et al. observed lower platelet reactivity based on Multiplate® aggregometry, compared to healthy controls, despite having higher levels of fibrinogen, fibrin degradation product, and plasminogen activator inhibitor-1 level and detected hypercoagulability by thromboelastometry. According to our results, eight patients on aspirin exhibited an increased platelet reactivity by the ASPI test. Significantly higher maximal clot firmness (MCF) was observed in the ECA-test; meanwhile, significantly lower IN-and tPA-CFT were found in low-responders compared to responders, indicating faster developing, more solid, and more massive clot formation despite antiplatelet treatment. A large randomized clinical trial (RECOVERY) proved that aspirin does not improve survival for hospital-treated COVID-19 patients. Therefore, there is an urgent need to identify the low- or non-responder patients to antiplatelet therapy, to provide a modified antiplatelet regime or alternative strategies (e.g., PAI-1 antagonists, tissue plasminogen activators, activated protein C, anti-P-selectin monoclonal antibodies) will be needed to combat with the thromboembolic complications of COVID-19.

Della-Morte et al. hypothesized plasminogen as the precursor of fibrinolysis. They supported our findings because they found that low levels of plasminogen strongly correlated with mortality. Kruse et al. noted the lower level of plasminogen, suggesting that it was integrated into the clot but unable to disintegrate effectively. It is due to the inhibitory effect of

alpha$_2$-antiplasmin, which provides thrombi resistant to plasmin; meanwhile, plasminogen activator inhibitor (PAI-1) might inhibit the activation of tissue plasminogen activator (tPA). The net effect of these mechanisms could result in the fibrinolysis shut-down phenomenon, leading to the lysis-resistant microthrombi formation in different organs (particularly in the lungs) and multi-organ dysfunction and failure. Notably, we aimed to explore predictors of impaired fibrinolytic response and found only fibrinogen with an OR of 3.55 as an independent predictor of hypofibrinolysis.

We detected reduced activated partial thromboplastin time (aPTT) in non-survivors, and a recently published Dutch study - evaluating ICU patients with COVID-19 – resulted in prolongation of the prothrombin time >3s and activated partial thromboplastin time >3 as independent predictors of thromboembolic complications. But in our cohort, the thromboembolic complications were only associated with reduced clotting time using the FIB-test.

D-dimer originates from the lysis of cross-linked fibrin (also known as fibrin degradation product, FDP) and indicates the activation of coagulation and fibrinolysis. Even though tPA lysis time (tPA LT) tended to be increased among aspirin low-/non-responders ($p=0.06$), the concentration of D-dimer did not show any difference between ASA responder and nonresponder subgroups. There was no significant difference in D-dimer level between survivors and nonsurvivors. However, larger studies reported D-dimer level as a strong predictor of mortality. For example, Zhang et al. declared that elevated D-dimer level is an independent factor of all-cause mortality among hospitalized patients with COVID-19. Furthermore, Corrado et al. found that nonsurvivors exhibited rapidly increasing D-dimer kinetics. Due to this cohort's meager survival rate, independent mortality predictors could not be analyzed here.

13. Limitation of the study

When interpreting the study results, it is crucial to consider the potential limitations because of

- small sample size
- our results must be confirmed on a larger sample size with different disease severity clusters
- sampling at multiple time points instead of a single sample collection would clarify more the kinetics and the variables in the outcome subgroups

- more rigid inclusion and exclusion criteria also limit the generalizability of this research.

14. Summary of novel findings

14.1. Novel biomarkers for recurrent cerebrovascular ischemic episodes

- neutrophil antisedimentation rate (NAR) independently predicted the recurrence of composite vascular events during 36-month follow-up in post-stroke patients taking clopidogrel as a prevention strategy
- platelet function test based on electric impedance aggregometry in the upper blood sample after one-hour gravity sedimentation revealed that the area under the curve value (AUC_{upper}) predicted recurrent ischemic stroke with high sensitivity and specificity during 36-month follow-up in post-stroke patients taking clopidogrel as a prevention strategy
- a more precise model was created when a ROC analysis was performed with the predicted probability of the combination of NAR and AUC_{upper}
- besides the total number of peripherally circulating microvesicles, endothelial-derived ($CD31^+$) and platelet-derived ($CD42a^+$) microvesicles were significantly higher in convalescent post-stroke patients compared to age-matched healthy controls
- the study observed a positive correlation between $PAC1^+$, and $CD42a^+$ PMVs, respectively, and the percentage of neutrophils in the lower blood sample after one-hour gravity sedimentation of the whole blood, indicating strong counterplay between the procoagulant potential of platelet-derived microvesicles and the thromboinflammatory cascade in the pathogenesis of recurrent stroke

14.2. Novel biomarkers for impaired fibrinolysis in severe COVID-19

- the authors found only fibrinogen with an OR of 3.55 as an independent predictor of impaired fibrinolytic response leading to thromboembolic complications in patients suffering from severe COVID-19

15. Scientometrics

Scientific papers (up to 31st of December 2022 based on MTMT)

Total: 6

English language papers: 4

Impact factors (up to 31st of December 2022 based on MTMT)

First author: 12.782

Cumulative: 14.523

List of publications

First author papers upon which this thesis relies

Schrick D, Ezer E, Tokes-Fuzesi M, Szapary L and Molnar T (2021) Novel Predictors of Future Vascular Events in Post-stroke Patients—A Pilot Study. *Front. Neurol.* 12:666994. doi: 10.3389/fneur.2021.666994 (Q1; IF: 5.581)

Schrick D, Molnár T, Tőkés-Füzesi M, Molnár A, Ezer E. Circulating Microvesicles in Convalescent Ischemic Stroke Patients: A Contributor to High-On-Treatment Residual Platelet Reactivity? *Front. Biosci. (Landmark Ed)* **2022**, 27(5), 158. https://doi.org/10.31083/j.fbl2705158 (Q2; IF (2021): 3.115)

Schrick D, Tőkés-Füzesi M, Réger B, Molnár T. Plasma Fibrinogen Independently Predicts Hypofibrinolysis in Severe COVID-19. Metabolites. 2021 Nov 30;11(12):826. doi: 10.3390/metabo11120826. PMID: 34940584; PMCID: PMC8708410. (Q2; IF: 5.581)

Other papers

Ezer E, **Schrick D**, Tőkés-Füzesi M, Szapary L, Bogar L, Molnar T. A novel approach of platelet function test for prediction of attenuated response to clopidogrel. Clin Hemorheol Microcirc. 2019;73(2):359-369. doi: 10.3233/CH-190580. PMID: 31156147; PMCID: PMC6971826 (Q2; IF: 1.741)