Hemorheological and hemostatic changes in acute and chronic vascular diseases

PhD Thesis

Doctoral School of Clinical Sciences

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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
ACTIVE-4a: Accelerating COVID-19 Therapeutic Interventions and Vaccines 4 Acute
ACS: acute coronary syndrome
ADAMTS13: A Disintegrin-like Metalloprotease domain with ThromboSpondin type 1 motif
ADP: adenosine-diphosphate
AF: atrial fibrillation
AHA: American Heart Association
ALAT: alanine-amino-transferase
AMP: adenosine-monophosphate
ANOVA: analysis of variance
APS: antiphospholipid syndrome
aPTT: activated partial thromboplastin time
ASA: acetylsalicylic acid
ASAT: aspartate-amino-transferase
ASPECTS: Alberta Stroke Program Early CT Score
ATTACC: Antithrombotic Therapy to Ameliorate Complications of COVID-19
AUC: area under the curve
BP: blood pressure
$CaCl_2$: calcium-chloride
CAHA: COVID-19-associated hemostatic abnormalities
cAMP: cyclic adenosine monophosphate
CAPRIE: Clopidogrel versus Aspirin in Patients at Risk in Ischemic Events Trial
CD: cluster of differentiation
CFT: clot formation time
CK: creatin-kinase
COVID-19: coronavirus disease-19
CT: computer tomography
CYP: Cytochrome P enzyme system
DALY: disability-adjusted life years
DAPT: dual antiplatelet therapy
DIC: disseminated intravascular coagulopathy
DOAC: direct-acting oral anticoagulants
DVT: deep vein thrombosis
e.g.: exempli gratia, meaning for example
ECA: ecarin-based assay
ECG: electrocardiography
ECLIA: electrochemiluminescence immunoassay
ECMO: extracorporeal membrane oxygenator

ED: emergency department
EDTA: ethylenediamine tetra-acetic acid
EESZT: Elektronikus Egészségügyi Szolgáltatási Tér
ELSO: Extracorporeal Life Support Organization
ESR: erythrocyte sedimentation rate
ESUS: embolic stroke of an undetermined source
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)
GBD: Global Durden of Death
GDP: gross domestic product
GGO: ground glass opacity
GP: glycoprotein
HbA1c: amount (%) of blood glucose attached to hemoglobin
HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Hgb: hemoglobin
H-IPF: high-immature platelet fraction
HMGB1: high-mobility group box 1
HPS: hemophagocytosis syndrome
HR: hazard ratio
HRCT: high-resolution CT
HRPR: high-residual platelet reactivity
hs-CRP: (high-sensitivity) C-reactive protein
HTPR: high on treatment residual platelet reactivity
IBM: International Business Machines
ICU: intensive care unit
IL: interleukin
INR: international normalized ratio
IPF: immature platelet fraction
IQR: interquartile ratio
IST: International Stroke Trial
IV: intravenous, invasive ventilation
KCl: potassium-chloride
l: liter
LAR: leukocyte antisedimentation rate
LCR: larce cell ratio
LDH: lactate-dehydrogenase

LDL-C: low-density lipoprotein concentration
LE: level of evidence
LMWH: low-molecular-weight heparin
LT: lysis time
LTD: limited liability company
M: mole
MATCH: Management of Atherothrombosis with Clopidogrel in High-Risk Patients Trial
MCF: maximal clot firmness
ml: milliliter
mPFT: modified platelet function test
MPO: myeloperoxidase
MPV: mean platelet volume
MRI: magnetic resonance imaging
mRS: modified Rankin Scale
MV: mechanical ventilation
MV: microvesicles
NAAT: nucleic acid amplification test
NaCl: sodium-chloride
NAR: neutrophil antisedimentation rate
NET: neutrophil extracellular traps
NHFO2: nasal high-flow oxygen therapy
NICE: National Institute for Health and Care Excellence
NIH: National Insitute of Health
NIHSSS: National Institutes of Health Stroke Scale Score
NIV: noninvasive ventilation
NLR: neutrophil-to-lymphocyte ratio
NSAID: nonsteroidal anti-inflammatory drug
NY: New York
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

POINT: Platelet-Oriented Inhibition in New TIA and Minor Ischemic Stroke Trial

RECOVERY: Randomised Evaluation of COVID-19 Therapy

REMAP-CAP: Randomized Embedded Multifactorial Adaptive Platform for Community-acquired Pneumonia

RNA: ribonucleic acid

ROC: receiver operating characteristic

ROTEM: rotational thromboelastometry

rtPA: recombinant tissue plasminogen activator

RT-PCR: (real-time) polymerase chain reaction

SAPT: solo antiplatelet therapy

SARS-CoV-2: severe acute respiratory syndrome – coronavirus 2

SD: standard deviation

SEM: standard error of meaning

SFL: fluorescent light

SIC: sepsis-induced coagulopathy

SOCRATES: Acute Stroke or Transient Ischemic Attack Treated with Aspirin or Ticagrelor and Patient Outcomes Trial

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

TP-R: thromboxane receptor

TRAP: thrombin receptor activating peptide-6

TT: thrombin time

TXA2: thromboxane A2

UF: unfractionated heparin

USA: United States of America

VKA: vitamin K antagonists

VTE: venous thromboembolism

vWF: vonWillebrand factor

vWF:Ag: vonWillebrand factor antigen

vWF:Rco: vonWillebrand factor ristocetin cofactor activity

WBC: white blood cell

YKL-40: mammalian chitinase matrix protein of specific granules in human neutrophils

µl: microliter

1. Prologue

1.1. Biomarker research

Nowadays, biomarkers are extensively used for diagnosis, prognosis, and treatment follow-up in patients with cardio- and cerebrovascular diseases[1]. Biomarkers are circulating molecules that provide insight into pathophysiological processes and aid in establishing, refining the prognosis, and guiding the treatment. Diagnostic biomarkers are applied to detect or confirm the presence of a disease of interest.

Diagnostic biomarkers are used as eligibility criteria for enrolment into clinical trials studying medical conditions. Prognostic biomarkers determine the likelihood of a clinical event, disease recurrence, or progression. These are especially important to detect future adverse clinical events. Susceptibility or risk biomarkers indicate a potential for developing a medical condition before clinical symptoms appear[2]. These biomarkers are applied to guide preventive strategies.

An ideal biomarker possesses high sensitivity, allowing 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 can help better understand underlying pathophysiological processes in special medical conditions, which can lead to new therapeutic perspectives potentially improving the outcomes[3].

1.2. Basic terms to describe diagnostic or prognostic tools

Sensitivity is the ability of a test to identify an individual with a specific condition. Sensitivity = true positives / (true positives + false negatives) x 100. The closer the value to 100%, the more sensitive the test. High-sensitivity tests are useful for screening purposes. Specificity is the proportion of individuals without the disease correctly classified as negatives. Specificity = true negatives / (true negatives + false positives) x 100. The higher and closer to 100% the specificity value, the lower the probability of a false positive screening result.

1.3. Concept of this thesis

My thesis is divided into two main topics: chronic vascular disease research (in post-stroke population) and acute viral disease (COVID-19) with vascular complications.

Acute and chronic thromboembolic diseases were my main interests during medical school, so I started my research work focusing on acute coronary syndrome at the Department of Interventional Cardiology. Later I joined the research group on stroke at the Department of Neurointensive Care.

I was enthusiastic about this topic because stroke is the leading cause of disability worldwide, and it significantly impacts a person's and family's quality of life, with significant economic implications. The emergence of stroke will increase worldwide, and successful recanalization strategies provide a chance for a better outcome for the victims. Such patients require an individual approach to secondary stroke prevention. However, this is not fully understood or even solved so far.

The COVID-19 pandemic put the scientific world into bloom, though it was challenging due to its novelty and many losses. I worked since the beginning at the COVID-19 intensive care unit, and I was involved in the new treatment strategies, which forced me to have a better insight into the pathophysiology of the disease. Many thromboembolic complications occurred during my work, so we decided to research COVID-19 complications, especially thromboembolisms.

2.
CHRONIC VASCULAR DISEASE: POST-STROKE HEMORHEOLOGICAL AND HEMOSTATIC RESEARCH

2.1. Introduction to stroke

A stroke (also called „brain attack") occurs when the blood supply is blocked to a part of the brain or a vessel burst in the brain; in either case, parts of the nerve tissue become damaged or die. ***Figure 1.*** illustrates the representation of ischemic stroke subtypes. A stroke is a medical emergency requiring prompt diagnosis and treatment to reduce brain damage. Diagnosis is upon clinical symptoms and radiological imaging. Red flag symptoms for stroke are sudden paraesthesia and paresis in the face, arm, or leg, especially if it is unilateral. Furthermore, confusion, aphasia, dizziness, loss of balance, lack of coordination, and sudden severe headache is often associated with acute illness. Usually, head (and neck) CT angiography is performed or MR angiography. The primary aim of the treatment is to restore cerebral blood flow as soon as possible to improve or resolve neurological deficits[4,5]. Acute treatment involves intravenous thrombolysis (within 4.5 hours of onset with the administration of tissue plasminogen activator drug (tPA); usually, alteplase is used). Patients can be candidates for mechanical thrombectomy (up to 24 hours after onset). The decision between therapies is established upon pre-stroke modified Rankin Scale (mRS)[6], the onset of symptoms, location of the occlusion, National Institutes of Health Stroke Scale Score (NIHSSS)[7] and Alberta Stroke Program Early CT Score (ASPECTS)[8].

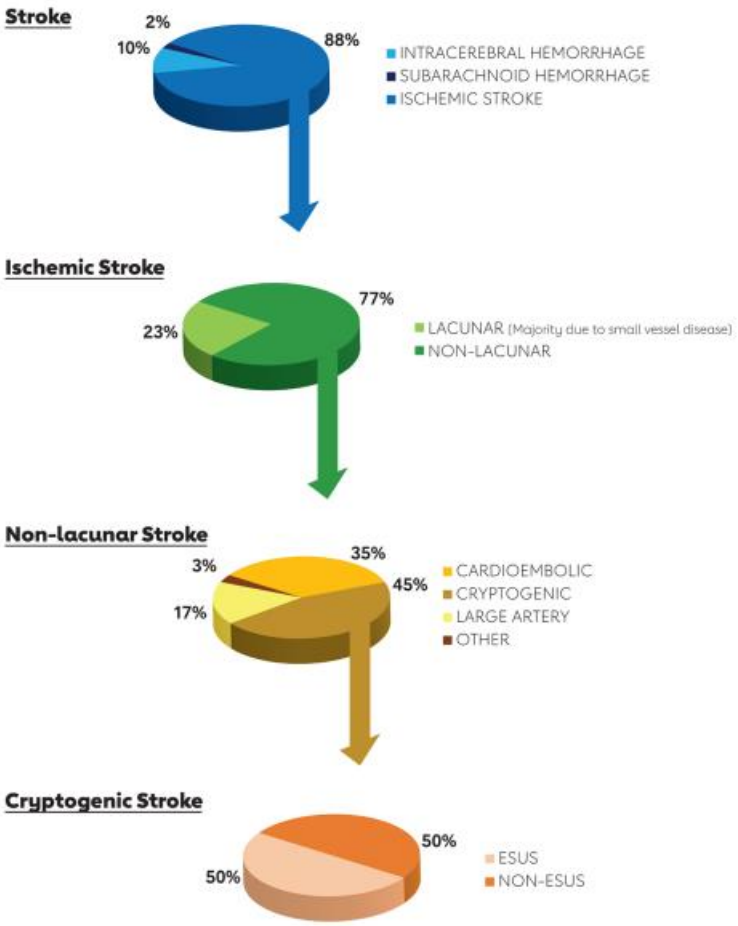

Figure 1. *Representation of ischemic stroke subtypes according to Kolominsky-Rabas et al.*[9] *and Gardener et al.*[10] *Definitions are from the TOAST (Trial of Org 10172 in Acute Stroke Treatment) classification scheme. ESUS means an embolic stroke of an undetermined source*

2.1.1. Epidemiology of stroke

From 1990 to 2019, absolute stroke incidents increased by 70%, the prevalence of stroke increased by 85%, death from stroke increased by 43%, and disability-adjusted life years (DALYs) due to stroke increased by 32%[11]. Up to the recent stroke statistics for the year 2022[12], 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. The most recent Global Burden of Death (GBD) in 2019, stroke burden showed as the leading cause of disability and the second cause of death and disability-adjusted life years lost worldwide.

2.1.2. Short and long-term complications and their importance

Due to the data mentioned above, there is an urgent need to reduce stroke incidence and improve outcomes in post-stroke patients at all geographical and stakeholder levels because stroke can lead to short- and long-term cognitive and physical impairments, even with extensive treatment, causing loss of independence and forces the need for long-term care. The estimated global cost of stroke is 1.12% of the worldwide GDP[13].

2.1.3. Secondary prevention of stroke

The specific recommendations for prevention strategies depend on each stroke subtype. When possible, the most important is to define the etiology and identify the treatment targets to reduce the recurrent ischemic episodes. It is the best achieved with individually tailored therapy. Lifestyle factors – including a healthy Mediterranean-style diet (with the level of evidence (LE) 2A[14]), regular activity (LE 1A[15]), and changing patient behavior - are essential for recurrent stroke risk reduction. Management of vascular risk factors remains critical. 50% of stroke patients suffer from hypertension, and normalizing blood pressure (goal BP <130/80Hgmm) helps lower the risk of recurrent stroke (LE 1A)[16,17]. Lipid-lowering therapy with statin and ezetimibe may be needed to achieve a goal of LDL-C ≤70mg/dl to reduce the risk of major cardiovascular events (LE 1A)[18]. Many patients with diabetes suffer a stroke as a microvascular complication of the disease. Meanwhile, approximately 20% of patients with stroke have undiagnosed diabetes. The goal for glycemic control is HbA1C ≤7%[19]. Antithrombotic therapy (using antiplatelet or anticoagulant agents) is recommended for nearly all patients if there is no contraindication. With very few exceptions (for example, atrial fibrillation), the combination of antithrombotic and anticoagulant therapy is not indicated. Dual antiplatelet therapy is neither recommended for long-term usage. ECG is a simple, noninvasive method of diagnosing atrial fibrillation (AF) in patients with stroke. A meta-analysis in 2014 found that the proportion of patients diagnosed with poststroke AF in the emergency department (ED) was 7.7%[20]. If AF is established, then anticoagulation is required, preferably with direct-acting oral anticoagulants (DOACs, anti-Xa agonists, direct thrombin inhibitors) or vitamin K antagonists (VKAs); the international normalized ratio (INR) goal for VKAs is 2.0-3.0[21].

To reduce the burden of recurrent ischemic stroke episodes, antiplatelet therapy is a critical component of secondary prevention (LE 1A)[22,23]. The most commonly discussed antiplatelets in practice guidelines are aspirin, aspirin-dipyridamole, clopidogrel, and ticagrelor.

Antiplatelet therapy should be started as soon as possible after brain imaging excludes hemorrhage within 24 hours of symptoms onset (LE 1B)[24]. For long-term secondary prevention, either aspirin (80-325mg/day, LE 1A) or clopidogrel (75mg/day, LE 1A), or combined aspirin-dipyridamole (25/200mg, LE 1B) seems to be the appropriate treatment; selection should be based on unique patient factors and clinical circumstances (LE 1A)[22,25].

2.1.4. Platelets and their roles in stroke

Thrombosis is due to a rupture of an atherosclerotic plaque or an embolism. 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[26]. Many studies suggested that platelets can be excessively activated in the acute convalescent phase of cerebral ischemia[27].

2.1.5. Antiplatelet drugs

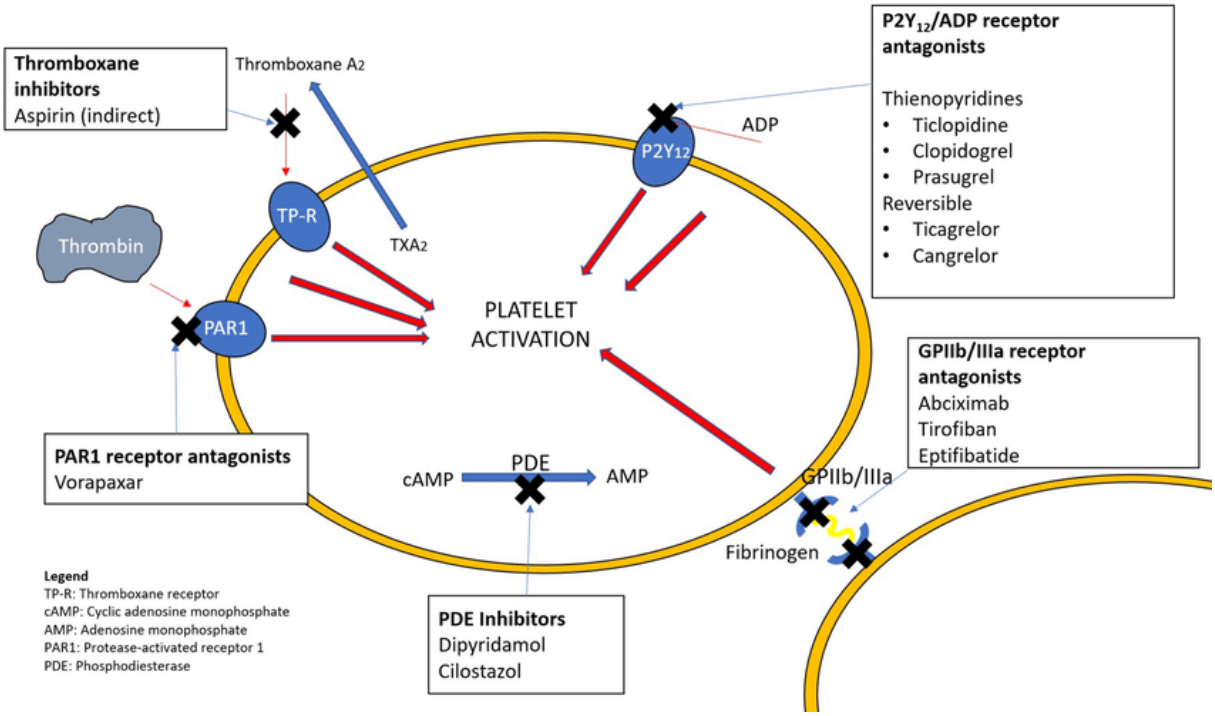

Figure 2. *Target receptors and mechanism of action of different antiplatelet agents*[28]

Mainly used antiplatelet drugs, their target receptors, and their mechanism of action are summarized and visualized in ***Figure 2.***

Aspirin or acetylsalicylic acid (ASA) irreversibly inactivates platelet cyclooxygenase, thus blocking thromboxane and prostaglandin synthesis. Thromboxane A_2 (TXA_2) is a potent

platelet activator; hence by blocking it, ASA can achieve the antiplatelet effect. International Stroke Trial (IST)[29] indicates a substantial benefit for acute initiation of aspirin after ischemic stroke to reduce the risk of further stroke. The optimal dose was established in the second Antithrombotic Trialists' Collaboration overview; it suggests that 75mg to 150mg/day is the optimal dose range to achieve the antithrombotic effect with minimized gastrotoxicity[30]. Gastrointestinal hemorrhage could be prevented by promptly diagnosing and treating Helicobacter pylori infection and proton pump inhibition.

Aspirin-dipyridamole is a platelet aggregation inhibitor agent that inhibits the platelet cAMP-phosphodiesterase, blocks adenosine reuptake from vascular and blood cells, and potentiates the effect of the antiaggregatory activity of prostaglandin I_2 (PGI_2). It has lower compliance (due to a twice-daily dosing regimen) and may cause headaches[31].

Clopidogrel is a thienopyridine compound whose active metabolite selectively and irreversibly inhibits the P_2Y_{12} receptor on platelets. Subsequently, via ADP mediated glycoprotein (GP)IIb/IIIa complex, inhibiting platelet aggregation. It was first tested in patients with cerebrovascular disease in Clopidogrel Versus Aspirin in Patients at Risk in Ischemic Events (CAPRIE) trial. They observed a relative risk reduction of 7.3% in post-stroke patients, favoring clopidogrel, which was nonsignificant (−5.7% to 18.7%; p=0.26)[32]. Aspirin and clopidogrel compared with clopidogrel alone after recent ischaemic stroke or transient ischaemic attack in high-risk patients (MATCH): randomized, double-blind, placebo-controlled trial[33]. There was no evidence of significant benefit for the combination of clopidogrel and aspirin, compared to the clopidogrel alone group (relative risk reduction, 6.4%; 95% CI, −4.6% to 16.3%; p=0.244), meanwhile primary intracranial bleeding and gastrointestinal bleeding occurred more frequently with dual antiplatelet therapy (DAPT). The Platelet-Oriented Inhibition in New TIA and Minor Ischemic Stroke (POINT) trial estimated that for every 1000 patients treated with DAPT for 90 days after the primary ischemic event, treatment would prevent fifteen ischemic strokes and cause five major hemorrhages[34].

Ticagrelor is a potent reversible P_2Y_{12} receptor antagonist. SOCRATES trial declared that ticagrelor was substantially more efficacious in large artery disease, compared to aspirin[35].

2.1.6. High residual platelet reactivity

The definition of drug resistance/nonresponsiveness to an antiplatelet agent is the failure of the drug to inhibit its target of action[36]. It should be based on laboratory techniques that can

quantify the activity of the target receptor before and after the administration of the drug. High residual platelet reactivity (HRPR) is defined as the high level of platelet reactivity present after receiving a loading dose of an antiplatelet agent. In most prospective studies, clopidogrel has been most extensively studied (due to its extensive metabolism, genetic polymorphism and loss of function mutation of CYP_2C_{19} system, drug-drug interactions at the level of CYP_3A_4 enzyme acivity[37,38]). Several tests assessing ex vivo platelet reactivity were used to identify high on-treatment platelet reactivity (HTPR); prevalence could vary depending on the definitions and assays used. Bonello et al. in 2010 defined consensus values for HTPR based on prior studies for the most widely used platelet function assays. The HTPR value - determined by ROC analyses - is >468 arbitrary aggregation units/min in response to ADP measured by Multiplate® Analyzer[36]. There is an independent correlation between HRPR and recurrent ischemia; the higher the residual platelet reactivity, the higher risk of cardio-cerebrovascular adverse events[39–41].

2.1.7. Prognostic tools for recurrent stroke

The highest risk for recurrent ischemic episodes is within 90 days after TIA or stroke. Prediction of recurrent stroke would be clinically useful. C-reactive protein (CRP) is the most predictive in post-stroke patients for a further vascular attack[42]. Segal et al. declared age/sex-adjusted HR=1.16, 1.01-1.34, p=0.042; fully-adjusted HR =1.21,1.02-1.43, p=0.03 for CRP predicting recurrent stroke[43]. Fibrinogen and D-dimer have been shown to predict long-term risk[44].

2.1.8. Novel biomarkers for recurrent ischemic episodes

Monitoring the effectiveness of medical treatment is essential to provide sufficient prevention[45]. It is known that there is heterogeneity in individual patient responses to antiplatelet therapy, and high residual platelet reactivity is associated with an increased risk for adverse events. Furthermore, genetic testing may demonstrate the genetic polymorphism of several cytochrome enzymes (especially CYP_2C_{19})[46]. The contribution of inflammatory markers in estimating prognosis after stroke is still undefined. The neutrophil-to-lymphocyte ratio (NLR) is emerging as an easily accessible, easily reproducible, and objective indicator for systemic inflammatory status; this ratio reflects the balance between neutrophils and lymphocytes in peripheral blood. It has been reported to be associated with coronary artery disease, peripheral arterial occlusive disease, and ischemic stroke as well[47–49]. In a recent cohort

study, IL-6 and YKL-40 proteins were independently associated with recurrent stroke and poor functional outcome[50].

2.2. Hypothesis and objectives

2.2.1. Antisedimentation of different cellular blood 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[51]. Later it was established that leukocyte antisedimentation rate could help in the early recognition of post-stroke infection[52] and postoperative complications[53]. 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[54]. Activation of neutrophil granulocytes is reflected by the neutrophil antisedimentation rate (NAR).

2.2.2. Platelet-associated 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[55]. 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), CD42b (GPIbβ), CD61(GPIIIa), CD62P (P-selectin)[56]. 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; it can also bind to another ligand such as thrombin, P-selectin, integrin $\alpha M\beta_2$, factor XI, and factor XII[57]. 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[58]. A significant increase in circulating PMVs was observed in patients with cardiovascular diseases[59]. Therefore PMVs might be essential biomarkers for identifying various recurrent cardio- and cerebrovascular diseases[60].

The aims of our research:

- to observe peripheral blood cell characteristics as predictors of recurrent ischemic episodes
- to find contributing factors to the outcome of post-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 patients 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

2.3. Methods and materials

2.3.1. Study design

We planned 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.

2.3.2. Subjects

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 (due to high material cost and time-consuming measurement). 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.

2.3.3. Exclusion criteria

Patients with acute infection and acute vascular events - such as stroke, transient ischemic attack, and acute coronary syndrome - cannot take place in our study. Further exclusion criteria were thrombocytopenia (<150G/l), congenital platelet abnormalities (e.g., Bernard-Soulier syndrome, Glanzmann thrombasthenia, platelet storage pool disorders, von Willebrand disease), congenital disorders of hemostasis (e.g., hemophilia A, B), anemia (Hgb <100g/l) and patients on regular medical therapy that interfere with coagulation (e.g., vitamin K antagonists, direct-acting oral anticoagulants, nonsteroidal anti-inflammatory drugs).

2.3.4. Sample collection and processing for platelet function test

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 with 3.2% (0.109M) Na_3-citrate, K_3-EDTA (Beckton Dickinson,

Diagon Ltd., Hungary) and hirudin (Starstedt S-Monovette®, containing 1.6ml hirudin) as an anticoagulant. Upon blood sampling, the first 3ml of blood was discarded. Blood samples were transported immediately for laboratory measurements and were processed within one hour.

2.3.5. Platelet and neutrophil antisedimentation rate

A modified whole blood gravity sedimentation technique was developed for studying platelet and neutrophil sedimentation properties[61]. 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 (schematic view of the process in ***Figure 3.***).

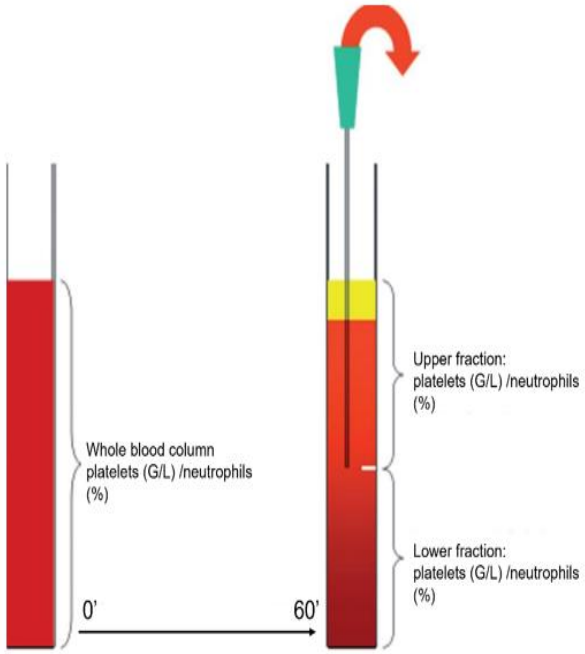

Figure 3. *Sample preparation, separation of the upper and lower blood sample fractions after one-hour gravity sedimentation. The figure is designed by D. Schrick*

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 a Sysmex XN 9000 integrated hematology analyzer (Sysmex Co., Kobe, Japan, 2017). Next, the platelet antisedimentation rate (PAR; %), leukocyte antisedimentation rate (LAR; %), and neutrophil antisedimantation rate (NAR; %) were – respectively – calculated based on this equation:

$$\frac{\text{cell count in upper fraction} - \text{cell count in lower fraction}}{\text{cell count in upper fraction} + \text{cell count in lower fraction}} \text{ x } 100$$

2.3.6. Electric impedance aggregometry (Multiplate® analyzer)

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 (Roche Diagnostics, Mannheim, Germany). Platelet aggregometry was uniformly carried out sixty minutes after blood sampling using adenosine-diphosphate (ADP; 6.5 M) as an agonist. As a novelty, not only whole blood, but after one-hour gravity sedimentation, the separated upper and lower half blood samples were simultaneously analyzed in each convalescent stroke patient taking clopidogrel. 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 – according to the manufacturer[62]. 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.

2.3.7. Sample preparation for microvesicles (MVs) analysis

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. Detailed MV measurement was described in a previously published article by our research team. Briefly, the samples for measurement were thawed on melting ice and pelleted at 18000×g for 10 minutes. The supernatant was carefully removed, leaving 25 µl of microvesicle-rich plasma at the bottom of the Eppendorf tube. MVs were suspended with gentle vortexing for 20 seconds in 1.0 ml Apo-binding buffer solution (10mmol/l HEPES, 5mmol/l KCl, 1mmol/l $MgCl_2$, 136mmol/l NaCl, pH=7.4; HEPES was obtained from Sigma-Aldrich Ltd., Budapest, Hungary) without $CaCl_2$. 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.

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	Recognize 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*

For labeling, 10µl MV in Ca^{2+} free buffer was incubated in 100µl Apo-binding buffer supplemented with 2.5mmol/l $CaCl_2$ with a total of 10 µl antibody, previously diluted to optimal labeling concentration. Staining was incubated for 30 minutes at room temperature in a dark chamber. All buffers were filtered through 0.2 µm membrane filters.

2.3.8. Microvesicles (MVs) measurement

Flow cytometric measurements and data analysis were performed on a Beckman-Coulter FC-511 cytometer with CXP software (version 2.3 Beckman Coulter Life Sciences, Indianapolis, USA). The MV's reference gate was defined with Megamix beads (Biocytex). 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[63]. 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 (Becton Dickinson) were used to determine the optimal labeling concentrations. All antibodies and annexin V were titrated. Labeling concentrations were defined by antibody staining of samples and sample-free buffers in the presence or absence of $CaCl_2$. Labeling was optimal if $CaCl_2$ labeled sample

measurement events were distinguishable from background, $CaCl_2$ free staining, and isotype controls.

The summarizing flowchart of sample preparation and further analytical processes are shown in ***Figure 4***.

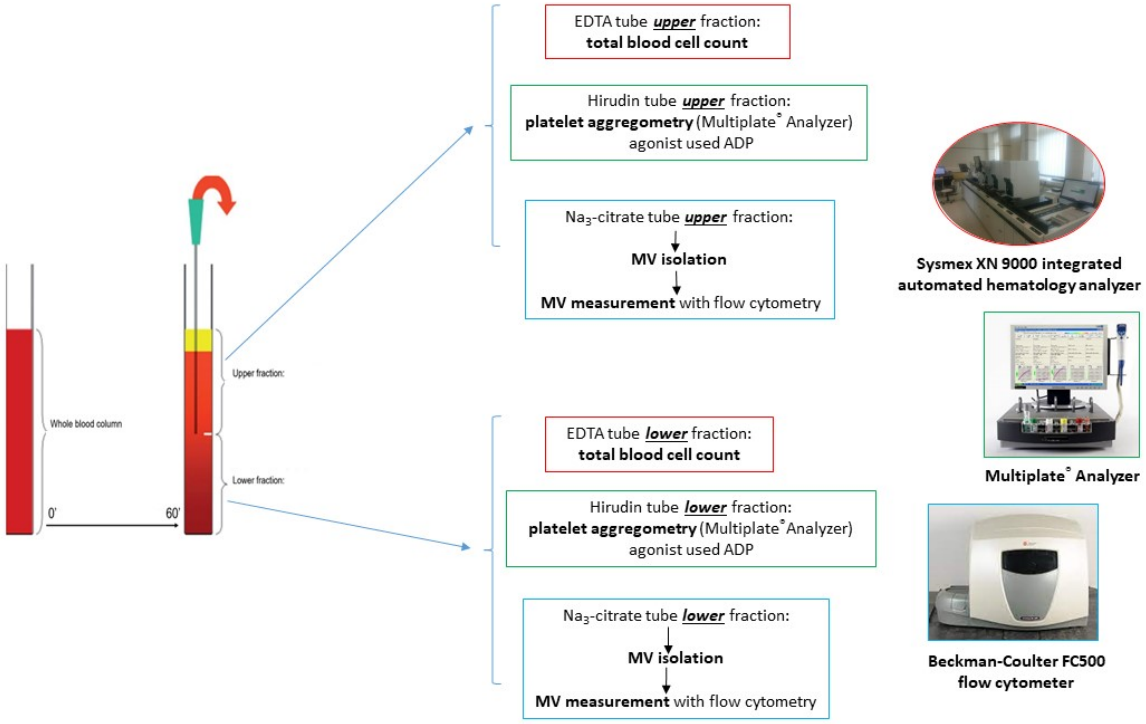

Figure 4. *Summary of sample preparation, separation of the upper and lower blood sample after 1-hour gravity sedimentation, and further analytical processes. This figure is designed by D. Schrick*

2.3.9. 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 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 patients with either ACS or recurrent ischemic stroke were presented at the Emergency Department and underwent careful clinical evaluation, then archived into an electronic database (e-MedSolution system or national e-health infrastructure (abbreviated as EESZT in Hungarian).

Data were evaluated by the SPSS software package (Version of 19 SPSS Inc, Chicago, USA and 23.0. IBM Corporation, Armonk, NY, USA). 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 $\chi 2$ test for categorical variables, where appropriate. Correlation analysis was performed by calculating Spearman's correlation coefficient (rho). Binary logistic regression, including age, PAR, H-IPF, LCR, MPV, and AUC in the upper sample, ESR and CRP as confounders, was used to explore the independent predictor of high residual platelet reactivity. A p-value<0.05 was considered statistically significant.

2.4. Results

2.4.1. Demography and baseline values for novel predictors of the future vascular events in post-stroke patients

A total of 52 convalescent stroke patients were prospectively recruited into the study. All patients suffered large vessel occlusion, diagnosed by neuroimaging. The demography and clinical data of the patients are summarized in ***Table 2***. 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 area under the curve (AUC) in the whole blood and the upper and lower blood samples after one-hour gravity sedimentation in the total population, and also in comparison between „uneventful" and „stroke + ACS" subgroups, as well as between „uneventful" and „stroke alone" subgroups are shown in ***Table 3***. The AUC_{upper} was significantly higher in patients with recurrent stroke compared to those with uneventful follow-up (p=0.003).

2.4.2. Independent predictors of recurrent ischemic episodes

Based on ROC analysis, the AUC_{upper} with a cut-off value ≥70 measured by the modified platelet function test (mPFT: electric impedance aggregometry after separation of the whole blood coloumn) was able to predict recurrent stroke events (p=0.01) with the best sensitivity and specificity. Composite vascular events (stroke + ACS) were independently predicted by neutrophil antisedimentation rate (NAR) with a sensitivity of 82% and specificity of 88% using multiple regression analysis, including the relevant covariates in ***Table 4.*** Neither recurrent stroke nor acute coronary syndrome was associated with high on-treatment platelet reactivity, defined by AUC > 53 measured by the Multiplate® Analyzer in the whole blood.

	Total population n=52	Uneventful n=41	Vascular events n=11	p-value
Age	66±8	66±8	66±9	0.937
Male; n	34	26	8	0.564
Hypertension; n	51	40	11	0.427
Diabetes mellitus; n	14	10	4	1.000
ESR	12 (8-18)	10 (8-16)	18 (14-29)	0.063
CRP	1.9 (0.7-4.6)	10 (8-16)	18 (14-29)	0.614
PLT	224 (200-260)	224 (207-251)	243 (171-300)	0.805
PAR	67.9 (63.1-73.4)	67.8 (62.9-73.5)	70 (64.6-72.6)	0.614
WBC	6.8 (5.8-8.0)	6.6 (5.8-7.9)	7.4 (5.5-10.6)	0.420
LAR	35.7 (23.7-46.3)	36.2 (24.7-46.4)	34.4 (24.0-43.5)	0.806
Neutrophils	61.8 (55.4-66.4)	62 (56-67)	58 (51-62)	0.317
NAR	-1.1 (-4.8-6.5)	0.9 (-3.9-7.2)	-5.2 (-6.8-(-4.7))	0.001

Table 2*. Demography and clinical data of the study population and comparison between patients with or without recurrent ischemia during 36-month follow-up. Vascular event = recurrent stroke, de novo acute coronary event. Abbreviations: ESR, eryhtrocyte sedimentation rate; CRP, C-reactive protein; PLT, platelet; PAR, platelet antisedimentation rate; WBC, white blood cell; LAR, leukocyte antisedimentation rate, NAR, neutrophil antisedimentation rate. Data are presented as median and 25th-75th percentiles, except age as mean ± SD*

	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 3. *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*

	β	p-value	OR	95% CI	
Age	0.071	0.353	0.931	0.801	1.082
AUC	-0.046	0.320	0.955	0.871	1.046
AUC_{upper}	-0.083	0.031	1.086	1.007	1.171
NAR	-0.489	0.032	0.613	0.392	0.960

Table 4*. Predictors of vascular events during the 36-month follow-up. Abbreviations: AUC, area under the curve; NAR, neutrophil antisedimentation rate*

2.4.3. Cut-off values of predictors

The ROC curves of variables predicting the recurrence of vascular events during 36 month follow-up period are shown in ***Figure 5.*** 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 5A***. 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 5B***. 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 5C***.

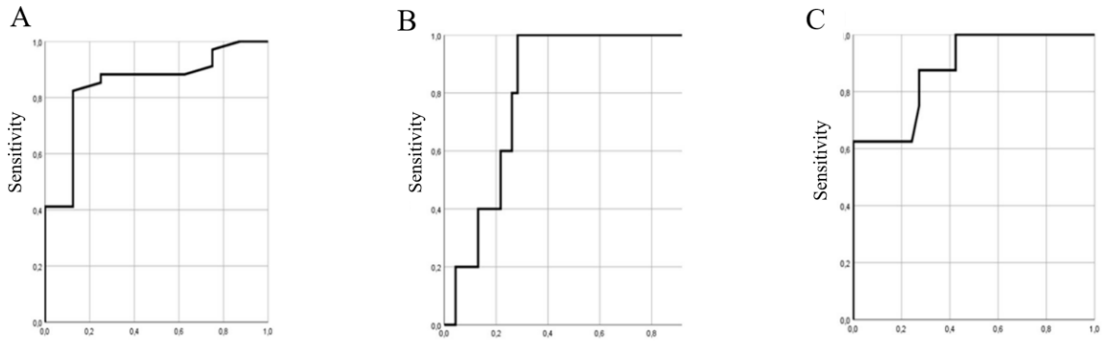

Figure 5. *ROC of variables predicting the vascular event recurrence during 36-month follow-up.* ***A****: ROC of neutrophil antisedimentation rate (NAR) with the area=0.847, p=0.002, 95%CI 0.703-0.992;* ***B****: ROC of* AUC_{upper} *with the area=0.813, p=0.023, 95%CI 0689-0.937;* ***C****: ROC of predicted probability of the combination of NAR and* AUC_{upper} *with area:0.881, p=0.001, 95%CI: 0.754-1.0*

2.4.4. Demography and baseline microvesicle values

Eighteen convalescent ischemic stroke patients taking 75mg/day clopidogrel and 20 age-matched healthy volunteers were enrolled in this prospective pilot study. Demographical data of patients and healthy subjects and baseline laboratory parameters are included in ***Table 5.*** There was no significant difference in age and gender. The total number of circulating microvesicles (p<0.001) and particularly the endothelial-derived $CD31^+$ (p=0.016) and platelet-derived $CD42a^+$ (P<0.001) MVs measured from whole blood were significantly higher in post-stroke patients compared to healthy people. Interestingly $CD62^+$ MVs showed no significant difference between groups. All of the considerable MV data from *Table 5.* are presented in ***Figure 6.***

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

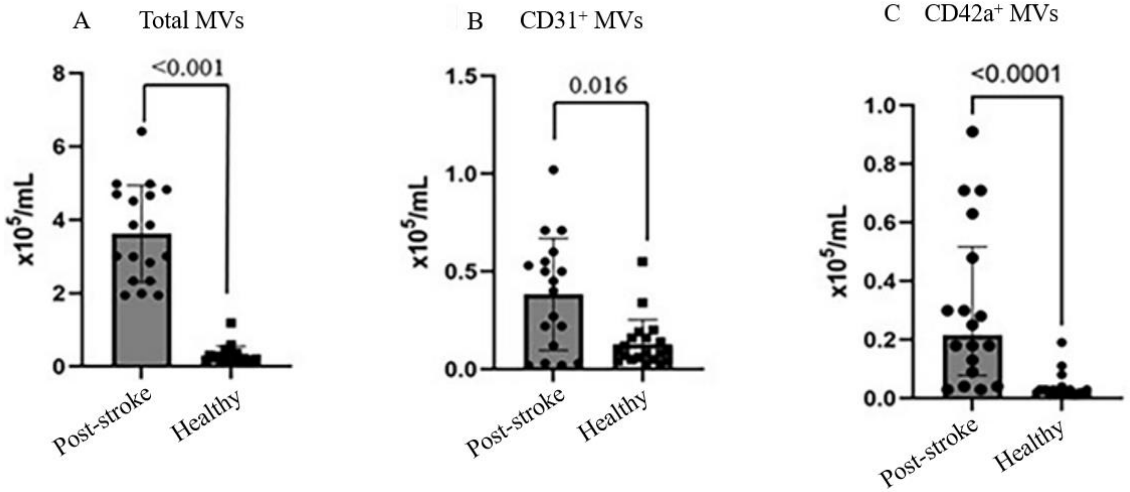

Figure 6. A: *Comparison of the total MVs count;* ***B****: CD31⁺ MVs;* ***C****: CD42a⁺ MVs in the whole blood of post-stroke patients and healthy subjects (Mann-Whitney U test)*

2.4.5. Associations between microvesicles and aggregometry data

We analyzed the correlation between MVs and aggregometry data. Platelet aggregation in the whole blood (expressed in the area under the curve, AUC) measured by Multiplate® in patients on clopidogrel 75mg daily - but not in age-matched healthy subjects – showed a significant negative correlation with the total number of MVs (x10^5/ml) in the lower blood sample after one-hour gravity sedimentation (r=-0.650, p=0.005), presented in ***Figure 7***. Nonetheless, we did not recognize any correlation between the AUC and the total count of MVs in the whole blood column.

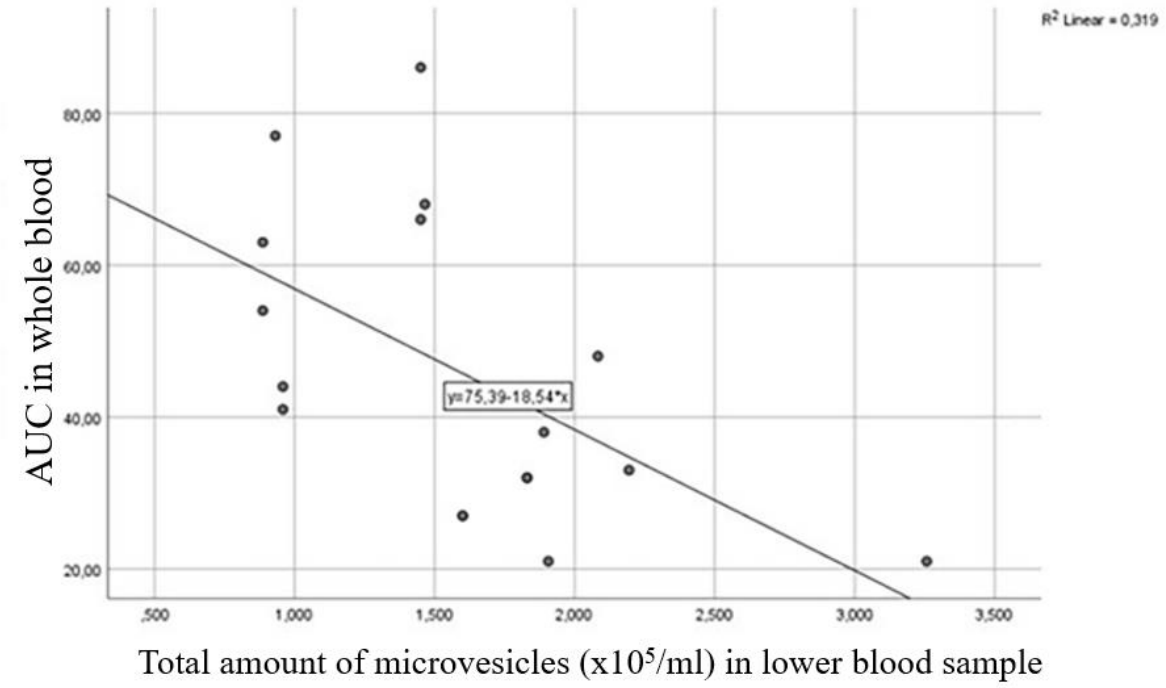

Figure 7. *Correlation between the area under the curve in whole blood measured by Multiplate® aggregometry and the total number of microvesicles in the lower blood sample after one-hour gravity sedimentation (Spearman correlation, p=0.005)*

2.4.6. Association between microvesicles and clopidogrel responsiveness in post-stroke patients

We explored the potential associations between microvesicles (MVs) and aggregometry data (area under the curve and velocity, respectively) obtained from clopidogrel good and low-responders (experiencing residual platelet reactivity despite antiplatelet drug administration) based on the previously defined cut-off value (AUC ≥53). 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), demonstrated in ***Figure 8.***

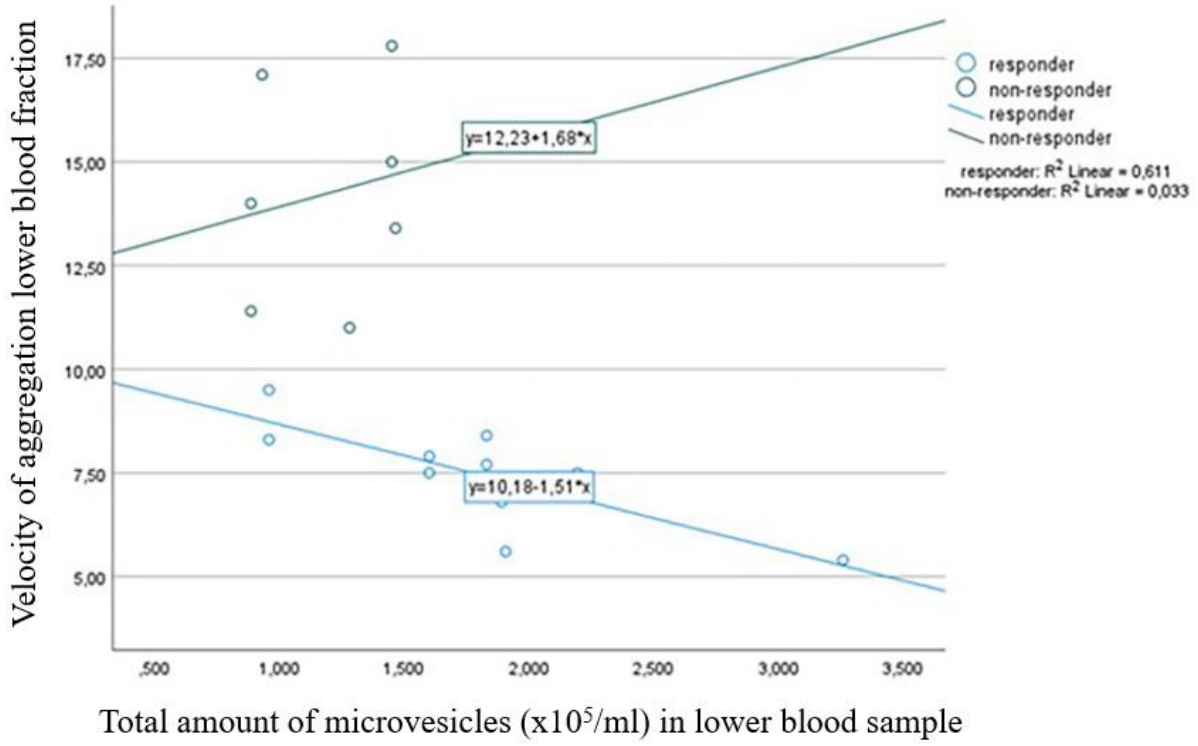

Figure 8. *Correlation between velocity measured by Multiplate® aggregometry and the total number of microvesicles (x10^5/ml) in the lower blood sample in clopidogrel responders and low-responders (high residual platelet reactivity state). The blue line indicates a negative correlation in responder patients (Spearman correlation, r=-0.801, p=0.005)*

2.4.7. Association between microvesicles and neutrophils in post-stroke patients

Activation-induced conformational epitope on CD41/CD61 complex positive (PAC-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), it is presented in ***Figure 9.***

On the contrary, this positive correlation dissipated when whole blood indices were analyzed. Additionally, the constitutional platelet marker positive (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), commenced in ***Figure 10***. Though, no significant correlation was found in the whole blood samples.

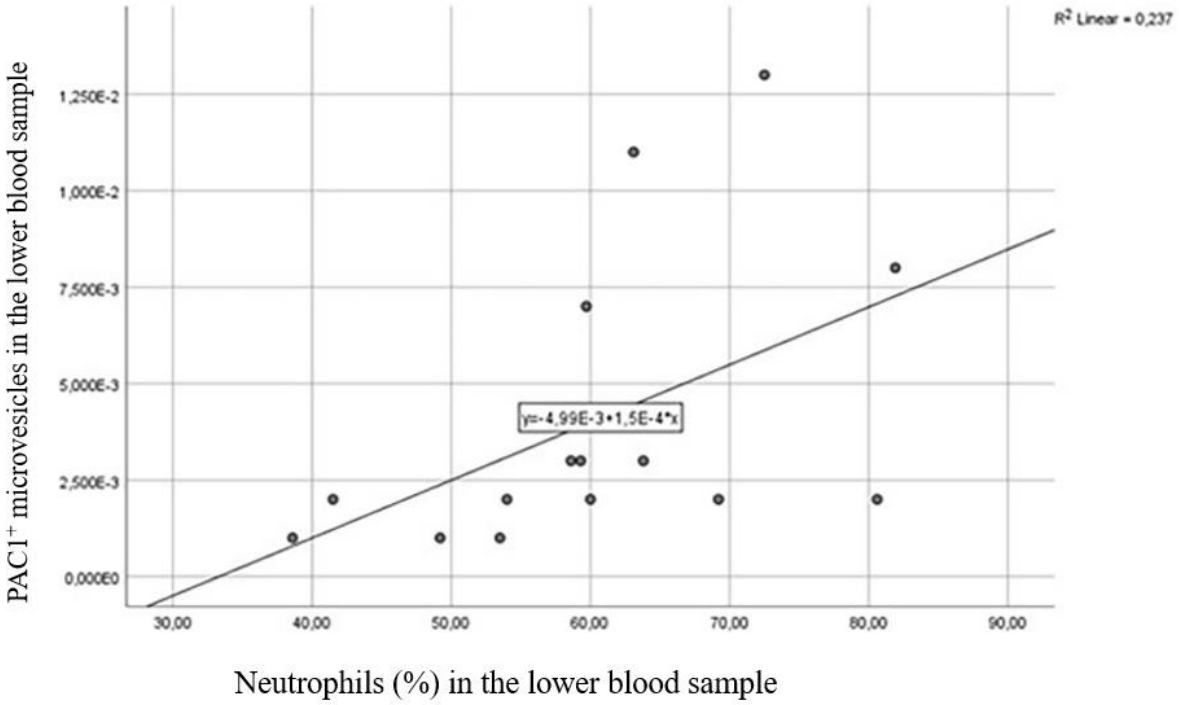

Figure 9. *Correlation between platelet-derived PAC1^{+} microvesicles (x10^{5}/ml) and neutrophils (%) in the lower blood sample (Spearman correlation, p=0.008)*

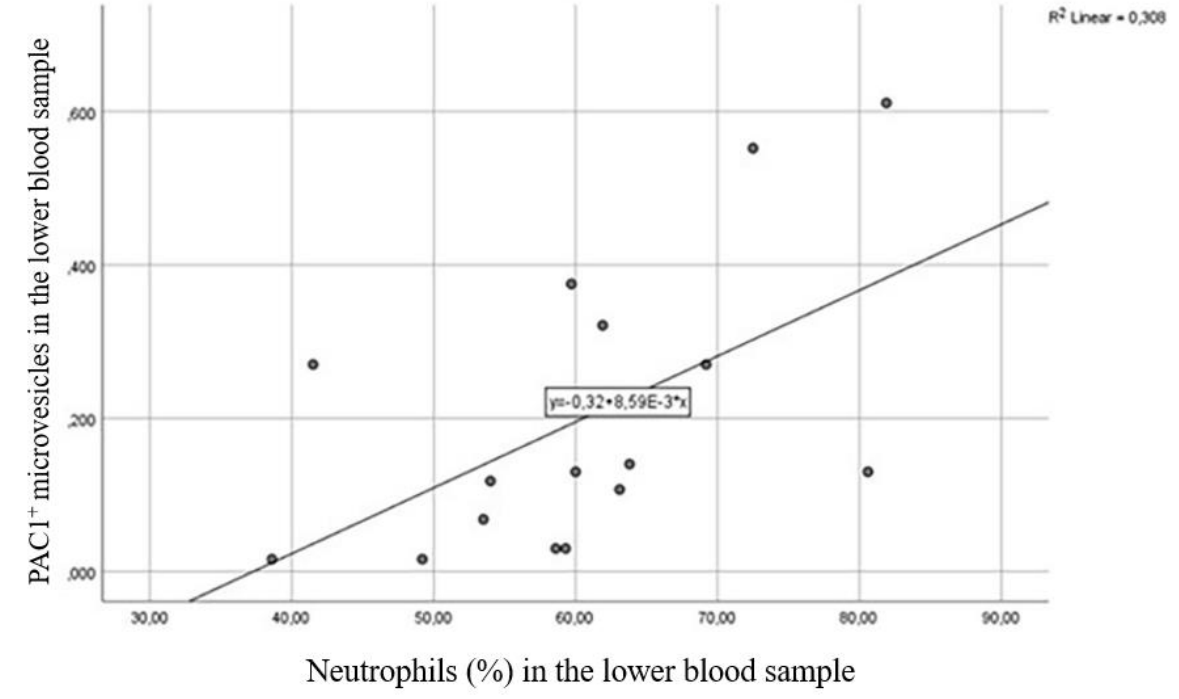

Figure 10. *Correlation between platelet-derived CD42a^{+} microvesicles (x10^{5}/ml) measured in the upper blood fraction and neutrophils (%) in the lower blood fraction (Spearman correlation, p=0.006)*

2.5. 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[64,65]. Our finding suggests neutrophils are important markers for stroke outcomes, as their predictive role was previously shown in patients with acute coronary syndrome[66]. Neutrophil activation (reflected by NAR) seems to be the most sensitive marker of recurrence of ischemic cerebral attacks in post-stoke patients on clopidogrel. Both animal and human clinical data support the pivotal role of activated peripheral blood cells in neuroinflammation after ischemic stroke[67,68]. The dynamic microcirculatory stall phenomenon in the hyperacute stage can contribute to ongoing penumbral bran injury[69]. 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)[70]. 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[71–74]. 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[75]. So, there is a huge need for 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. Further studies are needed to explore the characteristics of platelet subpopulations and their impact on post-stroke complications and outcomes. 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[76], 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 (AUC) and the total number of microvesicles in the whole blood, we discovered a negative correlation between $AUC_{whole\ blood}$ and the total number of microvesicles in the lower blood sample after one-hour gravity sedimentation. The majority of MVs are derived from platelets, and the differently activated platelet clusters were previously identified based on the motion during one-hour gravity sedimentation. Their „dust" (PMVs) might affect the clopidogrel responsiveness. 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[77]. 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 $PAC\text{-}1^+/CD61^+$, $CD62P^+/CD61^+$, and $CD31^+/CD61^+$ microvesicles were found in acute stroke patients indicating antiplatelet treatment failure[78,79]. 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. Moreover, in recent research, high levels of MVs with different origins were found predictive for estimating stroke severity and prognosis[80].

Another vital aspect is emerging evidence of platelets and PMVs and their crucial role in immune processes. Notably, we observed positive correlations between $PAC1^+$, and $CD42a^+$ PMVs, respectively, and the percentage of neutrophils in the lower blood sample after one-hour

gravity sedimentation of whole blood, indicating a strong counterplay between the procoagulant potential of platelet-derived microvesicles and thromboinflammatory cascade. 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[81]. Neutrophil extracellular traps (NETs) not only play a protective role after stroke but also have prothrombotic properties; laden with a citrullinated histone H3, CatG, NE, and myeloperoxidase (MPO), they can cause platelet mediated thromboinflammation in the neurovasculature[82]. Activated platelets upregulate damage-associated molecular pattern molecule high-mobility group box 1 (HMGB1) in multiple inflammatory diseases (such as the post-stroke state). It has also been shown to be a critical mediator of thrombosis by regulating platelet activation, granular secretion, adhesion, and spreading[82].

Based on our findings, platelets seems to be the crucial players in thromboinflammation and the pathogenesis of stroke. There are many ongoing types of research for developing viable drug discoveries targeting particular proteins and pathways involved in pathophysiological settings. Platelet-dependent thromboinflammation can be effectively targeted by an optimal inhibition of P_2Y_{12} receptors, blocking vWF-GPIb interactions.

2.6. Conclusion

Our research suggested that AUC_{upper} indicates a more precise definition of HRPR. The neutrophil antisedimentation rate reflects the inflammatory state in post-stroke patients. It is known that interactions between inflammation and stroke are multifaceted; a better understanding of such cellular and physiological mechanisms would lead to enhanced secondary prevention protocols, including immunomodulatory approaches, to reduce further ischemia. Based on this small, single-center pilot study, these novel markers may better predict ischemic events, leading to better risk stratification and providing individually tailored vascular therapy, including antiplatelet and anti-inflammatory regimens, in the secondary prevention of ischemic cerebrovascular diseases[83,84]. Our findings will be impactful in developing novel and potent therapies against stroke and help drive effective therapy management to prevent further burden on patients.

2.7. 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

2.8. Future perspectives

Our aim in the future to explore alterations in the movement of platelets and (neutrophils) in acute stroke, in order to begin the tailored antiplatelet regime as soon as possible after the ischemic attack. Furthermore, we plan to enroll more patients to provide more significant statistical base to our finding. Next to clopidogrel only, we will examine aspirin treatment as well.

3.
ACUTE VASCULAR DISEASE:
COVID-19 AND COAGULATION RESEARCH

3.1. Introduction to coronavirus disease

Coronaviruses are a highly diverse family of enveloped single-stranded RNA viruses; they infect humans, other mammals, and avian species. Coronaviridae is further specified into the subfamily of Orthocoronaviridae: alpha- and betacoronaviruses exclusively infect mammalian species. Meanwhile, gamma- and deltacoronviruses have a broader host range[85]. Transmission from human to human occurs via respiratory droplets and aerosols. First, the viruses bind to the nasal epithelial cells in the upper respiratory tract. It can get into the pulmonary epithelium if inhaled deeper by conducting airways. Inside the body, the virus binds to host receptors and enters into host cells through endocytosis or fusion. The virus is made up of four structural proteins: spike (S), membrane (M), envelope (E), and nucleocapsid (N) proteins (***Figure 11.***). Coronaviruses primarily infect human lungs via angiotensin-converting enzyme 2 (ACE2) receptors. The virus becomes incorporated into the cells, viral RNA is released into the cytoplasm, and replication begins. Novel virus particles bud out of the cellular surface membrane, propagating the infection.

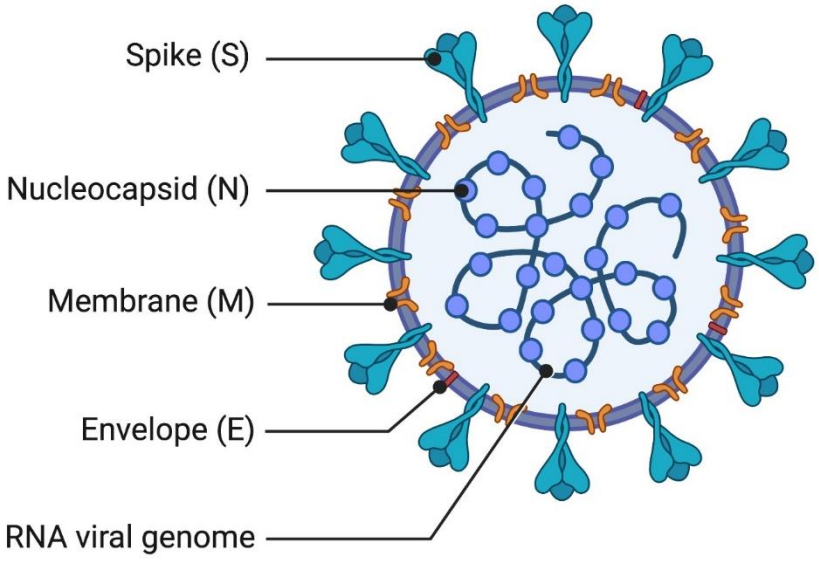

Figure 11. *Structure of SARS-CoV-2, image is made in Biorender*[86]

3.1.1. Epidemiology of COVID-19

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 30th of January 2020, the World Health Organization (WHO) declared that the SARS-CoV-2 outbreak constituted a public health emergency of international concern, after the first infection was diagnosed in Europe (Germany) and transmitted outside Asia. On the 11th of march 2020, WHO declared the novel coronavirus outbreak (COVID-19) a global pandemic[87]. The novel SARS-CoV-2 infection and COVID-19 pandemic became a relentless worldwide catastrophe of public health and socio-economical emergency[88]. Up to the latest data (while writing this thesis), more than >0.6billion cases and > 6 million death happened due to the infection globally. Gladly, > 13 billion preventive vaccines were administered to people worldwide. Data is visualized in ***Figure 12.***

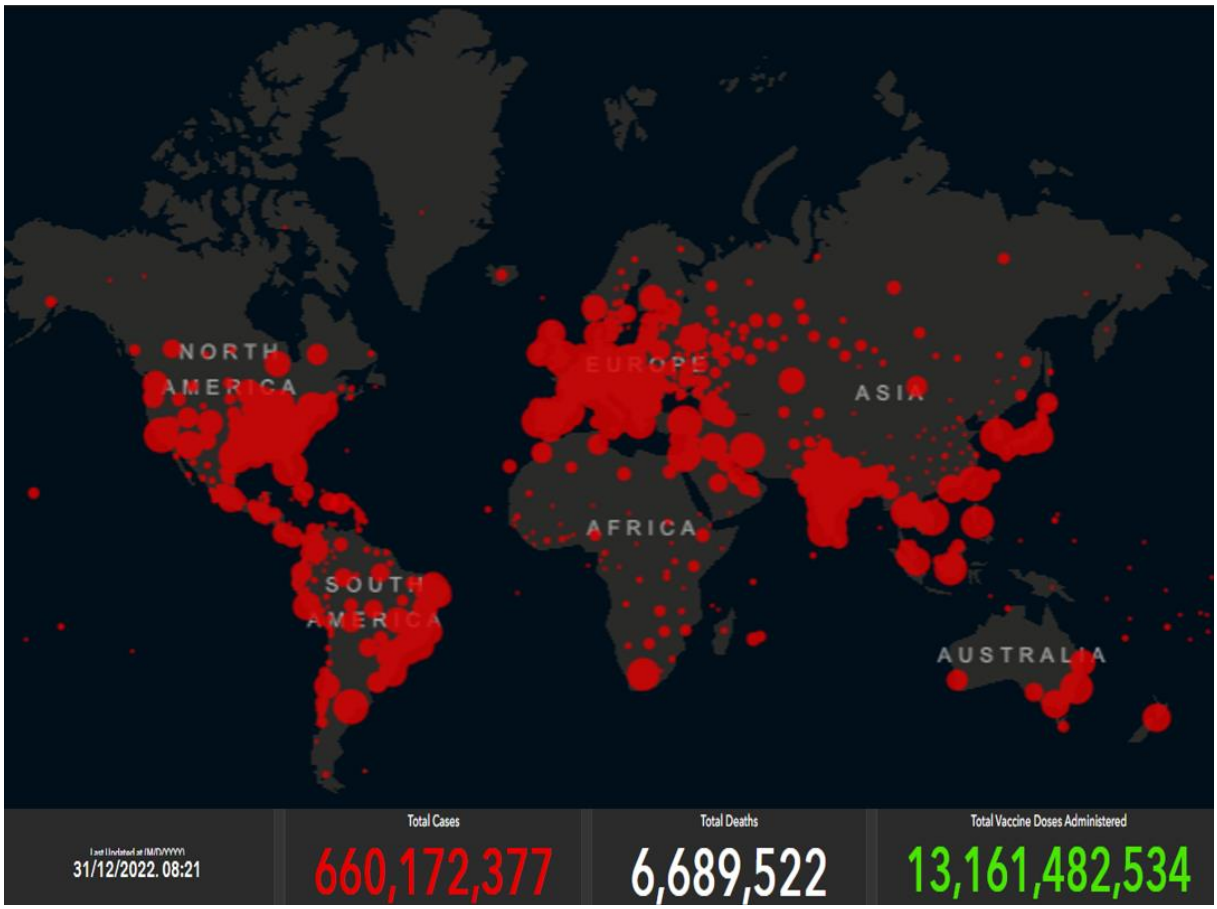

Figure 12. *COVID-19 dashboard by the Center for System Science and Engineering at John Hopkins University at the end of the year 2022*[89]

3.1.2. Diagnosis and clinical aspects of the disease

The incubation time (from exposure to the virus to symptom onset) 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 13.***

Clinical diagnosis is made upon clinical symptoms (fatigue, headache, fever, blocked nose, loss of smell or taste, sore throat, cough, shortness of breath, muscle aches, nausea, vomiting, diarrhea), blood tests (often presented with lymphopenia, elevated LDH, CRP, CK, ALAT, ASAT, D-dimer, NLR, and probably coagulation abnormalities), and radiological finding (chest X-ray: multifocal bilateral alveolar opacities, but preferably high-resolution CT (HRCT): ground glass opacities and reserved halo sign, lymphadenopathy). 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[90].

SARS-CoV-2 infection severity can be categorized as follows[91]:

- asymptomatic infection: individuals with positive nucleic acid amplification test (NAAT) or positive antigen test but who have no symptoms
- mild illness: upper respiratory tract infection without shortness of breath or abnormal chest imaging
- moderate illness: lower respiratory disease and SpO_2 ≥94% on room air at sea level
- severe illness: SpO_2 <94% on room air at sea level, and the ratio of arterial partial pressure of oxygen to fraction of inspired oxygen (PaO_2/FiO_2; Horowitz quotient) <300 mmHg, tachypnoea or lung infiltrates >50%
- critical illness: respiratory failure, septic shock and/or multi-organ dysfunction, failure

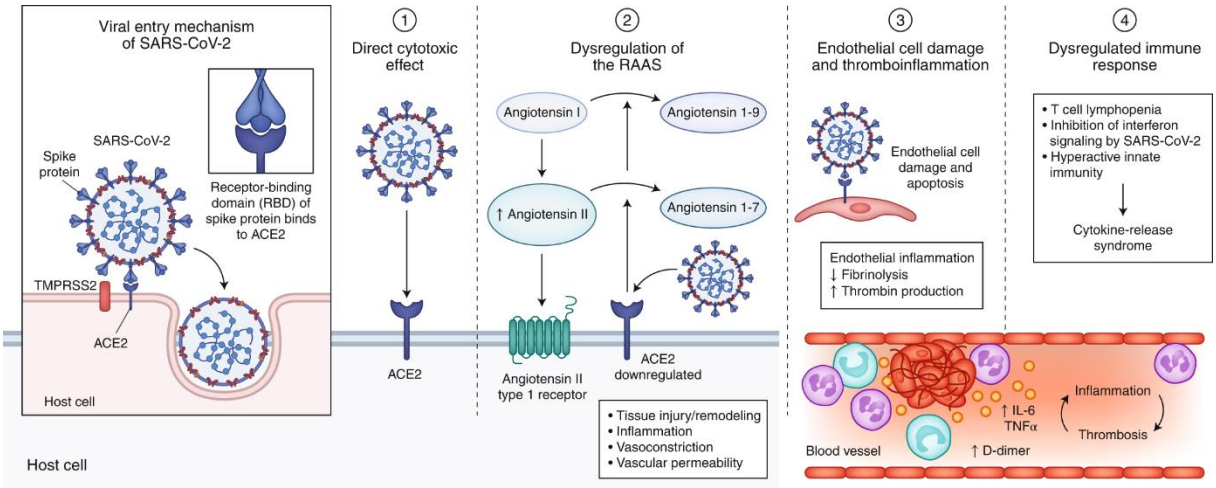

Figure 13. *Pathophysiology of COVID-19. (1) direct virus-mediated cell damage via ACE2 receptors on host cells (2) dysregulation of the renin-angiotensin-aldosterone system (RAAS) (3) endothelial damage and thromboinflammation (4) dysregulation of the immune response causing T cell lymphodepletion, and there is an increased production of proinflammatory cytokines, particularly IL-6 and TNFα. This image was initially published by Gupta et al.*[92]

3.1.3. Extrapulmonary manifestations of SARS-CoV-2 infection

The pulmonary manifestations of the SARS-CoV-2 infection, including pneumonia and ARDS, are well established[93]. Infection might promote multi-organ dysfunctions due to dysregulated immune response and cytokine tsunami. It is not negligible, that cardiological symptoms usually include myocarditis and acute cor pulmonale. Furthermore, hepatitis-like liver dysfunction, acute kidney injury, and dysregulated glucose homeostasis often combine with the disease, as well as neurological symtoms. Several studies have presented an increased risk for thromboembolic complications, like stroke and myocardial ischemia[94–96]. Merkel et al. declared that COVID-19 might increase the risk of acute ischemic stroke similar to the increased risk of 3.2-fold to 7.8-fold seen within the first three days after other respiratory tract infections[97,98]. The most common extrapulmonary manifestations are summarized in ***Figure 14.***[92,99]

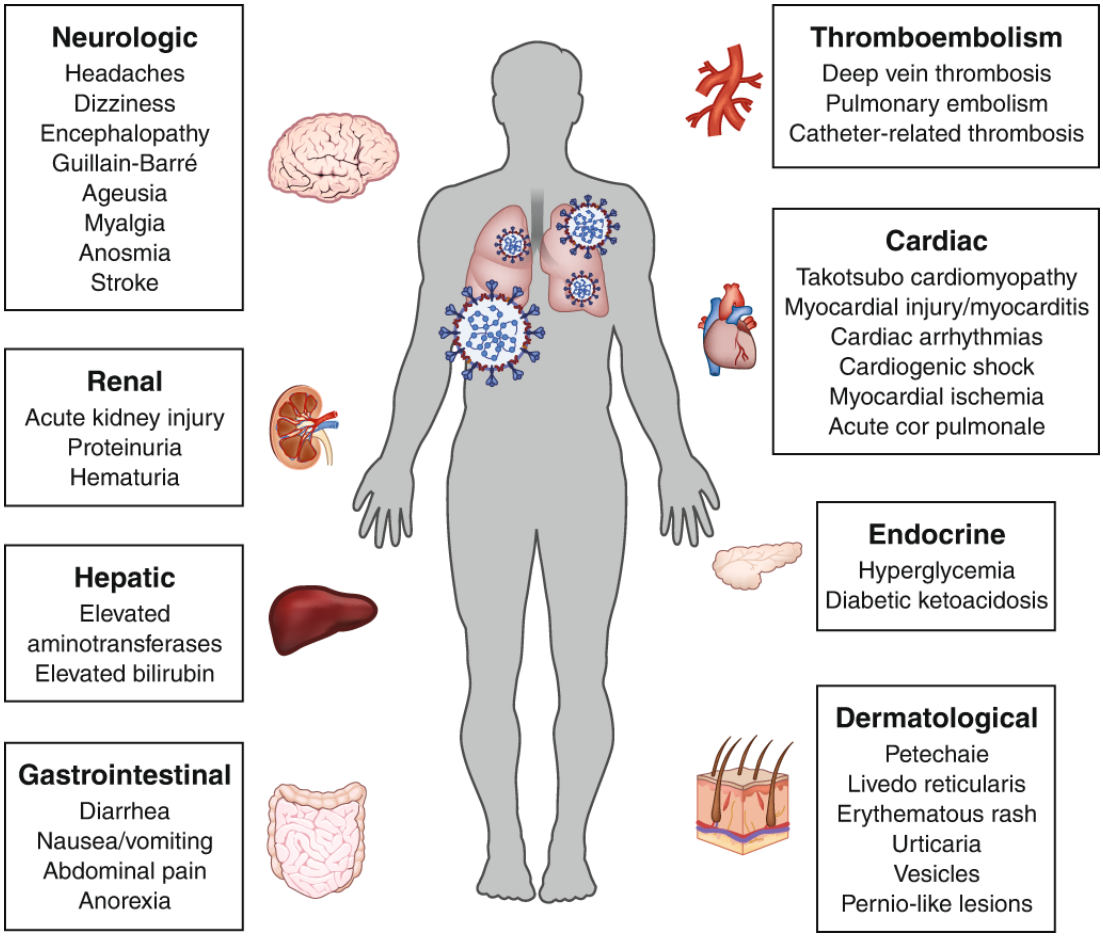

Figure 14. *Extrapulmonary manifestations of COVID-19. This image was initially published by Gupta et al.* [92]

3.1.4. COVID-19-associated hemorheological and hemostatic changes.

As previously mentioned, the angiotensin-converting enzyme 2 (ACE-2) has been identified as a functional receptor for coronaviruses. ACE-2 receptors are highly expressed in the cardiovascular system, so there are several cardiovascular, hemorheological and hemostatic complications of coronavirus infection[99]. Gap junctions provide direct communication between endothelial cells and pericytes via autocrine and paracrine signaling pathways to maintain vascular integrity. Pericytes have pivotal roles in vascular homeostasis and inflammatory processes. Most viral infections eventually promote apoptosis of the host cells, and SARS-CoV-2 induces apoptosis (pyroptosis is the right word for the highly inflammatory form of regulated cell death) via protein kinase B/Akt intracellular signaling pathway[100]. This mechanism has been described in endothelial cells as well. Furthermore, endoltheliitis is an immune response, leading to a generalized pulmonary hypercoagulable state[101,102]. Furthermore, the extensive cytokine response in ARDS promotes vascular leakage and results in intravascular

coagulopathy. It is widely known nowadays that platelets represent the interplay between the immune system and the hemostasis[103]. Pathogen-platelet interaction can trigger degranulation with subsequent platelet activation[104]. Most patients with mild-moderate infection have normal or even increased platelet count; however, in critically ill patients, thrombocytopenia is a common feature, and DIC-like symptoms and increased megakaryocyte count may be found in almost 70% of nonsuvivors[105]. In the autopsy case series of COVID patients, Rapkiewitz et al. described megakaryocytes in the vascular beds; this phenomenon appears to be a unique feature of the disease and may play an important role in their increased thrombotic risk[106]. Neutrophil extracellular traps (NETs) are large, extracellular, weblike structures, and they may play an essential role in end-organ injury and the phenotypic scheme of COVID-19. Their structures are ideal for binding activated platelets leukocytes, activating factor XI, and generating thrombin for fibrin production. Thus Nicolai et al. reported the microvascular thrombi containing NETs in the lung, heart, and kidneys. Immunothrombotic dysregulation could explain the multiorgan failure and the systemic hypercoagulability in severely ill patients with SARS-CoV-2 infection[107,108]. In summary, COVID-19 promotes a hypercoagulable state, termed COVID-19-associated hemostasis abnormalities (CAHA)[109]. CAHA has no consensus definition yet. ***Figure 15.*** presents a schematic view of the overlap-like syndromes of different coagulation abnormalities presented in infected patients, based on the work of Toshiaki et al[110].

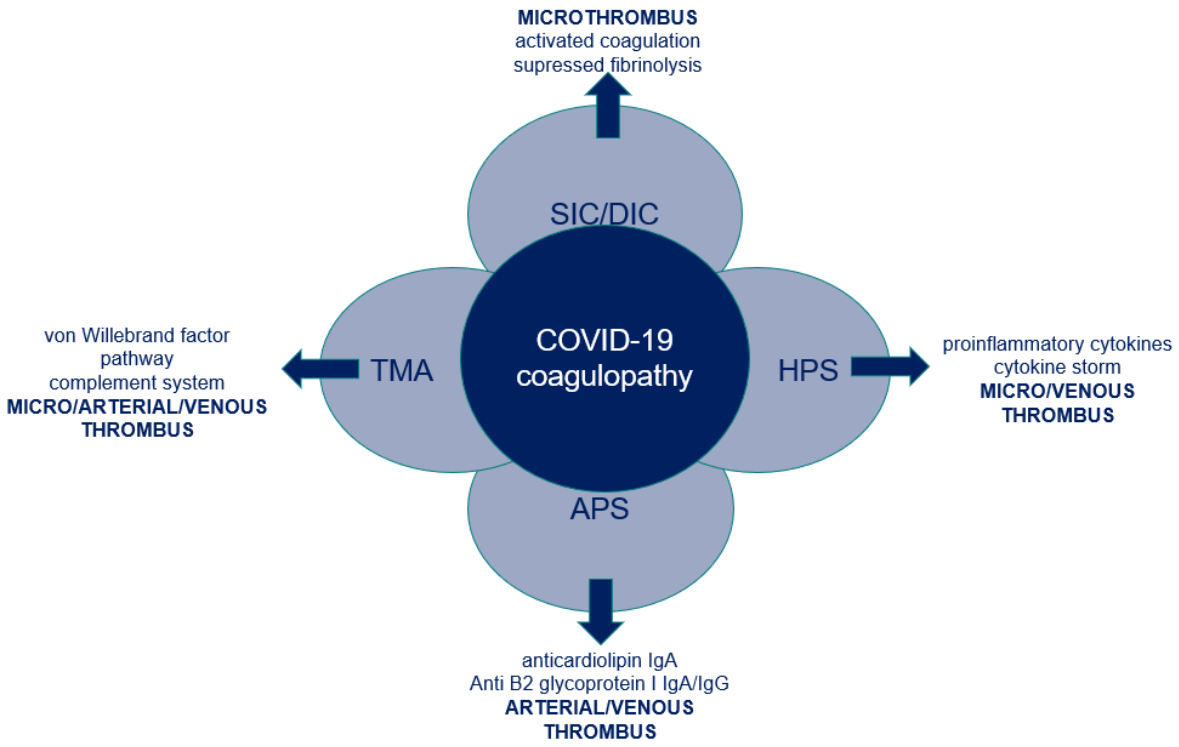

Figure 15. *COVID-19-associated coagulopathy. Special clinical features partially overlap with haemophagocytosis syndrome (HPS), antiphospholipid syndrome (APS), thrombotic microangiopathy (TMA), and sepsis-induces coagulopathy (SIC) / disseminated intravascular coagulation (DIC), however, it is not 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.*[110]

3.1.5. Complications of coagulation and their prevention

A broad spectrum of thrombotic and thromboembolic complications characterizes COVID-19 patients. The extent of these manifestations is correlated with the severity of the disease. The rate of thrombotic events is around 16% in hospitalized patients, varying between 11.5% in a non-intensive care unit (ICU) to 29.4% in severe patients, requiring intensive care settings[109]. Venous thromboembolism, including pulmonary embolism (PE) and deep vein thrombosis (DVT) varies according to the severity of the infection. The main risk factors are immobilization, hypoxia, and endothelial damage. Prevalence in severe COVID-19 patients (with ICU requirement) is 40% - according to Wang et al., despite thromboprophylaxis[111]. All hospitalized patients should undergo VTE risk stratification to provide adequate prevention. Most scientific societies recommend prophylaxis with daily subcutaneous low-molecular-weight heparins (LMWHs) or twice-daily unfractionated heparin (UFH). LMWH is the preferred choice because LMWHs have anti-inflammatory and immunomodulatory effects and more reliable pharmacokinetic and pharmacodynamic properties[111]. Therapeutic

anticoagulation and thrombolysis (if needed) are the cornerstones of VTE treatment[111]. In the case of previously acquired VTE, anticoagulant therapy (whether with VKAs and DOACs) should be discontinued in critically ill patients and must switch to LMWH therapy. Arterial complications, like acute coronary syndrome and less frequently acute ischemic stroke, acute limb ischemia, and splenic infarcts[112,113]. Among these patients antiplatelet therapy (DAPT in patients with MI, SAPT after stroke) is essential. Upon the most up-to-date guidelines, patients should be treated with aspirin and oral P_2Y_{12} inhibitors (clopidogrel, prasugrel, ticagrelor)[114]. It is important to note that patients on antithrombotic therapy per standard of care must continue their previously taken antiplatelet agents. However, some investigational antiviral agents may have drug-drug interactions. There are still several gaps in our knowledge of the optimal preventive strategies for arterial-venous thromboembolic complications. Coagulopathy with or without bleeding is less common than thrombosis in patients with COVID-19. Clinically relevant thrombocytopenia in critically ill patients and reduced fibrinogen levels are associated with an increased risk of bleeding complication[114]. It is recommended to keep platelet count $>25\times10^9$/l, and if bleeding is present, initial administration of fresh frozen plasma should be considered[114]. Critically ill patients with refractory ARDS and hypoxemia may need oxygenation support with an extracorporeal membrane oxygenator (ECMO). Patients on ECMO require full dose anticoagulation, targeted upon the Extracorporeal Life Support Organizations (ELSO) guidelines [114].

ATTACC/ACTIVE-4a/REMAP-CAP was a multiplatform (performed in nine and then in twenty countries), open-label RCT of therapeutic anticoagulation in noncritically ill, hospitalized patients with COVID-19[115]. It was established that therapeutic heparin increased the organ support-free days (OR 1.27; 95% CrI, 1.03–1.58; 99% posterior probability) and decreased the need for organ support but did not significantly affect major thrombosis or death (4% absolute difference, favoring therapeutic anticoagulation (95% CrI, 0.5–7.2))[114]. Meanwhile, in patients requiring intensive care, therapeutic anticoagulation did not reduce the duration of organ support or mortality (OR 0.83; 95% CrI, 0.67–1.03; 99.9% posterior probability of futility; OR <1.2), nonsignificantly more bleeding events occurred while fewer thrombotic events were established[115]. NIH treatment guidelines recommend a prophylactic dose of heparin if no contraindication exists (LE 1A). Guidelines for the usage of antithrombotic therapy in COVID-19 patients have been released by multiple organizations; including the American Society of Hematology, the International Society of Thrombosis and Hemostasis, the National Institute for Health and Care Excellence (NICE) and the Royal College of Physicians

as well[116,117]. REMAP-CAP revealed that using aspirin or a P_2Y_{12} inhibitor in critically ill patients did not reduce the number of organ support-free days or in-hospital mortality[118]. RECOVERY trial found that the use of aspirin was not associated with reduction in 28-day mortality or the risk of progressing to MV or death[119].

3.1.6. Prognostic tools for thromboembolic complications

Since the pandemic's beginning, several biomarkers associated with inflammatory and coagulation pathways were identified that correlate with or predict progression to severe disease and death. C-reactive protein (CRP) is a well-established biomarker of acute inflammatory states. Interleukin-6 (IL-6) is a predictive biomarker of more severe outcomes, but no specific correlation with thrombosis development was established. In contrast, CRP levels showed to be predictive of the risk of thrombosis[120]. D-dimer is the principal fibrin degradation product used as a biomarker of coagulation and fibrinolysis; it could screen for thrombosis and predict mortality[120]. Conventional plasma-based assays, like prothrombin time, activated partial thromboplastin time (aPTT), and anti-Xa assay, has been used to monitor the effectiveness of treatment with LMWH or UFH. In a retrospective study, personalized anticoagulation therapy was independently associated with a lower risk of COVID-19-related death (OR 0.040, p=0.043). Viscoelastic assays, such as thromboelastography (TEG, ClotPro®) and rotational thromboelastometry (ROTEM®), could provide a global assessment of dynamic changes in initiation-clot formation stability and lysis in the whole blood in COVID-19 patients; it can be used to assess hypercoagulability[120]. Post-mortem studies revealed that COVID-19 had shown a high incidence of „white" (platelet-fibrin) microthrombi in different organs. Lower platelet count ($<100 \times 10^9$/l) was more common in critically ill patients, indicating the difference between survivors vs. nonsurvivors. The immature platelet fraction also showed some utility in predicting disease severity, and MPV may also play an indicator role in platelet activation[121,122].

3.1.7. Novel biomarkers for thromboembolic complications

Tissue factor (TF) presentation on extracellular vesicles might show more specific information about coagulation disturbances; persistent NETosis and complement activation might be an important accelerator of thromboinflammation in severe conditions[123]. Endotheliitis is well represented by vWF and the ratio of vWF:Ag to ADAMTS13[124]. There are novel soluble biomarkers, like complement factors, associated with disease severity but not with the occurrence of thrombosis. Calprotectin showed an association with thrombosis and

critical illness[124]. Platelet activation can be detected by the soluble CD40L level, soluble P-selectin level, P-selectin extracellular expression, and arachidonic-acid/ADP-induced platelet aggregometry[125,126]. Circulating microRNA levels were correlated with disease severity, and specific circulating microRNA profiles seemed to have some prognostic utility[127]. Circulating ACE2 and soluble E-selectin (CD62E) levels were elevated, and new biomarkers of severe lung disorder with a vascular injury in critically ill patients[128].

3.2. 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

3.3. Methods

3.3.1. Study design

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. Written informed consent was obtained from patients or their close relatives (due to their severe condition) and healthy controls.

3.3.2. Subjects

Twenty-one patients with severe SARS-CoV-2 infection were retrospectively analyzed from a prospective database. 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). Twenty-one age-matched, healthy, SARS-CoV-2 RT PCR negative healthcare workers served as controls.

3.3.3. Eligibility and exclusion criteria

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 under 18 years old, or patients with congenital hemostatic abnormalities (platelet storage pool disorders, von Willebrand disease, hemophilia), severe thrombocytopenia (<50G/l), and anamnestic or recurrent malignant disease, pregnant women were excluded from the investigation.

3.3.4. Sample collection and processing

All ICU patients had a central venous catheter (in the jugular internal or subclavian vein). Blood samples were drawn into a closed system blood sampling tube with 3.2% Na_3-

citrate (Beckton Dickinson, Diagon Ltd., Budapest, Hungary), hirudin-containing tube (Sarstedt S-Monovette® 1.6ml hirudin), and K_3-EDTA (Becton Dickinson, Diagon Ltd., Budapest, Hungary) as anticoagulant and serum separator tubes without anticoagulant (native). Samples were processed within a maximum of one hour after collection. Healthy volunteers' blood was taken through an antecubital vein puncture with a 21G needle into a closed system.

3.3.5. Blood count, platelet count, high immature platelet fraction measurement

The total blood cell count from the whole blood and absolute neutrophil count were measured on Sysmex XN 9000 integrated automated hematology analyzer (Sysmex Co, Japan, 2017). The platelet number (PLT-F) 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).

3.3.6. Erythrocyte sedimentation rate (ESR)

The ESR test (Westergreen) measures how quickly red blood cells subside to the bottom of a test tube. The rate at which red blood cells settle is measured as the number of millimeters of clear plasma present at the top of the column after one hour (mm/h). According to Westergreen, we used a BD seditainer stand with an adjustable zero mark for manual determination of ESR. After swivelling the tube to mix the blood sample, the tubes were immediately placed in the stand to start the measurement. After one hour of sedimentation, the results were read visually.

3.3.7. Measurement of hemostasis parameters

3.3.7.1. Routine and special hemostasis tests

Activated partial thromboplastin time (aPTT), D-dimer, and fibrinogen (quantitively determined based on the Clauss method) were measured as part of the routine hemostasis parameters on ACL-TOP-750 analyzer (Werfen, Hungary) with APTT-SP (liquid, HemosIL® Werfen, Hungary), HemosIL D-dimer HS (Werfen Hungary) and Q.F.A thrombin (Bovine; HemosIL® Werfen, Hungary) reagent, respectively.

The special hemostasis tests were measured on an ACL-TOP-500 analyzer (Werfen, Hungary). 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, both with HemosIL® reagent. For quantitative measurement of plasminogen as the critical factor of fibrinolysis, we used an automated chromogenic assay (Plasminogen; HemosIL®). The quantitative determination of alpha$_2$-antiplasmin as an essential regulator of the fibrinolytic system was carried out with an automated chromogenic assay (Plasmin inhibitor; HemosIL®).

3.3.7.2. Platelet function tests

To monitor aspirin therapy, we performed a platelet function test from hirudin anticoagulated whole blood within one hour after collection on Cobas® Multiplate® analyzer (Roche Diagnostics, Mannheim, Germany) 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 (high concentration – 0.77mg/ml – ristocetin as an agonist). The level of aggregation was expressed as the area under the curve (AUC). AUC was calculated by the analyzer using the product of aggregation unit (AU) × time (minutes). 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-/nonresponders" with an AUC≥40 to aspirin therapy[129].

3.3.7.3. Viscoelastometric tests

Viscoelastometric testing was carried out by ClotPro® (DiaCare Solutions Ltd., Hungary) in vitro POC coagulation analyzer. 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 and control patients. 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. I want to note that EX-test, tPA-test, and FIB-test contain polybrene to neutralize heparin. 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 >393[130].

3.3.7.4. Measurement of hs-CRP, ferritin, and interleukin-6 (IL-6)

High sensitivity C-reactive protein (hs-CRP) was measured from patients' serum with Tina-quant® C-reactive protein reagent (Roche Hungary Ltd.) on Roche Cobas 6000 fully automated chemistry analyzer (Roche Diagnostics). This test is a two-reagent, immunoturbidimetric assay with a reference range of <10mg/l in serum in adults. For the measurement of ferritin and IL-6, we used Roche Elecsys® Ferritin and Elecsys® IL-6 (Roche Hungary Ltd.) reagents on a fully automated Cobas e801 analyzer (Roche Diagnostics). Both tests use the electrochemiluminescence immunoassay (ECLIA) technique which allows the in vitro qualitative detection of analytes in the sample. The expected values for ferritin were 30-400μg/l (in men, 20-60 years) and 13-150μg/l (in women, 17-60 years). The IL-6 assay, using a cut of 35pg/ml on admission, correctly identified 16 of 19 patients that required intubation.

3.3.8. 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, recurrent 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 (swelling of the affected extremity, shortness of breath, hemodynamic instability, shortness of breath, etc.), laboratory parameters

(LDH, D-dimer, GOT, GPT, Troponin T, etc.), and radiologically (CT-angiography, duplex Doppler ultrasound) 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.

3.4.Results

3.4.1. Demography and differences between patients and healthy subjects

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). Demographical data and past medical history laboratory results on admission are summarized in ***Table 6***. 76% of the patient had hypertension. Meanwhile, 57% of them were treated for diabetes mellitus. 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. However, their markers were not detected in the control population (not shown in the table).

We analyzed associations between ESR and acute phase proteins (such as hs-CRP and fibrinogen). We found a strong positive correlation between them in patients but not in healthy controls (***Figure 16.***). This reflects the ongoing inflammatory response in infected patients ($r = 0.812$, $p < 0.001$ and $r = 0.666$, $p = 0.001$). Furthermore, a positive correlation was observed between either von Willebrand factor or von Willebrand factor ristocetin cofactor activity and plasma level of D-dimer ($r = 0.683$, $p<0.001$; $r = 0.675$, $p<0.001$, respectively).

	Patients n=21	Controls n=21	p
Age (y)	69 (52-71)	67 (63-69)	0.22
Male (n)	12 (50%)	11 (52%)	0.757
BMI	27 (26-33)	24 (2326)	0.189
Hypertension	16 (76%)		
Diabetes mellitus	12 (57%)		
VTE/stroke	5 (24%)		
ACS	4 (19%)		
Heart failure	1 (5%)		
ESR (mm/h)	84 (36-107)	4 (4-10)	<0.001
Platelet count (G/l)	214 (114-355)	261 (249-265)	0.950
IPF (%)	8.7 (6.21-12.5)	7.6 (6.7-8.2)	0.753
H-IPF (%)	2.2 (0.6-4.1)	0.9 (0.8-1.0)	0.308
Fibrinogen (g/l)	5.1 (3.5-5.4)	3.2 (2.8-3.2)	0.059
D-dimer (mcg/l)	2296 (1415-6260)	495 (363-575)	<0.001
aPTT (s)	12.9 (12.4-14.0)	26.3 (24.9-27.8)	0.002
TT (s)	34.0 (31.2-36.7)	10.4 (10.2-10.8)	<0.001
vWF:Ag (%)	488 (412-605)	109 (97-109)	<0.001
vWF:Rco (%)	39 (353-568)	104 (97-109)	<0.001
Plasminogen (%)	86 (64-96)	106 (98-106)	0.028
$Alpha_2$-antiplasmin (%)	118 (107-126)	11 (116-121)	1.000
hs-CRP (mg/l)	90.5 (27.7-126.1)	1.2 (0.9-1.5)	<0.001

Table 6. *Demography, comorbidities, and admission laboratory parameters of the study population. Abbreviations used: BMI: body mass index; TIA: transient ischemic attack; DVT: deep vein thrombosis; ESR: erythrocyte sedimentation rate; IPF: immature platelet fraction, H-IPF: high-immature platelet fraction, aPTT: activated partial thromboplastin time, TT: thrombin time, vWF:Ag: von Willebrand factor antigen, vWF:RCo: von Willebrand factor ristocetin cofactor activity, hs-CRP: high-sensitivity C-reactive protein. Data are presented as count (%) or median (25th–75th percentiles)*

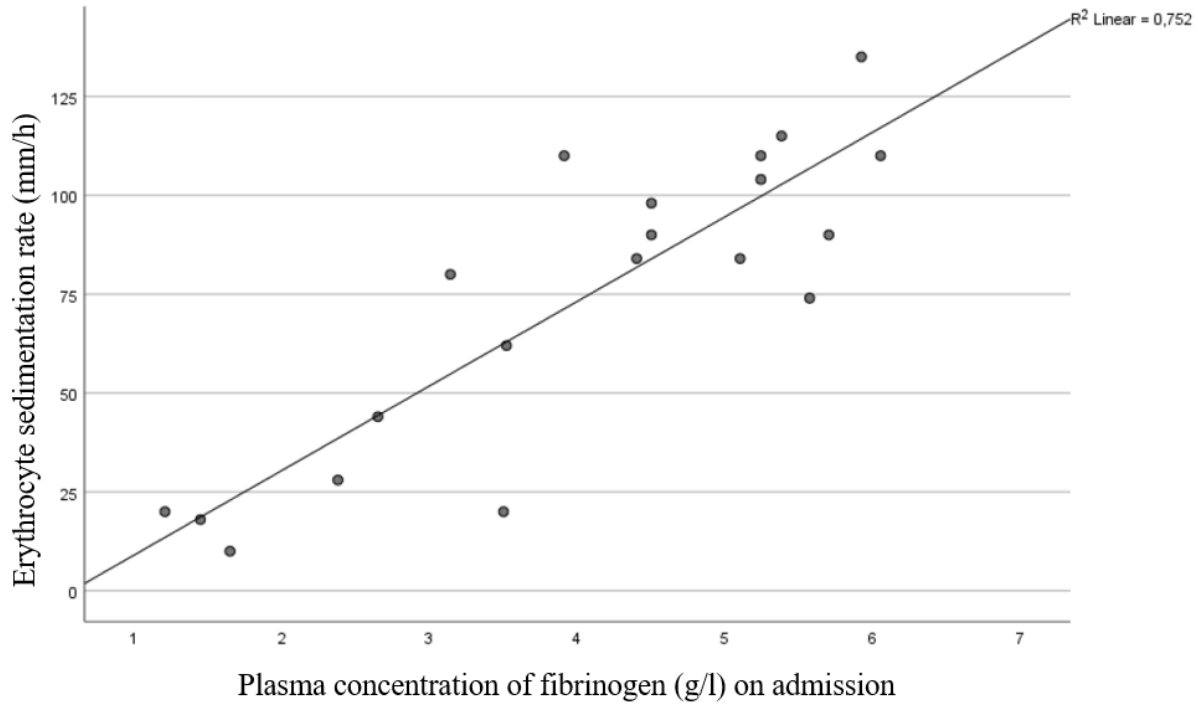

Figure 16. *Correlation between ESR and plasma fibrinogen level in COVID-19 patients on admission to ICU (p<0.001)*

3.4.2. Differences between the survivors and non-survivors

Sadly, only three patients were discharged from the hospital alive, while a total of 18 patients died despite intensive care and organ support. We did not find a significant difference in platelet count between survivors and nonsurvivors (p=0.008). On the contrary, 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 17.*** As an interest, we found that activated partial thromboplastin time (aPTT; sec) was lower in those who died compared to patients who left the hospital (13, 12.3–14.0 vs. 15.8, 14.95–26.35; p = 0.024). A significant negative correlation was seen between H-IPF (%) and plasma plasminogen (%) among non-survivors (r=-0.572, p=0.002), but this tendency was not presented in survivors, as shown in ***Figure 18.***

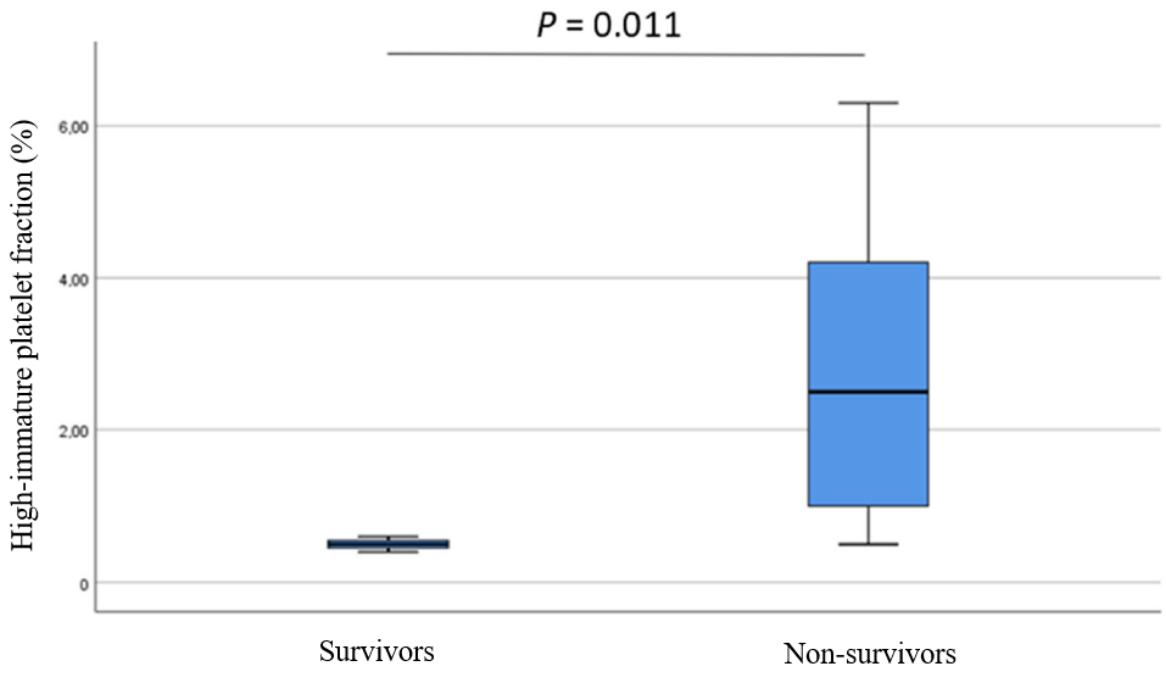

Figure 17. *High-immature platelet fraction (%) in survivors and non-survivors*

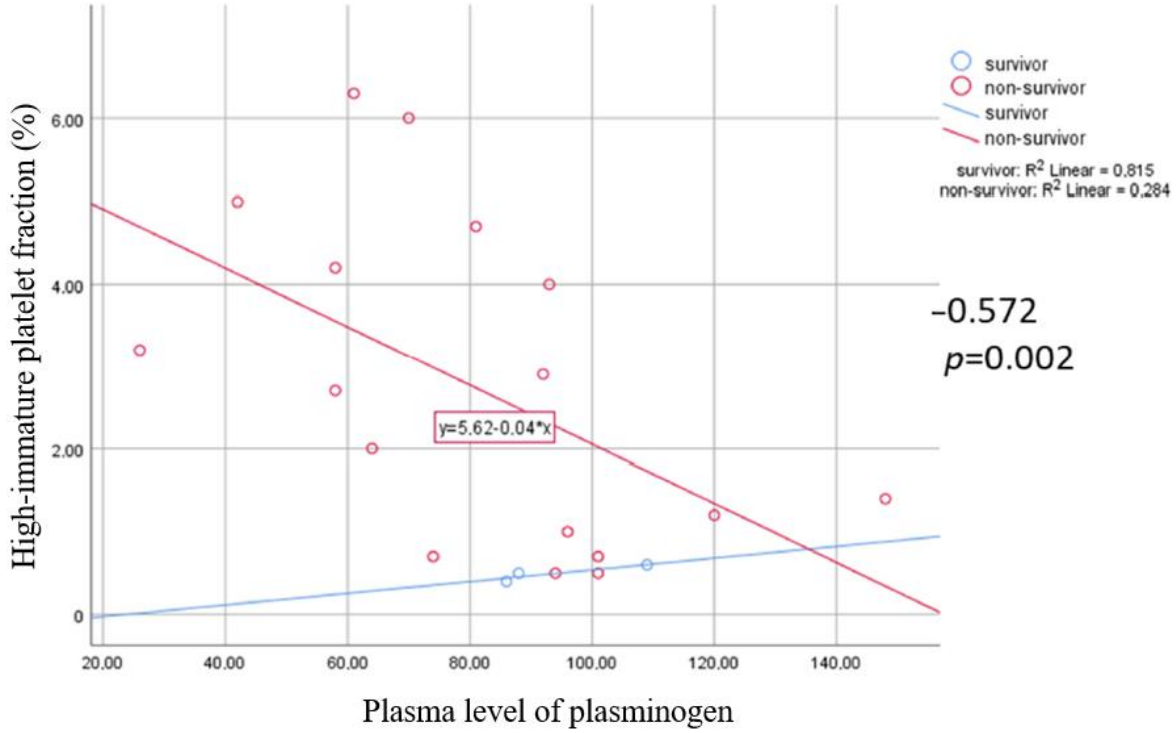

Figure 18. *Correlation of high-immature platelet fraction (H-IPF; %) and plasma plasminogen level in survivors (blue line) and non-survivors (red line). Rho and p indicate a negative correlation among the nonsurvival subgroup*

3.4.3 Responders and low-responders to aspiring among patients

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 19.*** below.

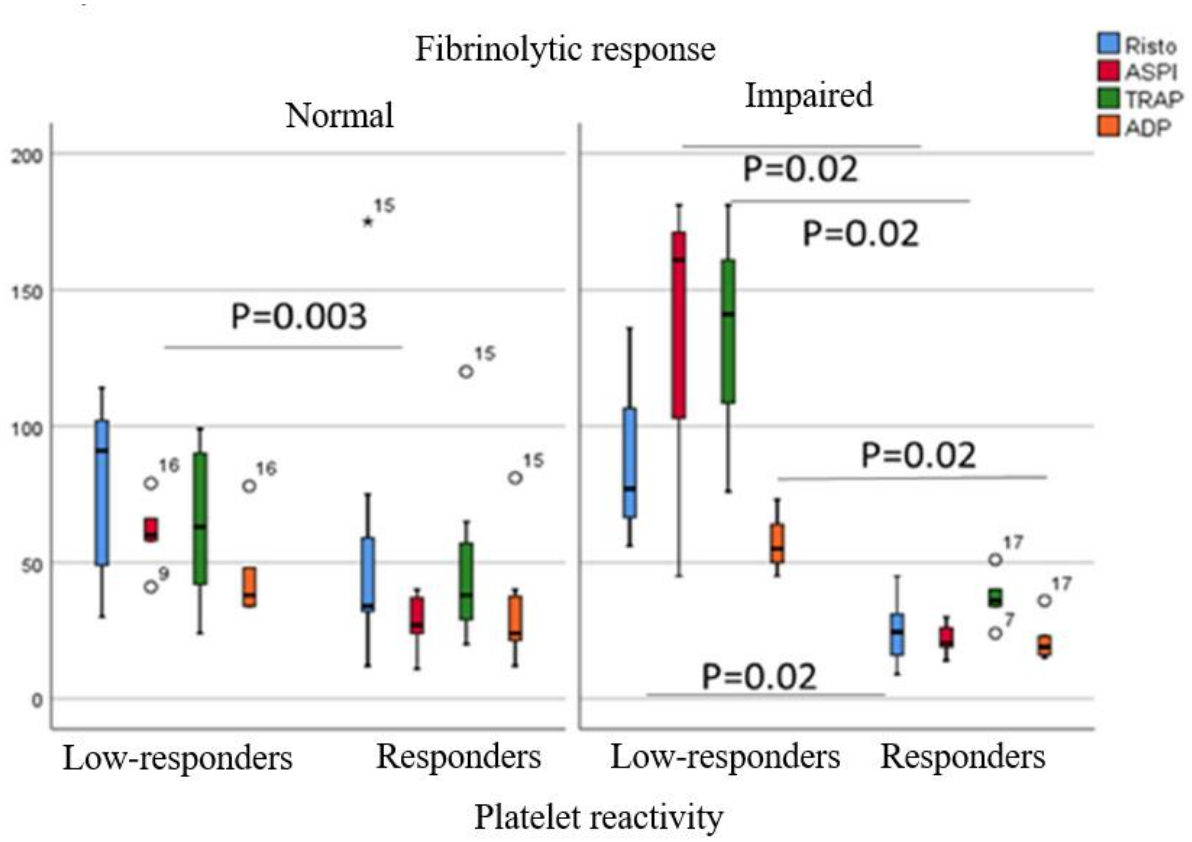

Figure 19. *Comparison of Risto-, ASPI-, TRAP, and ADP-tests (AUC) in patients with normal and impaired fibrinolytic response, dichotomized based on their responsiveness to aspirin (low-responders and responders). Performed by Mann-Whitney test (Asterix and white circles indicate extreme values)*

Neither thromboembolic events related to COVID-19 (p=0.071) nor survival rate (p=0.854) showed associations with high-on aspirin platelet reactivity status. Platelet count (p=0.03), ECA-A10 (p=0.008), and ECA-MCF (p=0.016) were significantly higher, while the tPA-CFT (p<0.001) was significantly lower among patients with high-on aspirin platelet reactivity. In addition, platelet count showed but positive correlation with AUC by Risto-

(0.500, p=0.021), ASPI- (0.500, p=0.021), TRAP- (0.760, p<0.001), and ADP-test (0.621, p=0.003). Meanwhile, H-IPF was negatively correlated with the AUC by TRAP- (-0.559, p=0.008) and ADP-tests (-0.530, p=0.013). The acute COVID-19-related thromboembolic events were defined as acute coronary syndrome (n=2), pulmonary embolism (n=4), and ischemic stroke (n=2). Both vWF antigen and vWF ristocetin cofactor activity showed positive correlations with H-IPF (p=0.016), and platelet count was significantly higher in patients with HAPR (p=0.03).

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 20.*** If we used the suggested AUC <71 as the cut-off value, the tPA lysis time (LT) tended to increase among ASA responders (p=0.06) compared to ASA low-responders, and eight of these patients showed the character of „fibrinolysis shut down" phenomenon.

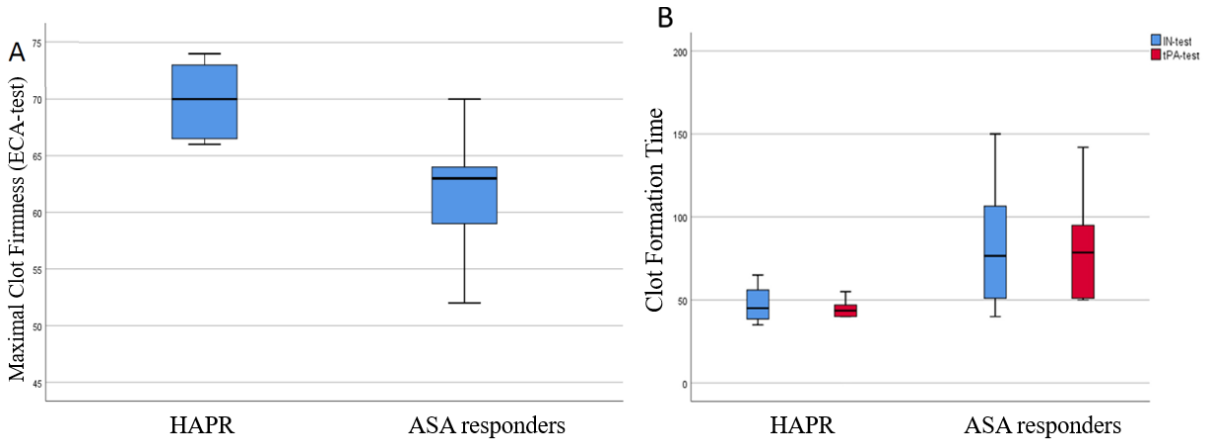

Figure 20. ***A****: Maximal clot firmness (MCF) measured by ECA-test in patients with high-on aspirin platelet reactivity (HAPR) and aspirin (ASA) responders (Mann-Whitney test, p=0.016);* ***B****: clot formation time (CFT) measured by IN- and tPA-test in patients with high-on aspirin platelet reactivity (HAPR) and aspirin (ASA) responders (Mann-Whitney test, p=0.039 and p<0.001, respectively)*

3.4.4. Characteristics of platelet count in COVID-19 patients

Patients were categorized into further subgroups based on the initial platelet count on admission (thrombocytopenia was declared as PLT <150 G/l, and normocythemia was stated as PLT >150 G/l). Both von Willebrand factor antigen (vWF:Ag) and von Willebrand factor ristocetin cofactor activity (vWF:RCo) were significantly higher in patients - separately from the platelet count – in comparison with healthy subjects (p<0.001). In contrast, the plasma level

of plasminogen - but not alpha$_2$-antiplasmin – was significantly lower among patients with thrombocytopenia, represented in ***Figure 21.***

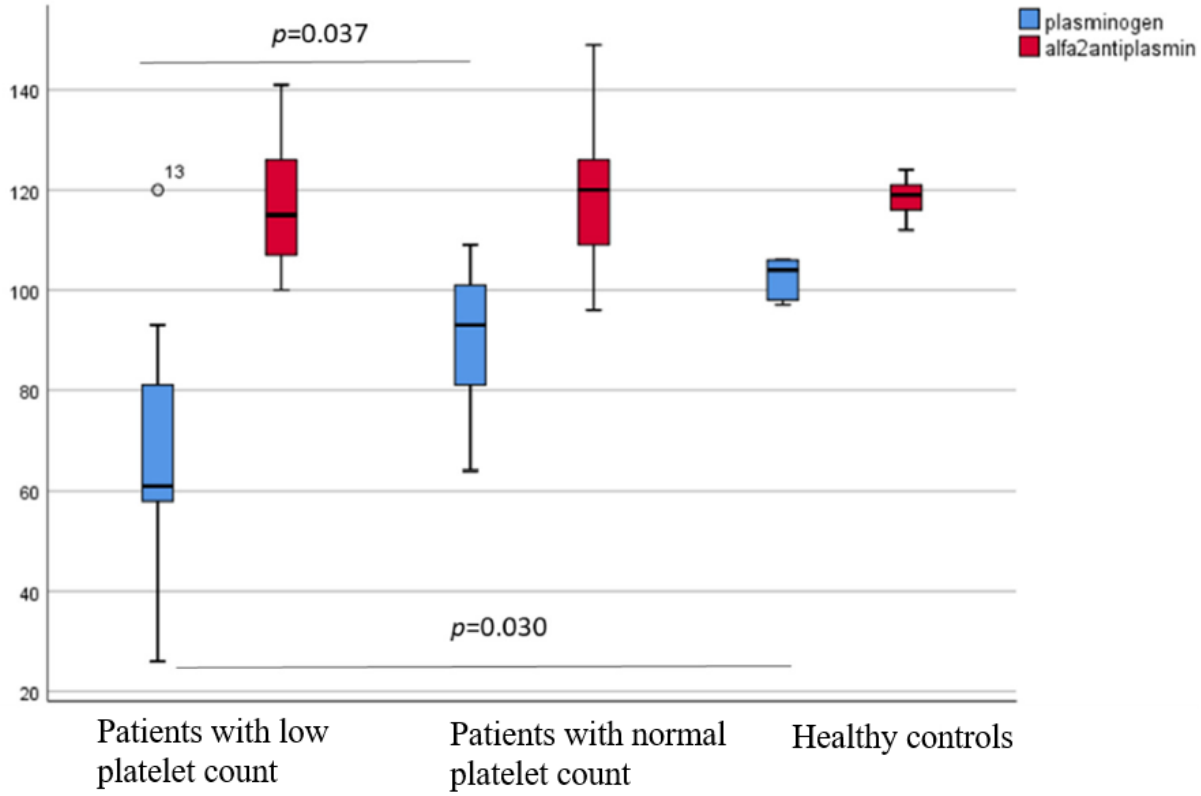

Figure 21. *Plasma plasminogen and alpha$_2$-antiplasmin levels (%) in patients with thrombocytopenia, normal platelet count, and healthy controls*

Additionally, significantly lower MCF values were detected in ECA- and IN-tests among patients with lower platelet counts (p=0.004, respectively), while higher FIB- and tPA-test results were observed in patients with normal platelet count in comparison with healthy subjects (P=0.014 and p=0.030, respectively) – it is visualized in ***Figure 22.***

Alongside, patients were dichotomized repeatedly based on their fibrinolytic response (to tPA). Thenceforth, the low platelet count subgroup was compared with the normal platelet count subgroup. In patients with impaired fibrinolytic response, the AUC measured by ADP and ASPI (P=0.030) were significantly higher than those with normal platelet count. Conversely, in the normal fibrinolytic subgroup of patients, only AUC measured by ADP-test showed a significant difference (p=0.040) – visually displayed in ***Figure 23.***

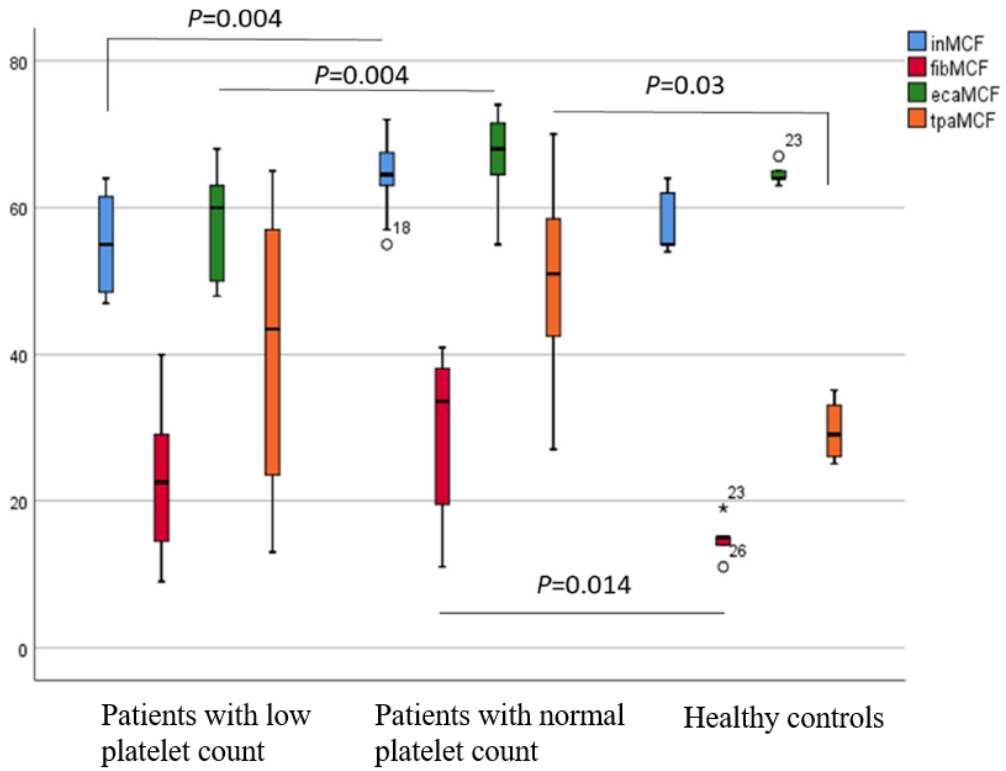

Figure 22. *Maximal clot firmness (MCF) measured by IN-, FIB-, ECA-, and tPA-tests in patients with low- and normal platelet count and healthy volunteers*

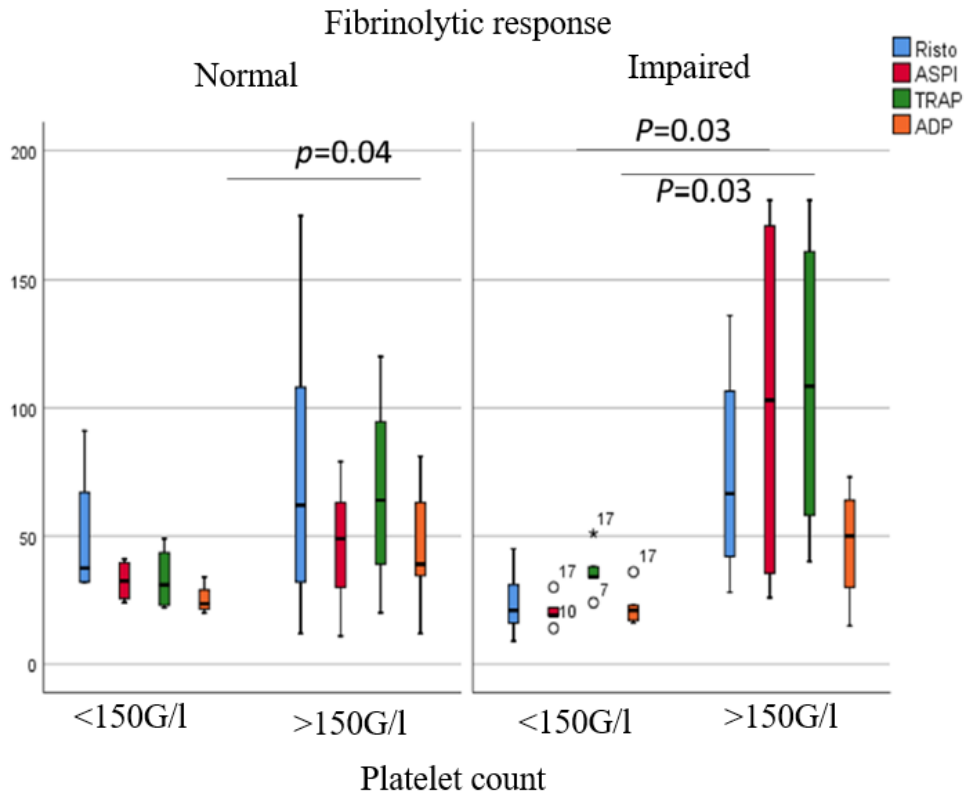

Figure 23. *Comparisons of AUCs measured by Risto-, ASPI-, TRAP- and ADP-tests in patients with low- and normal platelet count, patients were dichotomized upon their fibrinolytic response to tPA*

3.4.5. Independent predictors of impaired fibrinolytic response

We ran a separate analysis with hypofibrinolysis as the outcome of interest. Based on binary logistic regression analysis, including age, gender, fibrinogen level, D-dimer level, and aspirin responsiveness based on impedance electrode aggregometry by Multiplate®. Only fibrinogen (OR: 3.55, 95% CI: 1.33-9.47; p=0.010) proved to be an independent predictor of hypofibrinolysis in critically ill COVID-19 patients. 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 24.***

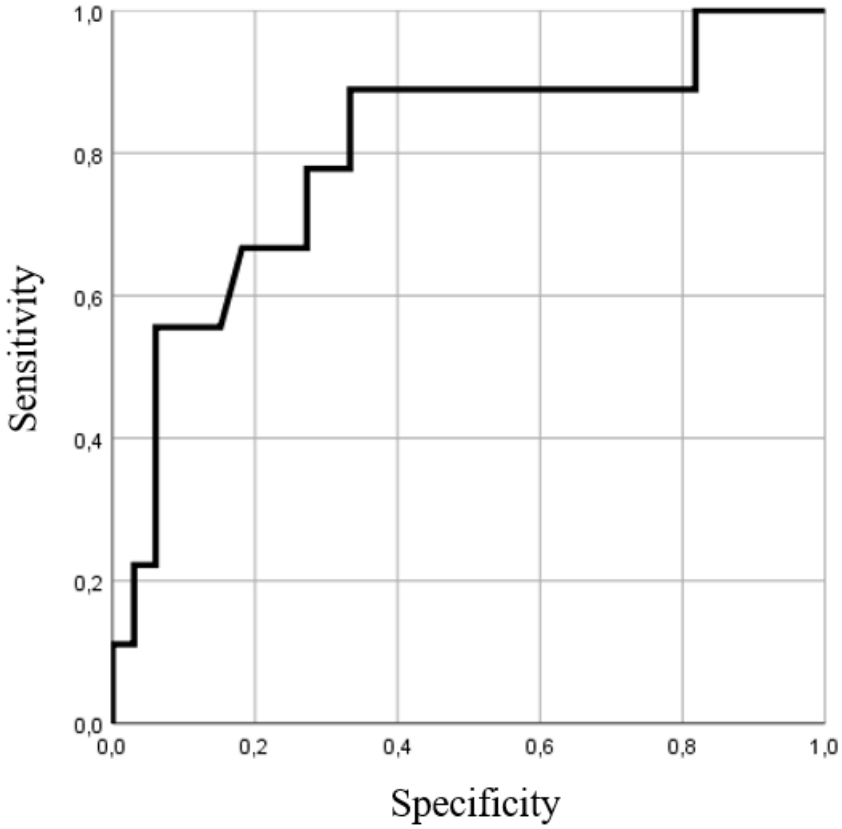

Figure 24. *Plasma fibrinogen level predict hypofibrinolysis with a sensitivity of 78% and a specificity of 73%*

3.5. Discussion

Regardless of the tiny 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[131–133]. 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[134]. 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. 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; increased activation and aggregation partially could be attributed to the increased level of MAPK intracellular signaling pathway activation and increased thromboxane production[135]. The presumed concept of platelet activation and aggregation was supported by the results of Bachler et al. as well[136]. 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[134]. 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[137]. 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. Based on our findings, besides platelet count and activation itself, the maximal clot firmness depends on various other influencing factors, like plasminogen and the von Willebrand factor. 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)[138]. 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.

Besides, ESR was significantly higher in severe SARS-CoV-2 infection and showed a strong positive correlation with fibrinogen concentration. Identical data were presented by Genry et al., who performed a meta-analysis involving twenty-one studies, showing that inflammatory markers such as ESR, CRP, procalcitonin, serum ferritin, IL-6, and IL-2R were significantly higher in patients with critical and fatal COVID-19[139]. Another systematic review and meta-analyses detected that ESR positively correlated with disease severity[140].

Wide-spectrum of researches support CRP as an appealingly sensitive systemic marker of acute-phase response in infection, inflammation, and tissue damage[141]. Elevated serum CRP level has been suggested in many studies as a reliable indicator of the presence and severity of SARS-CoV-2 infection[142,143].

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[144]. 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[144]. Furthermore, Corrado et al. found that nonsurvivors exhibited rapidly increasing D-dimer

kinetics[145]. Due to this cohort's meager survival rate, independent mortality predictors could not be analyzed here.

We detected reduced activated partial thromboplastin time (aPTT) in nonsurvivors, 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[146]. In our cohort, the thromboembolic complications were only associated with reduced clotting time using the FIB-test.

In fact, many patients with COVID-19 died due to thromboembolic complications of the disease. Della-Morte et al. hypothesized plasminogen as the precursor of fibrinolysis[147]. They supported our findings because they found that low levels of plasminogen strongly correlated with mortality. Recently, plasminogen was suggested to play a pivotal role in controlling the complex mechanisms beyond infection and its complication, so that it might be a useful prognostic marker and a potential therapeutic target as well[148].

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[149]. Accordingly, we also found a positive correlation between H-IPF (%), vWF antigen, and activity among patients with high-on aspirin platelet reactivity. 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[149].

3.6. Conclusion

In contrast with the responder subgroup, a faster-developing, more solid, and more extensive clot formation was observed in aspirin low-/nonresponder COVID-19 patients. Based on viscoelastic measurements, the clot seemed more resistant to lysis in the low-/nonresponders (more extended LT), suggesting that this cluster of patients might belong to the hypofibrinolytic subgroup, presenting the fibrinolysis shut-down phenomenon, but this statement still requires further validation. Nevertheless, several pathophysiological aspects should be considered in viscoelastic studies because activation of the endothelium and platelets entering the clot is not

registered in vitro. Though our results suggest the necessity of an individual approach regarding antiplatelet and anticoagulant therapy might be pivotal to preventing thromboembolic complications and decreasing mortality.

3.7. 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.

3.8. Future perspectives

While vaccines have been developed and efforts have been performed to control the spread of the virus have been partially successful, COVID-19 continues (maybe with less severe cases) and has a significant public health threat as well. It is impossible to predict with certainity whether there will be future pandemics or not. Still, we must prepare for them due to the world's increasing interconnectedness through international travel and trade, allowing diseases to spread more quickly. Destruction of natural habitats may also lead to a new pandemic. Because of this constant threat, it is pivotal to understand disease pathogenesis in more detail. We plan to present unpublished findings in further scientific paper to provide a better understand to the disease, and its complications.

4. Summary of novel findings

4.1. 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

4.2. 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

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List of publications

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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)

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frontiers in Neurology

ORIGINAL RESEARCH
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Novel Predictors of Future Vascular Events in Post-stroke Patients—A Pilot Study

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Introduction: A modified platelet function test (mPFT) was recently found to be superior compared to impedance aggregometry for selection of post-stroke patients with high on-treatment platelet reactivity (HTPR). We aimed to explore some peripheral blood cell characteristics as predictors of recurrent ischemic episodes. The predictive value of mPFT was also assessed in a cohort followed up to 36 months regarding recurrent ischemic vascular events.

Methods: As a novelty, not only whole blood (WB), but after 1-h gravity sedimentation the separated upper (UB) and lower half blood (LB) samples were analyzed including neutrophil antisedimentation rate (NAR) in 52 post-stroke patients taking clopidogrel. Area under the curve (AUC, AUC_{upper} and AUC_{lower}, respectively) was separately measured by Multiplate in the WB, UB and LB samples to characterize *ex vivo* platelet aggregation in the presence of ADP. Next, the occurrence of vascular events (stroke, acute coronary syndrome, ACS) were evaluated during 36-month follow-up.

Results: A total of 11 vascular events (stroke $n = 5$, ACS $n = 6$) occurred during the follow-up period. The AUC_{upper} was significantly higher in patients with recurrent stroke compared to those with uneventful follow-up ($p = 0.03$). The AUC_{upper} with a cut-off value $\geq$70 based on the mPFT, was able to predict all stroke events ($p = 0.01$), while the total vascular events were independently predicted by NAR with a sensitivity of 82% and specificity of 88%.

Conclusions: A combination of NAR reflecting the inflammatory state and AUC_{upper} indicating HTPR may provide a better prediction of recurrent ischemic events suggesting a better selection of patients at risk, thus providing an individually tailored vascular therapy.

Keywords: recurrent stroke, vascular event, platelet function, platelet reactivity, outcome

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INTRODUCTION

Despite successful recanalization strategies either with thrombolysis or using endovascular treatments for acute ischemic stroke, the eventual outcome of patients is far from desirable (1). Among many factors, some peripheral blood cells may play a pivotal role in post-procedural microcirculatory alterations contributing to the outcome (2, 3). A higher incidence of recurrent

cerebral ischemia was described in post-stroke patients with high on-treatment platelet reactivity (HTPR) (4, 5). Numerous tests assessing *ex vivo* platelet reactivity were used for identification of patients at risk for HTPR (6). However, the prevalence of HTPR was shown to vary depending on the definition and assay used (7). A modified platelet function test (mPFT) was recently found to be superior compared to conventional Multiplate Electrode Aggregometry for selection of post-stroke patients with HTPR (8).

Therefore, we aimed to explore some peripheral blood cell characteristics including platelets and neutrophils as predictors of recurrent ischemic episodes and factors contributing to the outcome. The predictive value of the mPFT as a point-of-care test (POCT) was also compared to conventional Multiplate Electrode Aggregometry in a cohort followed up to 36 months regarding recurrent ischemic vascular events.

MATERIALS AND METHODS

Subjects

The study protocoll was approved by the University of Pecs Clinical Centre Regional and Institutional Research Ethics Comittee (8). Written informed consent was obtained from each patient. A total of 52 patients (age: 66 ± 8 years, male: 31) on antiplatelet therapy (75 mg clopidogrel once daily) due to secondary stroke prevention were prospectively recruited into this study. The selected patients with previous anterior circulation large artery atherothrombosis were on regular medical check-up at the Outpatient Clinic of the Department of Neurology. Fasting venous blood samples were taken *via* a 21G peripheral venous canula from each patient and healthy subjects. Patients were instructed to take their daily clopidogrel at least 2 h prior to blood sampling. Exclusion criteria were acute infection and acute vascular events, such as acute ischemic stroke (AIS), transient ischemic attack (TIA), acute myocardial infarction (AMI), acute coronary syndrome (ACS), thrombocytopenia (platelet count <150G/l), congenital platelet abnormalities, congenital disorder of haemostatis (e.g., hemophilia), anemia and patients on medical therapy influencing blood coagulation (e.g., oral anticoagulants, novel oral anticoagulants, non-steroid antiinflammatory drugs). The comorbidities, medications and smoking status were also recorded. Besides, the baseline erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), and total blood count were measured. Next, the incidence of vascular events (ACS and recurrent ischemic stroke) in the total study population was evaluated in a 36-month follow-up. ACS was defined by using the ACC/AHA guidelines (shortly: based on clinical history, ECG results, levels of cardiac markers, and the results of stress testing). Each recurrent ischemic stroke was confirmed by neuroimaging (CT or MRI). All patients with either ACS or recurrent ischemic stroke were presented at the Emergency Department and underwent a careful clinical evaluation then archived by an electronic database.

Blood Sampling

Venopuncture was performed from the cubital vein after short time strangulation of the arm with 21G BD vacutainer needle. The total blood count was measured after taking into vacutainers with EDTA (REF: 368856, 5.4md EDTA). Whole blood for platelet aggregometry was also taken into hirudin containing tube for Multiplate Electrode Aggregometry.

Platelet Antisedimentation Rate, Neutrophil Antisedimentation Rate

Modified whole blood gravity sedimentation technique was developed for studying platelet and neutrophil sedimentation properties (8). After 1-h gravity sedimentation, the upper and lower half of the venous blood column was separately removed from the EDTA sedimentation tube and transferred to another EDTA tube for further analysis. An automatic cell counter system (Sysmex XN 9000, Sysmex Co, Japan, 2017) was applied to measure the upward floating (ascending) and sinking (non-ascending) cells in the separated samples. Next, the platelet antisedimentation rate (PAR, %), leukocyte antisedimentation rate (LAR,%) and neutrophil antisedimentation rate (NAR, %) were, respectively, calculated based on the equation:

$$\frac{\text{cell count}_{\text{upper}} - \text{cell count}_{\text{lower}}}{\text{cell count}_{\text{upper}} + \text{cell count}_{\text{lower}}} \text{X100}$$

Multiplate Electrode Aggregometry

Platelet function test in the whole blood was perfomed from a hirudin containing tube with a Multiplate® Analyzer (Roche Diagnostics, Mannheim, Germany). Another hirudin containing tube was used for sedimentation, similarly to whole blood sedimentation in the EDTA-tube. After 1-h gravity sedimentation, the blood coloumn was devided into upper and lower samples. Platelet aggregometry was uniformly performed 60 min after blood sampling using adenosine diphosphate (ADP; 6.5 M) as agonist. As a novelty, not only whole blood, but after 1-h gravity sedimentation the separated upper and lower half blood samples were simultaneously analyzed in each post-stroke patient taking clopidogrel. Aggregation level was expressed as the area under the curve (AUC). AUC was calculated by a Multiplate® Analyzer using the product of aggregation unit (AU) × time (minutes) (9). After ADP stimulation, the normal aggregation range was expected as AUC: 53–122 according to the manufacturer (9). Based on the whole blood AUC, patients on clopidogrel were categorized as responder cases with AUC <53 and resistant cases representing HTPR with an AUC ≥53 (10).

Statistical Analysis

Data were evaluated using SPSS software package (Version 19.0, SPSS Inc, Chicago, USA). Categorical data were summarized by means of absolute and relative frequencies (counts and percentages). Quantitative data were presented as median and 25th−75th percentiles, as well as mean ± SD. The Kolmogorov-Smirnov test was applied to check for normality. Chi-square test for categorical data and Student-*t* test for continuous data were used for analysis of demographic and clinical factors. Non-parametric Mann-Whitney *U* test was used for not normally distributed parameters. Correlation analysis was performed calculating Spearman's correlation coefficient (rho). A *p*-value <0.05 was considered statistically significant.

TABLE 1 | Demography and clinical data of the total population, and comparison between patients without vs. with recurrent vascular events during 36-month follow-up.

	Total population $n = 52$	Uneventful $n = 41$	Vascular events $n = 11$	*p*-value
age	66 ± 8	66 ± 8	66 ± 9	0.937
male, *n*	34	26	8	0.564
hypertension, *n*	51	40	11	0.601
diabetes mellitus, *n*	14	10	4	0.427
smoking, *n*	11	9	2	1.000
ESR	12 (8–18)	10 (8–16)	18 (14–29)	0.063
CRP	1.9 (0.7–4.6)	1.8 (0.7–5.0)	2.2 (1.4–3.35)	0.614
PLT	224 (200–260)	224 (207–251)	243 (171–300)	0.805
PAR	67.9 (63.1–73.4)	67.8 (62.9–73.5)	70.0 (64.6–72.6)	0.614
WBC	6.8 (5.8–8.0)	6.6 (5.8–7.9)	7.4 (5.5–10.6)	0.420
LAR	35.7 (23.7–46.3)	36.2 (24.7–46.4)	34.4 (24.0–43.5)	0.806
neutrophil	61.8 (55.4–66.4)	62 (56–67)	58 (51–62)	0.317
NAR	−1.1 (−4.8–6.5)	0.9 (−3.9–7.2)	−5.2 [−6.8–(−4.7)]	0.001

Vascular events, recurrent stroke, and de novo acute coronary event; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; PLT, platelet; PAR, platelet antisedimentation rate; WBC, white blood cell; LAR, leukocyte antisedimentation rate; NAR, neutrophil antisedimentation rate. Data are presented as median and 25th−75th percentiles, except age as mean ± SD.

TABLE 2 | Area under the curve (AUC) in the whole blood, and AUC in the upper and lower samples after 1-h gravity sedimentation in the total population and comparison between uneventful vs. stroke + ACS as well as uneventful vs. recurrent stroke subgroups.

	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)	18 (14–23)	17 (13–20)	0.567
	Total population $n = 52$	**Uneventful $n = 41$**	**Stroke events $n = 5$**	***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 (14–22)	17 (11–19)	0.763

AUC, area under the curve measured by Multiplate analyzer; AUC_{upper}, AUC in the upper sample after 1-h gravity sedimentation; AUC_{lower}, AUC in the lower sample after 1-h gravity sedimentation.

TABLE 3 | Predictors of vascular events during 36-month follow-up.

	β	*p*-value	OR	95% CI	
age	−0.071	0.353	0.931	0.801	1.082
AUC	−0.046	0.320	0.955	0.871	1.046
AUC_{upper}	−0.083	0.031	1.086	1.007	1.171
NAR	−0.489	0.032	0.613	0.392	0.960

AUC, area under the curve measured by Multiplate analyzer; AUC_{upper}, AUC in the upper sample after 1-h gravity sedimentation; NAR, neutrophil antisedimentation rate; OR, odds ratio, 95%CI, 95% confidence interval. Binary logistic regression analysis.

RESULTS

A total of 52 convalescent ischemic stroke patients were prospectively enrolled into this pilot study. All patients have been previously suffered from large vessel occlusion. The demography and clinical data of the study population is summarized in **Table 1**. A total of 11 vascular events (stroke n=5, ACS n=6) occured during 36-month follow-up. Of the antisedimentation rate indices, only NAR showed significant difference between "uneventful" vs. "vascular events" groups (**Table 1**). It is noteworthy that no difference was observed between the baseline blood count parameters (platelet, leukocyte, neutrophil), while a trend-like difference was observed in the ESR (**Table 1**). The AUC in the whole blood, and in the upper and lower samples after 1-h gravity sedimentation in the total population, and also a comparison between uneventful vs. stroke + ACS as well as uneventful vs. recurrent stroke alone subgroups are shown in (**Table 2**). The AUC_{upper} was significantly higher in patients with recurrent stroke compared to those with uneventful follow-up ($p = 0.03$) (**Table 2**).

Independent Predictors

Based on ROC analysis, the AUC_{upper} with a cut-off value ≥70 measured by the mPFT was able to predict recurrent stroke events ($p = 0.01$) with the best sensitivity and specificity. Moreover, the total vascular events (stroke+ACS) was independently predicted by NAR with a sensitivity of 82% and specificity of 88% using a multiple regression analysis including relevant covariates (**Table 3**). Neither recurrent stroke nor ACS showed association with HTPR status defined by AUC>53 measured by the Multiplate in the whole blood.

Cut-Off Values of Predictors

The ROC curves of variables predicting recurrence of vascular events during follow-up are shown in **Figure 1**. In this cohort, NAR with a cut-off ≥ −0.431 independently predicted 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) (**Figure 1A**). Furthermore, ROC of platelet function test based on impedance aggregometry in the upper blood sample after 1-h gravity sedimentation revealed, that AUC_{upper} with a cut-off ≥ 70 predicted recurrent 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) (**Figure 1B**). Finally, a more precise model was created, when a ROC analysis was performed with predicted probablity of the combination of NAR and PFT_{upper} (Area:0.881, $p = 0.001$, 95%CI:0.754–1.0) (**Figure 1C**).

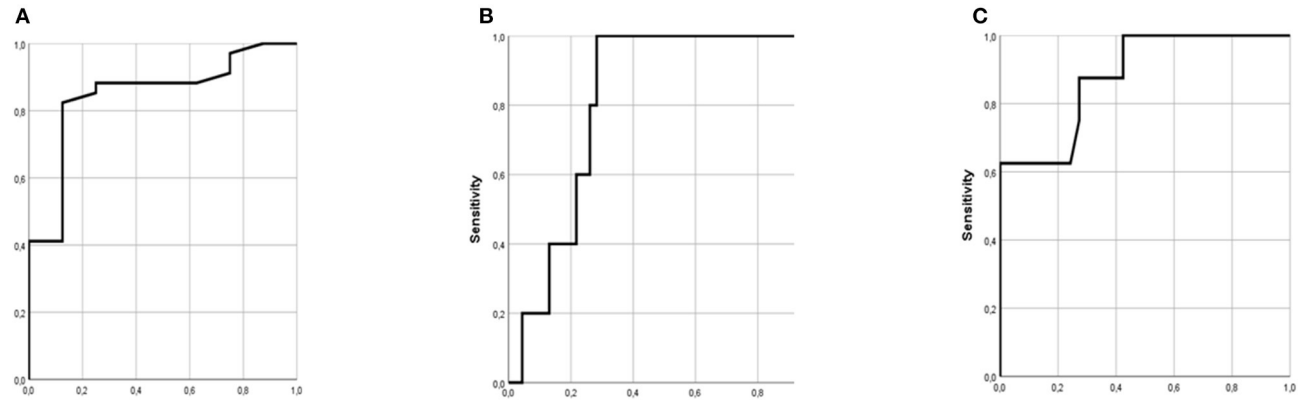

FIGURE 1 | ROC curves of variables predicting recurrence of vascular events during follow-up. **(A)** ROC of neutrophil antisedimentation rate (NAR) (Area: 0.847, $p = 0.002$, 95%CI: 0.703–0.992). **(B)** ROC of platelet function test based on impedance aggregometry in the upper blood sample (AUC_{upper}) after 1-h gravity sedimentation (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} (Area: 0.881, $p = 0.001$, 95%CI: 0.754–1.0).

DISCUSSION

Activation of neutrophils reflected by NAR was shown here as the most sensitive marker of recurrence of ischemic cerebral episodes in post-stroke patients taking clopidogrel. Both, animal and clinical data support the pivotal role of activated peripheral blood cells (e.g., neutrophils, monocytes, platelets) in neuroinflammation due to ischemic stroke (2, 3, 11). One side, the dynamic microcirculatory stall phenomenon in the hyperacute stage can be a contributing factor to ongoing penumbral brain injury (2, 12), on the other side the sustained detrimental effects of activated leukocytes in the systemic circulation carries a constant risk in patients with chronic inflammatory state (e.g., vascular diseases) (13). Interestingly, a downward motion of neutrophils during 1-h gravity sedimentation expressed by a negative value of NAR was observed in those patients who suffered from composite vascular events during 36-month follow-up. In contrast, an upward motion of both, leukocytes and platelets proportionally to their activation was described previously in acute ischemic stroke (3), post-stroke infection (14) and burn patients (15). Neither LAR, nor PAR was found to be predictive for future vascular events in convalescent stroke patients suggesting that leukocytes and platelets exert their actions predominantly in the acute phase of stroke. Our finding also suggests that neutrophils are important markers of stroke outcome as their predictive role was recently shown in patients with acute coronary syndrome (16).

Numerous data highlight that a high proportion of patients with cardiovascular diseases have *ex vivo* HTPR on their prescribed antiplatelet regimen (4, 5, 7). Although several studies show an increased rate of recurrent cerebrovascular ischemic events in patients presenting HTPR, the diagnostics of HTPR has been unsolved so far (4, 17). Here, the state of clopidogrel resistance based on Multiplate electrode aggregometry from the whole blood was not able to predict recurrent stroke. However, a higher AUC (≥70 as a cut-off value) from the separated upper blood sample after 1-h gravity sedimentation emerged as a novel independent predictor of future stroke episode in our study. This observation suggests that the upward motion of platelets might be associated with increased thrombotic tendency. Further studies are needed to explore the characteristics of this subpopulation of platelets and their impact on post-stroke complications and outcome. When the combination of NAR and PFT_{upper} was used in the statistical model, the predicted probability of a future vascular event was even more accurate.

In summary, while AUC_{upper} indicates more precise definition of HTPR, NAR rather reflects the inflammatory state in post-stroke patients (18). Based on this small, single-center pilot study, these novel markers may provide a better prediction of recurrent ischemic events leading to a better selection of patients at risk and providing an individually tailored vascular therapy including antiplatelet and anti-inflammatory regimens (17, 19).

LIMITATIONS

This is a small prospective cohort with a 36-month follow-up focusing primarily on recurrent coronary and cerebral ischemic episodes which required hospitalization. However, the silent ischemic lesion recurrence on MRI was not explored here. Therefore, a large, adequately sized, prospective multicenter study is needed to determine whether these novel assessments of HTPR in conjunction with pharmacogenetic and neuroimaging (diffusion weighted imaging, DWI) data, improves our ability to predict the risk of recurrent vascular events in patients with cardiovascular diseases. Although the interaction between inflammation and ischemic stroke is multifaceted, a better understanding of such mechanisms may lead to enhanced secondary prevention including immunomodulatory approaches and more precise antiplatelet therapy (20, 21).

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Local Ethics Committee of the University of Pécs. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

DS, EE, LS, and TM conceived, designed and coordinated the study, participated in acquisition, and interpretation of data. DS and TM drafted the manuscript. MT-F performed the laboratory measurements. MT-F and TM participated in the statistical analysis. All authors read and approved the manuscript.

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Original Research

Circulating Microvesicles in Convalescent Ischemic Stroke Patients: A Contributor to High-On-Treatment Residual Platelet Reactivity?

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Abstract

Introduction: Exploration of novel and effective antiplatelet strategies for the secondary prevention of ischemic stroke is utmost. Some platelet derived microparticles (PMVs) in convalescent stroke subjects were found to be predictive for the next vascular event. Patients with high-on-treatment platelet reactivity (HTPR) had a significantly higher risk for ischemic stroke. Here, we aimed to explore associations among circulating microparticles and responsivness to antiplatelet (clopidogrel) therapy. **Methods**: A total of 18 patients on clopidogrel therapy due to secondary stroke prevention were rospectively recruited into this study. Twenty age-matched healthy subjects served as controls. Flow cytometric measurements of microparicles (MVs) and data analysis were performed on Beckman-Coulter FC-500 cytometer with CXP software. Besides, platelet aggregometry data were revealed. Both measurements were performed in whole blood and from the lower and upper blood fractions separated after 1-hour gravity sedimentation by the analogy with erythrocyte sedimentation rate. **Results**: The total number of circulating MVs, and particularly the platelet derived $CD42^+$ and $PAC\text{-}1^+$ were significantly higher in post-stroke patients ($p < 0.001$). The platelet aggregation in the whole blood (area under the curve, AUC) showed a significant negative correlation with the total number of MPs in the lower blood sample after 1-hour gravity sedimentation ($r = –0.650$, $p = 0.005$). Next, we analyzed associations among MPs and aggregometry data obtained from clopidogrel responders and non-responders. Both, area under the curve (AUC) and velocity in the whole blood showed opposite correlation with the total number of MVs in the lower blood sample after 1-hour gravity sedimentation. Importantly, a significant negative correlation was observed for the velocity ($r = –0.801$, $p = 0.005$), but not for the AUC in responders. Platelet derived $CD42^+$ and $PAC\text{-}1^+$ MVs showed positive correlations with neutrophils in the lower blood sample ($p = 0.008$ and $p = 0.006$ respectively). **Conclusions**: Circulating MVs may allow to monitor the response to antiplatelet therapy in post-stroke patients. In addition, the link between platelet derived MVs and neutrophil granulocytes might become therapeutic targets in the future.

Keywords: microvesicles; platelet; neutrophil; ischemic stroke; antiplatelet therapy; clopidogrel

1. Introduction

Stroke is a highly prevalent condition that puts a significant burden on most societies. It is the leading cause of adult disability, the second leading cause of dementia and the fourth leading cause of death worldwide [1]. The prevalence of stroke and stroke-related costs will undoubtedly rise as the ratio of aging population increases worldwide, making prevention and identification of early signs of recurrent ischemic episodes a priority [2]. Peripheral circulating microvesicles (MVs) are small particles (0.1–1.0 micrometer in diameter) derived from the membrane blebs of activated cells, it can be found in synovial fluid, tear, liquor, saliva, urine, breastmilk and in bronchoalveolar lavage as well due to peripheral microcirculation [3]. Circulating platelet MVs (PMVs) are the most abundant type of MVs found in human circulation and they express various platelet surface markers such as CD42a, CD42b, CD61, CD62P [4,5]. Microvesicles have multiple biological functions: (i) antigen presentation; (ii) intercellular communication; (iii) immune reaction and (iv) RNA and protein delivery. Thus, they enable intercellular communication by delivering lipids, proteins and genetic material to nearby or distant cells and modulating the functions of these cellular targets [6,7]. There is growing evidence that MVs are playing a pivotal role in regulation of hemostasis, inflammation and angiogenesis [8]. Previous studies suggest that a large increase in circulating platelet derived microvesicles (PMVs) have been observed in patients with cardiovascular disease [9]. Therefore, PMVs might be important biomarkers and tools in the identification of the risk of various recurrent cerebrovascular diseases [10]. Platelet antisedimentation rate (PAR) reflects the percentage of platelets

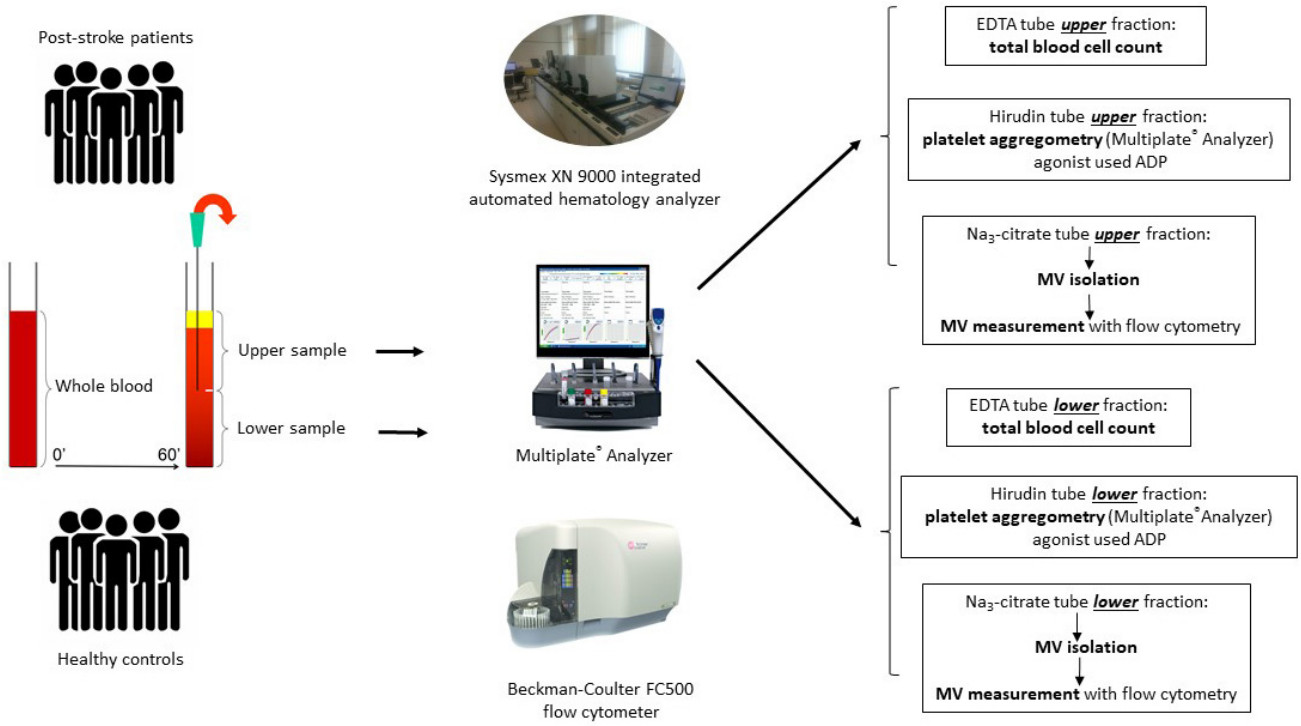

Fig. 1. Chart of sample preparation, separation of the upper and lower blood sample after 1-hour gravity sedimentation and further analytical process.

crossing the midline of the blood column upwards during 1-hour gravity sedimentation [11]. One of our previous studies concluded that the PAR value was able to discriminate clopidogrel non-responders from responders [11]. Activation of neutrophils reflected by neutrophil antisedimentation rate (NAR) proved to be a sensitive predictor of recurrent ischemic cerebral episodes in post-stroke patients on clopidogrel [12].

The aim of this prospective pilot-study was to explore: (i) differences of circulating MVs of different origin, comparing convalescent ischemic stroke patients vs healthy controls; (ii) whether the function of platelets correlate with certain MVs in patients on antiplatelet (clopidogrel) therapy; (iii) which PMVs show association with the high-on-treatment residual platelet reactivity (non-responder state).

2. Methods

2.1 Subjects

The study protocol was approved by the University of Pécs Clinical Centre Regional and Institutional Research Ethics Committee (Ref. number: 6735, Clinical Trial No: NTC03679858). All procedures were performed in accordance with the ethical guidelines of the 1975 Declaration of Helsinki. Written informed consent was obtained from both patients and healthy volunteers. A total of 18 patients (median age at 66 years, 12 males) on antiplatelet therapy (75 mg clopidogrel once daily) due to secondary stroke prevention were prospectively recruited into this study. The selected patients with previous anterior circulation large artery atherothrombosis were on regular medical check-ups at the Outpatient Clinic of the Department of Neurology at the University of Pécs.

2.2 Blood Sampling

Venous blood samples were drawn with 21G needle after short strangulation from an antecubital vein into closed blood collection system tubes with 3.2% (0.109 M) Na_3-citrate, K_3-EDTA (Becton Dickinson, Diagon LTD Hungary) and hirudin (Sarstedt S-Monovette® 1.6 mL Hirudin) as anticoagulant. Upon blood collection the first 3 mL blood was discarded. The blood samples for measurements were transported immediately to laboratory and were processed within 1 hour.

2.3 Neutrophil Count (%), Electric Impedance Aggregometry (Multiplate® Analyzer)

The modified whole blood gravity sedimentation technique was developed for studying platelet and neutrophil sedimentation properties [11]. After a 1-hour gravity sedimentation, the upper and lower half of the venous blood column were separately removed from the EDTA and hirudin containing tubes and transferred into another EDTA and hirudin tube for further analysis (Fig. 1). The total blood cell count and the neutrophil (%) count were measured from the whole blood and after 1-hour gravity sedimentation from the upper and lower part of the blood on a Sysmex XN 9000 integrated automated haematology analyser (Sysmex Co., Kobe, Japan, 2017).

Table 1. The selected CD markers for MV measurement, cellular origin, fluorescent dye and the manufacturer specification.

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

Abbreviations: Cy5, Cychrome5; FITC, fluorescein isothiocyanate; PE, phycoerythrin; Ig, immunoglobulin.

Platelet function test was performed in the whole blood and after 1 hour of sedimentation from the upper and lower part of the hirudin anticoagulated blood with a Multiplate® Analyzer (Roche Diagnostics, Mannheim, Germany). Platelet aggregometry was uniformly carried out 60 minutes after blood sampling using adenosine diphosphate (ADP; 6.5 M) as agonist [12]. Aggregation level was expressed as the area under the curve (AUC). AUC was calculated by a Multiplate® Analyzer using the product of aggregation unit (AU) $\times$ time (minutes). After ADP stimulation the normal aggregation range was expected as AUC: 53-220 — according to the manufacturer. Based on the whole blood AUC, patients on clopidogrel were categorized as responders with AUC $<$53 and non-responders with AUC $\geq$53.

2.4 Microvesicles (MVs) Measurement and Sample Preparation

After a one-hour sedimentation the upper and lower part of the citrated blood were centrifuged at 2500 $\times$ g for 20 minutes at room temperature. The supernatant was transferred into a new test tube and centrifuged at 2500 $\times$ g for further 20 minutes to obtain cell-free plasma. The top of the cell-free plasma was transferred into an Eppendorf tube and was immediately frozen on liquid nitrogen and stored at –80 °C until further use. MVs measurement was described in our previous article [13]. Briefly, the samples for measurement were thawed on melting ice and pelleted at 18000 $\times$ g for 10 minutes. The supernatant was carefully removed, leaving 25 μL of MV rich plasma at the bottom of the Eppendorf tube. MVs were suspended with gentle vortexing for 20 seconds in 1.0 mL Apo-binding buffer (10 mmol/L HEPES, 5 mmol/L KCl, 1 mmol/L $MgCl_2$, 136 mmol/L NaCl, pH = 7.4 HEPES was obtained from Sigma-Aldrich Ltd., Budapest, Hungary, other analytical grade reagents were obtained from Reanal Ltd., Budapest, Hungary) without $CaCl_2$. The selected CD markers, their cellular origin, the fluorescent dye used for labelling and the manufacturer specification for our MV measurements are summarized in Table 1. For sample labelling 10 μL MV in Ca^{2+} free buffer was incubated in 100 μL Apo-binding buffer supplemented with 2.5 mmol/L $CaCl_2$ with a total of 10 μL antibody, previously diluted to optimal labelling concentration. Staining was incubated for 30 minutes at room temperature in a dark chamber. All buffers were filtered through 0.2 μm membrane filters.

Flow cytometric measurements and data analysis were performed on a Beckman-Coulter FC-500 cytometer with CXP software (Version 2.3. Beckman Coulter Life Sciences, Indianapolis). The MV's reference gate was defined with Megamix beads (Biocytex). Side scatter, forward scatter and fluorescence channels were set in a logarithmic scale. MV size gate was determined between 0.5 μm and 1.0 μm size range. Events in the MV gate were further discriminated by labelling with Annexin [14]. MVs were defined as Annexin V positive events in the MV gate with fluorescence intensity above the isotype control. For determination of the MV number, known concentration (1 $\times$ 10^6/mL) of 3 μm diameter microbeads (Becton Dickinson) were used. To determine the optimal labelling concentrations all antibodies and Annexin V were titrated. Labelling concentrations were defined by antibody staining of samples and sample-free buffers in the presence or absence of $CaCl_2$. Labelling was considered optimal if $CaCl_2$ labelled sample measurement events were clearly distinguishable from background, $CaCl_2$ free staining, as well as from isotype controls.

2.5 Statistical Analysis

Statistical analysis was performed using SPSS version 23.0 (IBM Corporation, Armonk, NY, USA). Summary statistics of the participants were constructed using frequencies and proportions for categorical data and as mean and standard deviation (SD) for continuous variables. Conformity of data to normal distribution was determined by histogram and Kolmogorov–Smirnov test. The between-group difference was calculated with χ^2, Fisher's exact and Mann–Whitney U tests in line with suitability. Data with nonparametric distribution were presented as median and interquartile range (IQR). Correlations of microvesicle counts with aggregometry data (area under the curve, AUC; velocity of the slope of aggregation) were tested by linear regression using Spearman correlation coefficient (Rho). The significance level was considered as $p < 0.05$.

Table 2. Demography and baseline laboratory parameters.

Observed parameters	Post-stroke patients (n = 18)			Healthy controls (n = 20)	*p*-value
Age		66 (60–70)		57 (49–63)	0.078
Male/Female		2/6		10/10	0.298
MVs ($\times 10^5$/mL)	whole blood	upper sample	lower sample	whole blood	
Total MVs	3.43 (2.34–4.70)	1.79 (1.37–2.82)	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 (0.03–0.155)	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 (0.03–0.10)	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

MVs, microvesicles ($\times 10^5$/mL); CD31, endothel derived microvesicle; CD42a, glycoprotein Ib-V-IX complex on platelets; CD41, glycoprotein IIb/IIb integrin on platelets; PAC-1, fibrinogen binding site after activation of glycoprotein IIb-IIIa complex on platelets. Data are presented as absolute values or median (25th–75th percentiles), *p*-values indicate comparison of MVs in the whole blood.

3. Results

3.1 Demography and Baseline MV Values

A total of 18 convalescent ischemic stroke patients on clopidogrel (all patients suffered from large vessel occlusion) and 20 age-matched healthy subjects were recruited into this study prospectively. Demography of patients and healthy controls and baseline laboratory parameters of the study population are summarized in Table 2. There was no significant difference in regard to age and gender. The total number of circulating microvesicles ($p < 0.001$), and particularly the endothelial-derived $CD31^+$ ($p = 0.016$) and platelet derived $CD42a^+$ ($p < 0.001$) MVs measured in the whole blood were significantly higher in post-stroke patients compared to healthy subjects. Interestingly, $CD62P^+$ (P-selectin) positive MVs showed no significant difference between groups. All significant MV data from Table 2 are presented as Fig. 2.

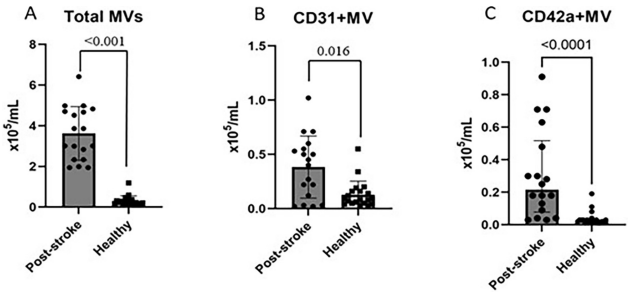

Fig. 2. Comparison of the total count of MVs (2A), the $CD31^+$ MVs (2B) and the $CD42a^+$ MVs (2C) in the whole blood of post-stroke patients and healthy subjects (Mann-Whitney U test).

3.2 Associations between MVs and Aggregometry Data

We analysed the correlation between MVs and aggregometry data. The platelet aggregation in the whole blood (area under the curve, AUC) measured by Multiplate® in patients taking clopidogrel, but not in age-matched healthy controls, showed a significant negative correlation with the total number of MVs ($\times 10^5$/mL) in the lower blood sample after 1-hour gravity sedimentation ($r = -0.650$, $p = 0.005$; Fig. 3). Nevertheless, we did not observe any correlation between the AUC and the total number of MVs in the whole blood.

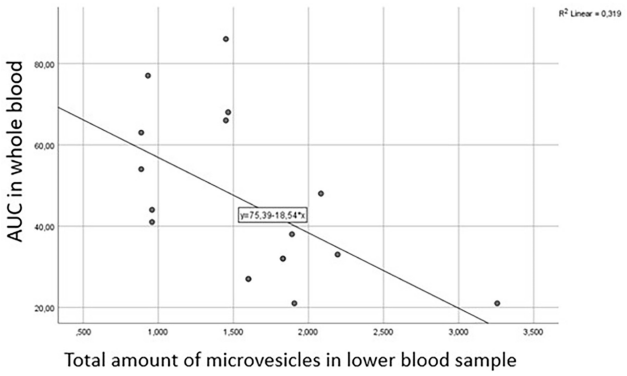

Fig. 3. Correlation between the area under the curve (AUC) in whole blood measured by Multiplate® and total number of microvesicles ($\times 10^5$/mL) in the lower blood sample after 1-hour gravity sedimentation in patients (Spearman correlation, $p = 0.005$).

3.3 Associations between MVs and Clopidogrel Responsiveness in Patients

Next, we explored the potential associations between MVs and aggregometry data (area under the curve, AUC and velocity respectively) obtained from clopidogrel responders and non-responders based on the previously defined cut-off value (AUC: 53). Both the AUC and the velocity in the whole blood showed negative correlation with the total number of MVs ($\times 10^5$/mL) in the lower blood sample

after 1-hour gravity sedimentation. Importantly, a significant negative correlation was observed for the velocity (r = –0.801, p = 0.005), but not for the AUC in responders (n = 11) (Fig. 4).

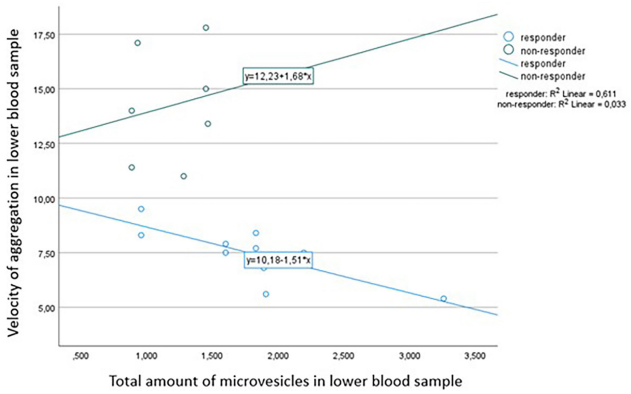

Fig. 4. Correlation between velocity measured by Multiplate® aggregometry and total number of microvesicles ($\times 10^5$/mL) in the lower blood sample in clopidogrel responders and non-responders respectively. Blue line indicates a negative correlation in responders (Spearman correlation, r = –0.801, p = 0.005).

3.4 Associations between MVs and Neutrophils in Patients

Activation-induced conformational epitope on CD41/CD61 complex positive (PAC-1$^+$) MVs in the lower blood sample showed a significant positive correlation with the percentage of neutrophil granulocytes in the lower blood sample after 1-hour gravity sedimentation (r = 0.634, p = 0.008; Fig. 5). In contrast, this positive correlation disappeared when whole blood indices were analysed. Furthermore, the constitutive platelet marker (CD42a$^+$) positive MVs measured in the upper blood fraction showed a significant correlation with the percentage of the neutrophils in the lower blood sample (r = 0.652, p = 0.006; Fig. 6). Nevertheless, no significant correlation was found in the whole blood samples.

4. Discussion

In accordance with a recently published meta-analysis [15], we found that the total number of circulating microvesicles, endothelial-derived CD31$^+$ and platelet derived CD42a$^+$ microvesicles were significantly higher in convalescent post-stroke patients when compared to age-matched healthy controls. Wang *et al.* [15] observed that pooled concentrations of total MVs (TMVs), endothelial-derived MVs (EMPs), platelet-derived MVs (PMVs), leukocyte-derived MVs (LMVs) and monocyte-derived MVs (MMVs) were significantly increased in ischemic stroke patients compared to the non-cerebrovascular disease controls [15].

We presumed that the quality (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 aggregometry reflected by AUC and the total number of MVs in the whole blood of post-stroke patients, we discovered a negative correlation between $AUC_{\text{whole blood}}$ and the total number of MVs in the lower blood sample after 1-hour gravity sedimentation. Considering that the majority of the total MVs derived from platelets (PMVs) and the differently activated platelet clusters were previously identified based on their motion during 1-hour gravity sedimentation by the analogy of ESR, it seems plausible that sinking platelets and their 'dust' (PMVs) could affect the clopidogrel responsiveness. Supporting our hypothesis, Kafian *et al.* [16] described elevated levels of circulating PMVs in patients with HTPR during clopidogrel treatment, indicating ongoing platelet activation despite the antiplatelet therapy.

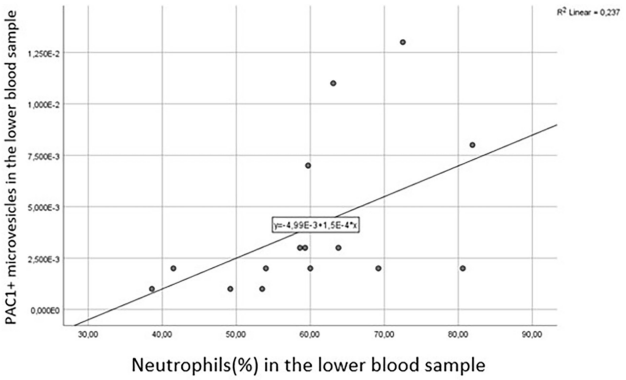

Fig. 5. Correlation between platelet derived PAC1$^+$ MVs ($\times 10^5$/mL) and neutrophils (%) in the lower blood sample (Spearman correlation, p = 0.008).

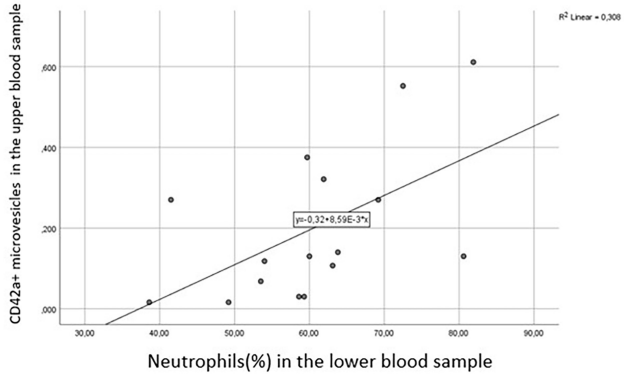

Fig. 6. Correlation between platelet derived CD42a$^+$ MV number ($\times 10^5$/mL) measured in the upper blood sample and neutrophils (%) in the lower blood sample (Spearman correlation, p = 0.006).

Interestingly, Rosinska *et al.* [17] revealed no relationship between circulating microvesicle number and platelet aggregation in post-stroke patients on aspirin (ASA), suggesting that residual platelet reactivity is not af-

fected by MVs in the presence of ASA. Nevertheless, elevated concentrations of PAC-1$^+$/CD61$^+$, CD62P$^+$/CD61$^+$ and CD31$^+$/CD61$^+$ microvesicles were found in acute stroke patients with treatment failure [17]. Accordingly, we observed negative correlation between the velocity of platelet aggregation and total MV count measured in the lower blood sample after 1-hour gravity sedimentation, suggesting that this sample separation technique might be suitable for discrimination of clopidogrel responders from non-responders. Moreover, in a recent study high levels of MVs with different origins were found to be linked to stroke severity and prognosis [18]. It should be noticed that patients with acute ischemic stroke were not investigated in our study. However, based on these preliminary results the advantage of the blood sedimentation technique may provide deeper insights into the behaviour of blood cell components in the acute phase of ischemic stroke. Considering the poor patient outcome despite successful recanalization, besides other factors (e.g., optimal perfusion of the ischemic penumbra), a personalized antiplatelet treatment strategy is vital. Therefore, extensive translational research will be needed in this field.

Another important aspect that arises in connection with platelets and PMVs is their role in immune processes. Importantly, we observed positive correlations between PAC1$^+$, CD42a$^+$ PMVs respectively and the percentage of neutrophil granulocytes in the lower blood sample after 1-hour gravity sedimentation indicating the relationship between the procoagulant potential of platelet derived MVs and the thromboinflammatory cascade [19]. Our data was supported by Michelson *et al.* [20], who identified platelet–neutrophil complexes as markers for platelet activation [20]. An increasing number of animal and human studies [21–24] recognise that neutrophils can contribute to venous and arterial thrombosis or 'immunothrombosis' due to hypercoagulability via the release of neutrophil extracellular traps (NETs) as a procoagulant surface in a septic state as well as in chronic vascular disorders.

Limitations of our study are the following: (i) small sample size; (ii) variance in time elapsed between the index event and the blood sampling; (iii) only patients taking clopidogrel were recruited, but other antiplatelet agents would be worth investigating in the future.

5. Conclusions

Based on our findings, the identification and quantification of circulating MVs may allow us to monitor the response to antiplatelet therapy with P2Y12 antagonists (e.g., clopidogrel), providing a novel opportunity to identify non-responder patients thus allowing an individually tailored antiplatelet strategy. Besides, links among platelet derived MVs, particularly CD42a$^+$ and PAC-1$^+$, and other players in the thrombotic cascade, such as neutrophil granulocytes, might become therapeutic targets in the future.

Author Contributions

DS, EE and TM conceived, designed and coordinated the study, participated in acquisition, and interpretation of data. DS drafted the manuscript. MT-F performed the laboratory measurements. MT-F and TM participated in the statistical analysis. AM, TM took part in manuscript revision. All authors read and approved the manuscript.

Ethics Approval and Consent to Participate

This study was conducted according to the guidelines of the Declaration of Helsinki and approved by Local Ethics Committee of the University of Pécs (Ref. number: 6735, Clinical Trial No: NTC03679858). Informed consent was obtained from all subjects involved in the study.

Acknowledgment

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.fbl2705158.

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metabolites

MDPI

Article

Plasma Fibrinogen Independently Predicts Hypofibrinolysis in Severe COVID-19

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Abstract: High rates of thrombosis are present in patients with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Deeper insight into the prothrombotic state is essential to provide the best thromboprophylaxis care. Here, we aimed to explore associations among platelet indices, conventional hemostasis parameters, and viscoelastometry data. This pilot study included patients with severe COVID-19 (n = 21) and age-matched controls (n = 21). Each patient received 100 mg aspirin therapy at the time of blood sampling. Total platelet count, high immature platelet fraction (H-IPF), fibrinogen, D-dimer, Activated Partial Thromboplastin Time, von Willebrand factor antigen and von Willebrand factor ristocetin cofactor activity, plasminogen, and alpha2-antiplasmin were measured. To monitor the aspirin therapy, a platelet function test from hirudin anticoagulated whole blood was performed using the ASPI test by Multiplate analyser. High on-aspirin platelet reactivity (n = 8) was defined with an AUC > 40 cut-off value by ASPI tests. In addition, in vitro viscoelastometric tests were carried out using a ClotPro analyser in COVID-associated thromboembolic events (n = 8) ($p = 0.071$) nor the survival rate ($p = 0.854$) showed associations with high on-aspirin platelet reactivity status. The platelet count ($p = 0.03$), all subjects. COVID-19 patients presented with higher levels of inflammatory markers, compared with the controls, along with evidence of hypercoagulability by ClotPro. H-IPF (%) was significantly higher among non-survivors (n = 18) compared to survivors ($p = 0.011$), and a negative correlation ($p = 0.002$) was found between H-IPF and plasminogen level in the total population. The platelet count was significantly higher among patients with high on-aspirin platelet reactivity ($p = 0.03$). Neither the ECA-A10 ($p = 0.008$), and ECA-MCF ($p = 0.016$) were significantly higher, while the tPA-CFT ($p < 0.001$) was significantly lower among patients with high on-aspirin platelet reactivity. However, only fibrinogen proved to be an independent predictor of hypofibrinolysis in severe COVID-19 patients. In conclusion, a faster developing, more solid clot formation was observed in aspirin 'non-responder' COVID-19 patients. Therefore, an individually tailored thromboprophylaxis is needed to prevent thrombotic complications, particularly in the hypofibrinolytic cluster.

Keywords: COVID-19; platelet; IPF; hemostasis; aggregometry; viscoelastic test

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1. Introduction

The COVID-19 pandemic, caused by SARS-CoV-2, is a contagious potentially life-threatening disease that has caused more than five million deaths worldwide [1]. Clinical characteristics of the disease can range from mild upper tract respiratory infection to multiple organ disfunction (MODS), multiple organ failure (MOF), and fatal hypoxemic respiratory failure [2,3]. There is a great body of evidence that COVID-19 significantly affects the coagulation system, contributing to hypercoagulable states and thrombotic events. The reason for such alterations is multifactorial, including the activation of the thrombo-inflammatory cascade and endothelial dysfunction [4,5]. There is a great need to identify novel markers to stratify disease severity and predict the outcome of disease.

Such attempts can not only provide deeper insight into the pathological process, but also allow more accurate triage and faster therapeutic interventions. Based on this concept, a faster correction of abnormal coagulation parameters might be associated with improved prognosis in infected patients [6–8]. In addition to primary hemostasis, platelets play an important role in inflammatory and immune responses. According to a recent study, platelet count per se provide valuable data in the assessment of disease severity and outcome [9]. Zhao et al. reported that an early decrease in blood platelet count was associated with poor prognosis in COVID-19 patients [10]. Hypothetically, an infection-induced cytokine storm or the virus itself directly affects bone marrow via CD13/CD66a and destroys cells and inhibits hematopoiesis [9,11,12]. In contrast, SARS-CoV-2-associated thrombocythemia has been reported too [12,13]. Platelet indices, such as immature platelet fraction (IPF, %), are valid indicators of thrombopoiesis level [14]. IPF represents young cells that have recently been released into the circulation and contain a higher concentration of ribonucleic acid than mature platelets [14]. Recently, IPF was reported as a novel early predictive marker for disease severity in patients with COVID-19 [15]. Critically ill COVID-19 patients have impaired fibrinolysis. The hypofibrinolytic state due to decreased fibrinolytic response may contribute to COVID-associated thromboembolic events. The decreased fibrinolytic response was recently defined as a lysis time (LT) >393 s. The aim of this clinical study was to explore associations among platelet indices and conventional hemorheological parameters in patients with severe SARS-CoV-2 infection and their impact on the clinical outcome [16].

The aim of this clinical study was to explore associations among platelet indices and conventional hemorheological parameters in patients with severe SARS-CoV-2 infection. In addition, the changes of platelet reactivity and fibrinolytic response contributing to the etiology of an increased thrombotic risk associated with COVID-19 were also examined here.

2. Results

2.1. Patients and Healthy Subjects

A total number of 21 COVID-19 patients (male: 12) and 21 age-matched SARS-CoV-2 PCR-negative control subjects were enrolled into this prospective observational study. All patients had SARS-CoV-2 PCR positivity, and they required intensive care with oxygen therapy with or without ventilator support. The demography, past medical history (co-morbidities), and clinical data of the study population are summarized in Table 1. Patients were compared to an age-matched control group (69 years; IQR: 52–71 vs. 67 years; IQR 63–69; $p = 0.222$). The gender ratio was the same in both study groups. There was no significant difference in their body mass index (BMI). A total of 76% of enrolled patients had hypertension and 57% of them was treated for diabetes (T2DM). Not surprisingly, significantly higher erythrocyte sedimentation rates (ESR), D-dimer levels, von Willebrand factor antigen and von Willebrand factor ristocetin cofactor activities ($p < 0.001$) were observed on admission to the ICU. Furthermore, serum levels of IL-6 and ferritin also exceeded the normal laboratory range in patients, but these markers were not measured in the controls (not shown in the table).

We analyzed associations between erythrocyte sedimentation rate (ESR) and acute phase proteins, such as fibrinogen and hs-CRP. We found strong positive correlations between ESR and plasma fibrinogen levels, as well as serum hs-CRP concentration in patients, but not in healthy controls, reflecting the ongoing inflammatory response in severe SARS-CoV-2-infected patients ($r = 0.812$, $p < 0.001$ and $r = 0.666$, $p = 0.001$) (Figure 1).

Table 1. Demography, comorbidities, and admission laboratory parameters of the total study population.

	Patients n = 21	**Controls n = 21**	***p***
Age (y)	69 (52–71)	67 (63–69)	0.222
Male (n)	12 (50%)	11 (52%)	0.757
BMI	27 (26–33)	25 (24–26)	0.189
Hypertension	16 (76)		
Diabetes mellitus	12 (57)		
Thromboembolic event (stroke/TIA, DVT)	5 (24)		
Myocardial infarct	4 (19)		
Heart failure	1 (5)		
ESR (mm/h)	84 (36–107)	4 (4–10)	<0.001
Platelet (g/L)	214 (114–355)	261 (248–265)	0.950
IPF (%)	8.7 (6.1–12.5)	7.6 (6.7–8.2)	0.753
H-IPF (%)	2.2 (0.6–4.1)	0.9 (0.8–1.0)	0.308
Fibrinogen (g/L)	5.1 (3.5–5.4)	3.2 (2.8–3.2)	0.059
D-dimer (µg FEU/L)	2296 (1415–6260)	495 (363–575)	<0.001
APTT (s)	12.9 (12.4–14.0)	26.3 (24.9–27.8)	0.002
TT (s)	34.0 (31.2–36.7)	10.4 (10.2–10.8)	<0.001
vWF:Ag (%)	488 (412–605)	109 (97–109)	<0.001
vWF:RCo (%)	399 (353–568)	104 (97–109)	<0.001
Plasminogen (%)	86 (64–96)	104 (98–106)	0.028
Alpha-2-antiplasmin (%)	118 (107–126)	119 (116–121)	1.000
hs-CRP(mg/L)	90.5 (27.7–126.1)	1.2 (0.9–1.5)	<0.001

BMI: body mass index; TIA: transient ischemic attack; DVT: deep vein thrombosis; ESR: erythrocyte sedimentation rate; IPF: immature platelet fraction, H-IPF: high-immature platelet fraction, APTT: activated partial thromboplastin time, TT: thrombin time, vWF:Ag: von Willebrand factor antigen, vWF:RCo: von Willebrand factor ristocetin cofactor activity. hs-CRP: high-sensitivity C-reactive protein. Data are presented as count (%) or median (25th–75th percentiles).

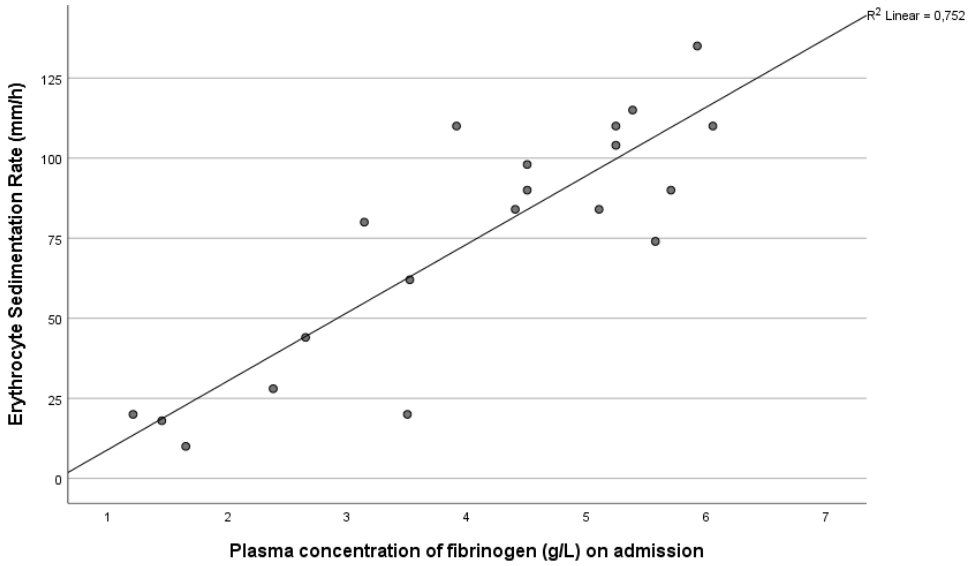

Figure 1. Correlation between erythrocyte sedimentation rate (ESR) and plasma fibrinogen level in COVID-19 patients on admission ($p < 0.001$).

A strong positive correlation was observed between von Willebrand factor antigen and plasma level of von Willebrand factor ristocetin cofactor activity in patients with severe COVID-19 (r = 0.966, $p < 0.001$). Furthermore, a positive correlation was seen between either von Willebrand factor antigen or von Willebrand factor ristocetin cofactor activity

and plasma level of D-dimer (r = 0.683, $p < 0.001$; r = 0.675, $p < 0.001$, respectively—data not shown).

2.2. Non-Survivals vs. Survivals

A total of 18 patients died during intensive care, while 3 patients were discharged from hospital alive. The total platelet count showed no difference between non-survivors and survivors ($p = 0.08$). In contrast, H-IPF (%) showed significant differences when the non-survival vs. survival subgroups were compared (2.5, 1.0–4.2 vs. 0.5, 0.45–0.55; $p = 0.011$) (Figure 2). Interestingly, we detected that activated partial thromboplastin time (APTT, sec) was lower in those who died compared to survivors (13, 12.3–14.0 vs. 15.8, 14.95–26.35; $p = 0.024$).

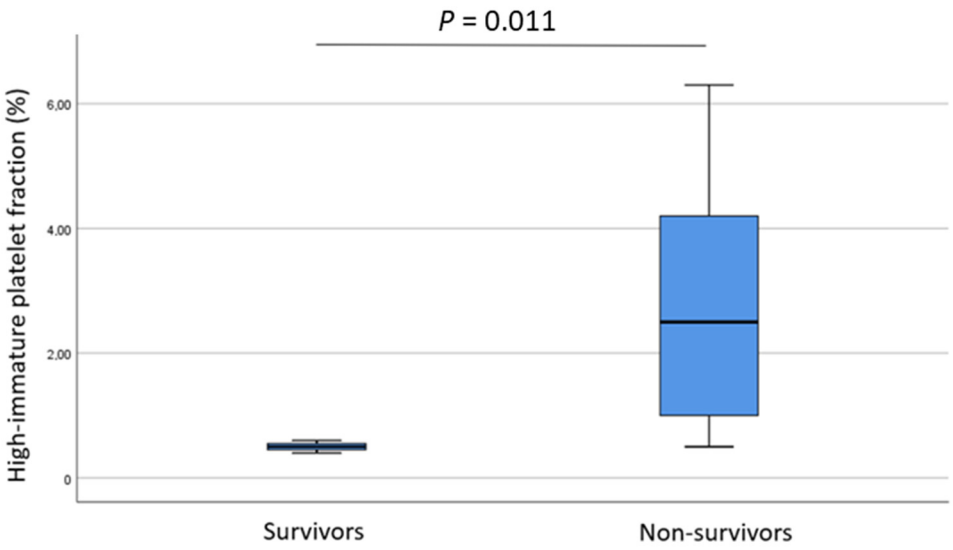

Figure 2. High-immature platelet fraction (%) in survivals and non-survivals.

A significant negative correlation was observed between H-IPF (%) and plasma plasminogen (%) among non-survivors (r = −0.572, $p = 0.002$), but not in survivors (Figure 3).

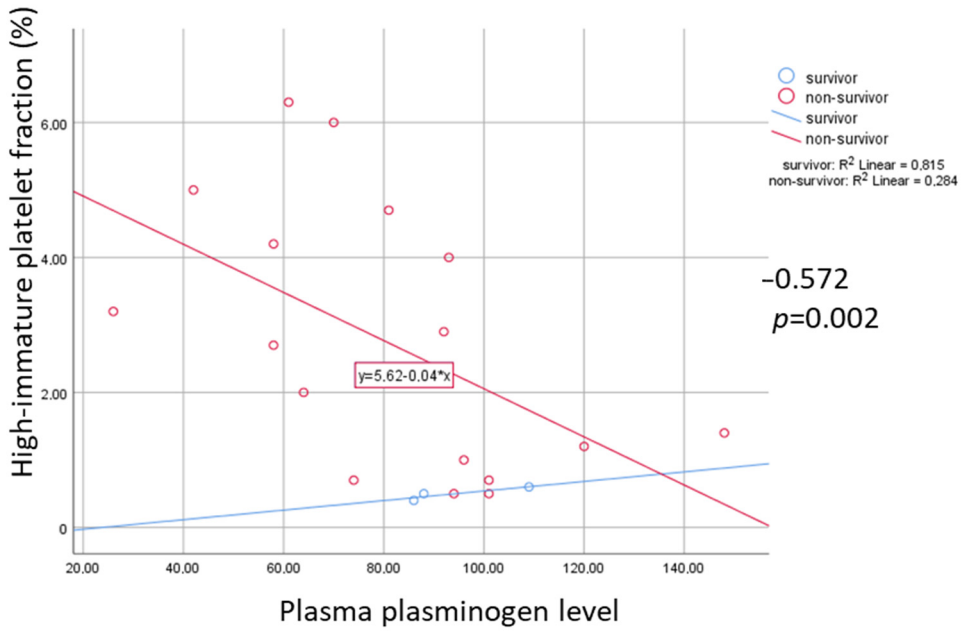

Figure 3. Correlation of high-immature platelet fraction (H-IPF, %) and plasma plasminogen level in survivals and non-survivals. Rho and p indicate negative correlation among non-survivals.

2.3. 'Responders' vs. 'Non-Responders'

Despite aspirin alone or in combination with enoxaparin, eight patients developed symptomatic thrombosis during their ICU stay. Patients were divided into two subgroups based on their ex vivo platelet reactivity measured by a Multiplate analyzer. High on-aspirin platelet reactivity was found in eight COVID patients using the AUC > 40 cut-off value by the ASPI test [17]. Next, COVID patients were dichotomized based on their fibrinolytic response; in patients with impaired fibrinolytic response, the AUC measured by ASPI, Risto, TRAP and ADP tests showed significant differences when aspirin responders and non-responders were compared (all $p = 0.024$, respectively) (Figure 4). Neither the thromboembolic events related to COVID-19 ($p = 0.071$), nor survival rate ($p = 0.854$) showed associations with high on-aspirin platelet reactivity status. The platelet count ($p = 0.03$), the ECA-A10 ($p = 0.008$), and ECA-MCF ($p = 0.016$) were significantly higher, while the tPA-CFT ($p < 0.001$) was significantly lower among patients with high on-aspirin platelet reactivity. In addition, the platelet count showed positive correlations with the AUC by Risto, ASPI, TRAP and ADP tests (0.500, $p = 0.021$; 0.500, $p = 0.021$; 0.760, $p < 0.001$; 0.621, $p = 0.003$, respectively), while H-IPF negatively correlated with the AUC by TRAP and ADP tests (−0.559, $p = 0.008$; −0.530, $p = 0.013$, respectively). The acute COVID-related thromboembolic events were acute coronary syndrome (n = 2), pulmonary embolism (n = 4), and ischemic stroke (n = 2). Both vWF antigen and vWF ristocetin cofactor activity showed positive correlations with H-IPF (both $p = 0.016$), and platelet count was significantly higher among patients with high on-aspirin platelet reactivity ($p = 0.03$) (data not shown).

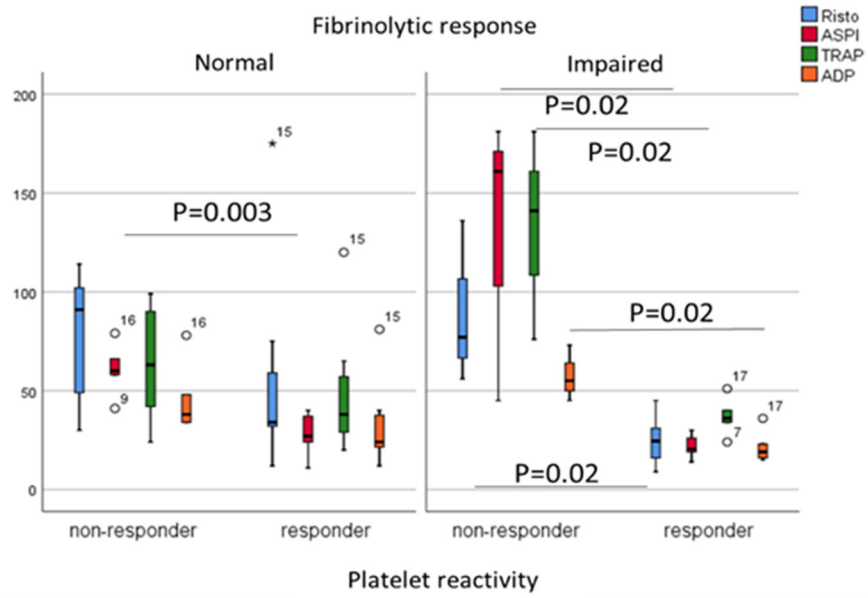

Figure 4. Comparisons of Risto, ASPI, TRAP and ADP tests (AUC) in patients with normal and impaired fibrinolytic response dichotomized based on their responsiveness to aspirin (responder vs. non-responder status). Mann-Whitney test (Asterix and white circles indicate extreme values).

Maximal clot firmness (MCF) was significantly higher measured by the ECA test ($p = 0.016$) in patients with high on-aspirin platelet reactivity (n = 8) compared to the 'responder' subgroup (n = 13), indicating larger and more solid clots despite 100 mg aspirin treatment. (Figure 5A,B). When the manufacturer's AUC < 71 cut-off value was used in comparison, the tPA lysis time (tPA LT) tended to increase among aspirin 'responder' COVID-19 patients ($p = 0.06$) compared to 'non-responders', and eight of these patients showed features of the 'fibrinolysis shut-down' phenomenon.

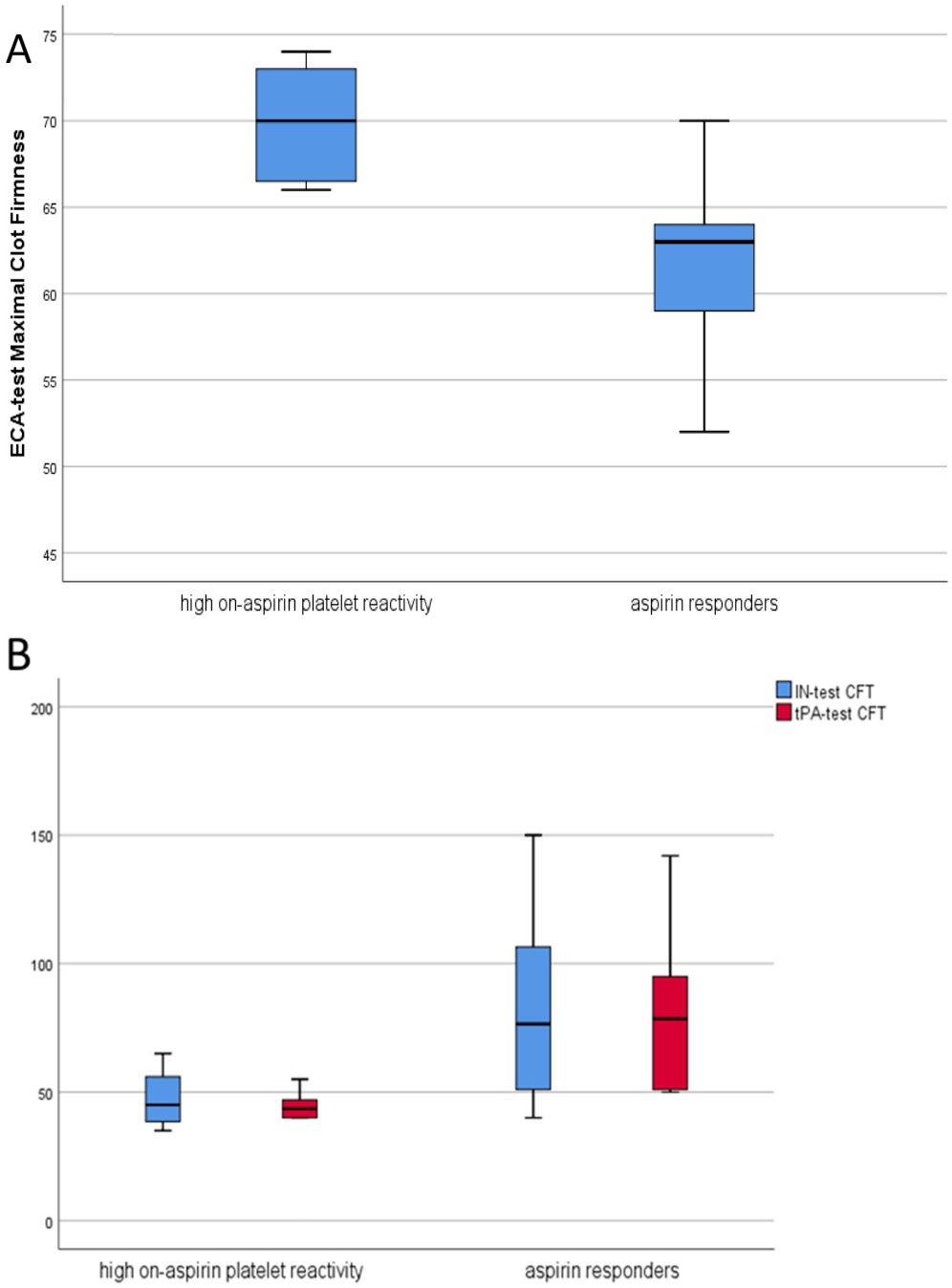

Figure 5. (**A**) Maximal clot firmness measured by ECA test in patients with high on-aspirin platelet reactivity vs. aspirin *'responders'* (Mann–Whitney test, $p = 0.016$); (**B**) Clot formation time measured by IN and tPA test in patients with high on-aspirin platelet reactivity vs. aspirin *'responders'* (Mann–Whitney test, $p = 0.039$ and $p < 0.001$, respectively).

2.4. Admission Platelet Count in COVID-19 Patients

Next, patients were divided into two subgroups based on their platelet count on admission (thrombocytopenia <150 g/L; and normocythemia >150 g/L). Both von Willebrand factor antigen (vWF:Ag) and von Willebrand factor ristocetin cofactor activities (vWF:RCo) were significantly higher in COVID-19 patients independently from their platelet count on admission compared to healthy subjects ($p < 0.001$) (data not shown). In contrast, the plasma level of plasminogen, but not alpha-2-antiplasmin, was significantly lower among COVID-19 patients with thrombocytopenia (<150 g/L) than in patients with normal platelet counts (>150 g/L) (Figure 6). In addition, significantly lower MCF values were observed

in the IN and ECA tests among patients with lower platelet counts (both $p = 0.004$), while significantly higher FIB and tPA test values were detected in patients with normal platelet counts compared to healthy controls ($p = 0.014$ and $p = 0.03$, respectively) (Figure 7).

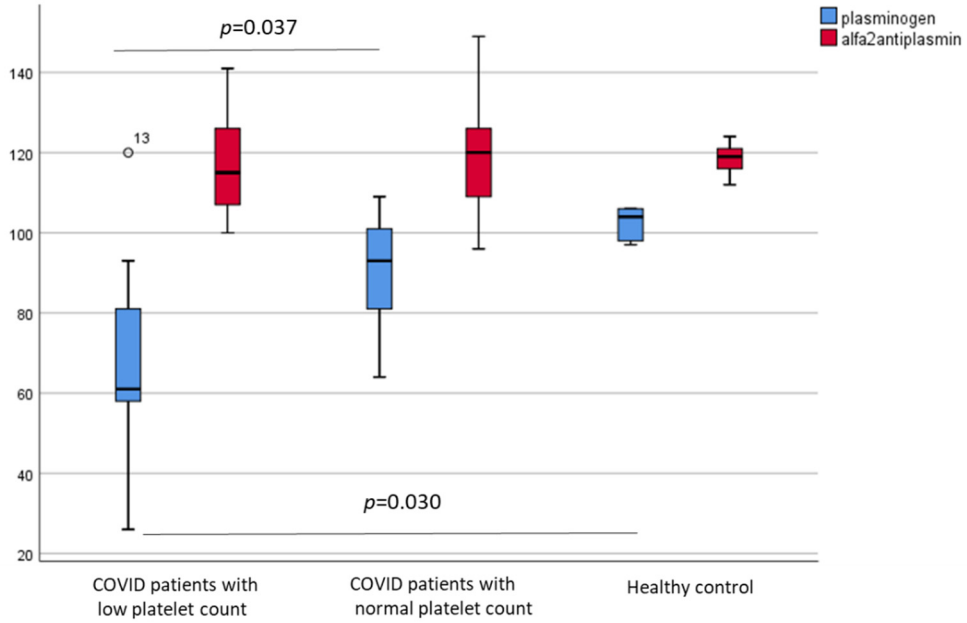

Figure 6. Plasminogen and alpha-2-antiplasmin levels in patients with low platelet counts (<150 g/L), with normal platelet counts (>150 g/L), and healthy controls.

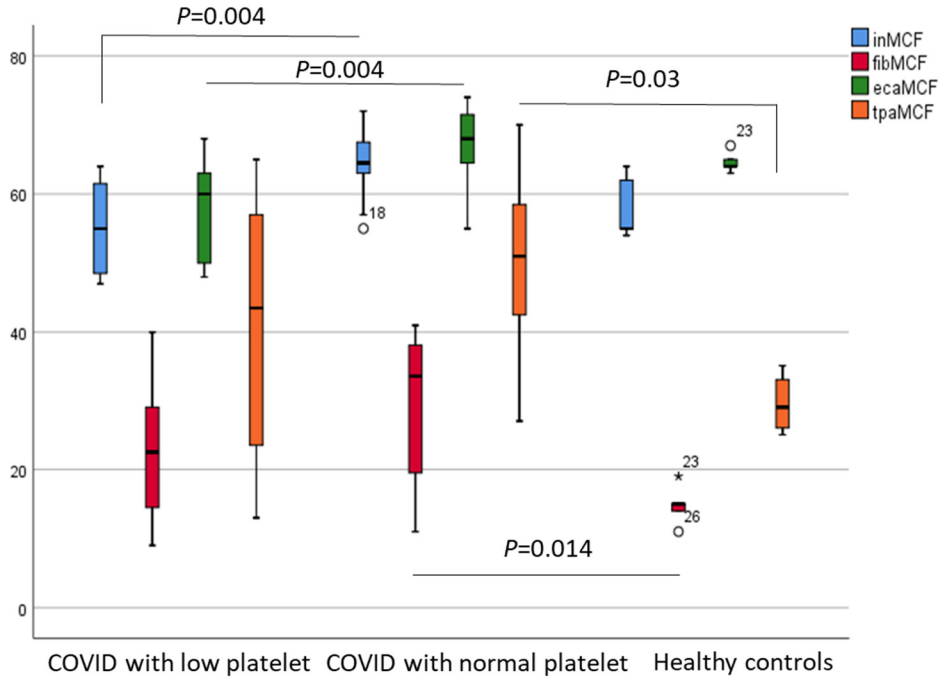

Figure 7. Maximal clot firmness MCF) by IN/FIB/ECA/tPA test respectively in patients with low platelet counts (<150 g/L), with normal platelet counts (>150 g/L), and healthy controls.

Next, COVID patients were dichotomized based on their fibrinolytic response again. Thereafter, the low platelet vs. normal platelet subgroups were compared. In patients with impaired fibrinolytic response, the AUCs measured by ASPI and TRAP tests ($p = 0.03$) were significantly higher in patients with normal platelet counts. In contrast, in the normal fibrinolytic group, only the ADP test showed significant differences ($p = 0.04$) (Figure 8).

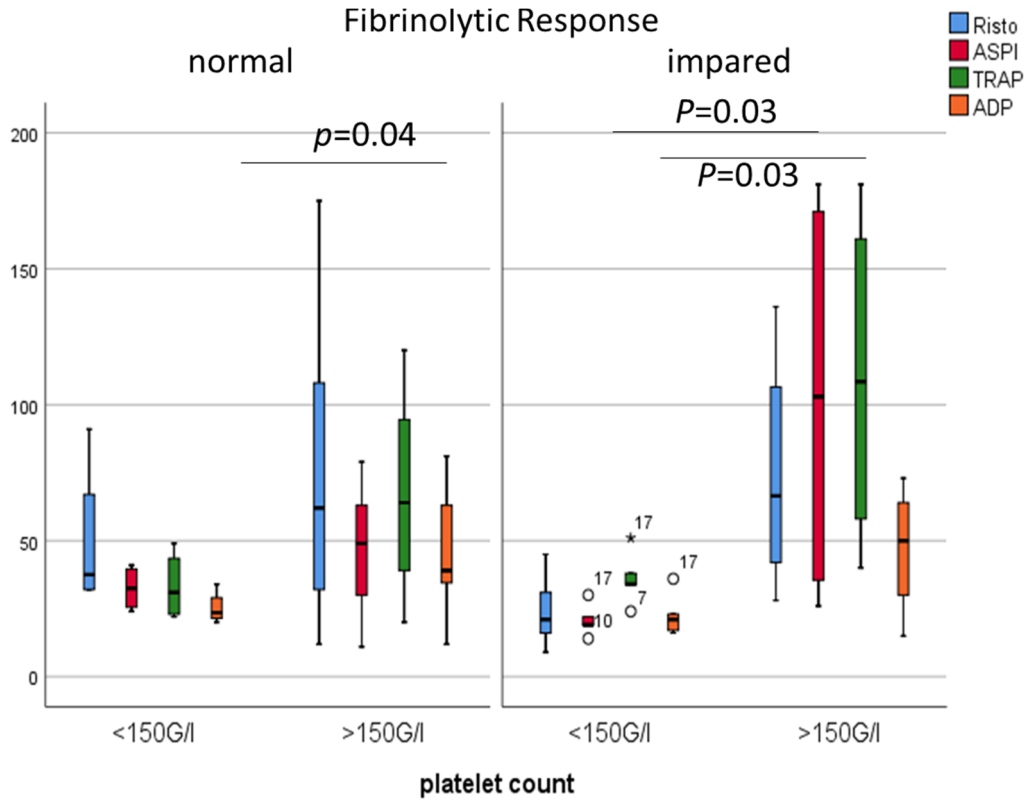

Figure 8. Comparisons of Risto, ASPI, TRAP and ADP tests (AUC) in patients with normal and impaired fibrinolytic response, dichotomized based on their platelet count (g/L).

2.5. Independent Predictors of Ompaired Fibrinolytic Response

A separate analysis was run with hypofibrinolysis as the outcome of interest. Based on binary logistic regression analysis including age, gender, D-dimer, fibrinogen, and aspirin responsiveness based on impedance electrode aggregometry by Multiplate, only fibrinogen (OR: 3.55, 95% CI: 1.33–9.47, $p = 0.01$) proved to be an independent predictor of hypofibrinolysis. The ROC analysis of plasma fibrinogen level as a predictor of hypofibrinolysis in severe COVID patients revealed a cut-off value of 3.86 g/L (AUC of 0.800, 95% CI: 0.623–0.976; $p = 0.006$) with a 78% sensitivity and 73% specificity.

3. Discussion

Despite the small number of included patients, an elevated level of H-IPF (%) was found to be predictive of the fatal outcome here. Welder et al. found that elevated percentages of IPF at presentation was predictive of the length of hospitalization, the need of ICU admission, and mechanical ventilation [15]. Importantly, a higher H-IPF level was associated with lower plasminogen levels in those COVID-19 patients who died. This finding is indirectly supported by Bertolin et al., who reported increased plasminogen activator inhibitor 1 (PAI-1) activity in COVID-19 patients, that may be due to the consumption of plasminogen in association with hypercoagulability [18]. Taken together, endothelial dysfunction (elevated vWF level), with the release of fibrinolysis inhibitor PAI-1, and hyperimmune response (increased ESR, CRP, ferritin, and IL-6) with younger (higher H-IPF) activated platelets seem to be significant contributors to thrombogenesis in COVID-19. Importantly, in our cohort, the aspirin non-responder patient group presented with not only higher platelet count, but also increased platelet reactivity based on either Risto, ASPI, TRAP or ADP tests in the hypofibrinolysis (LT > 393 s) group revealed by ClotPro (likewise by Bachler et al.) [16]. Bertolin et al. also observed lower platelet reactivity based on Multiplate aggregometry compared to healthy controls, despite having higher levels of D-dimer, fibrinogen, and PAI-1, and hypercoagulability by thromboelastometry [18]. In

accordance, eight COVID-patients on aspirin exhibited an increased platelet reactivity via the ASPI test (referred to in this article as 'non-responders'). Significantly higher maximal clot firmness (MCF) was observed in ECA tests; meanwhile, significantly lower IN-, and tPA-CFT were found in 'non-responders' compared to 'responders', indicating faster developing, larger and more solid clots despite aspirin treatment. A large randomized clinical trial (*RECOVERY*) found that aspirin did not improve survival for patients hospitalized with COVID-19 [19,20]. Therefore, there is an urgent need to identify aspirin low/non-responders providing a modified antiplatelet regime or alternative strategies (e.g., activated protein C, PAI-1 antagonists, and tissue plasminogen activators) to combat thrombosis in this disease.

Based on our findings, besides platelet count itself, the MCF depends on several other factors such as plasminogen and the von Willebrand factor. Kruse et al. observed lower levels of plasminogen, suggesting that it was integrated into the clot, but unable to disintegrate it effectively, presumably by the inhibitory effect of alpha2-antiplasmin, which makes thrombi resistant to plasmin; meanwhile, plasminogen activator inhibitor (PAI-1) inhibited the activation of tissue plasminogen activator (tPA). The net effect of these may result in the 'fibrinolysis shut-down' phenomenon, leading to lysis-resistant microthrombi formation in different organs, particularly in the lungs [21]. Importantly, we aimed to explore predictors of impaired fibrinolytic response and found only fibrinogen with an OR: 3.55 as an independent predictor of hypofibrinolysis.

Moreover, ESR was found to be significantly higher in patients with severe SARS-CoV-2 infection and it showed strong positive correlation with fibrinogen concentration. Similar data were shown by Henry et al., who performed a meta-analysis involving 21 studies showing that inflammatory markers such as ESR, CRP, serum ferritin, IL-6, procalcitonin, and IL-2R were significantly elevated in patients with severe and fatal COVID-19 [22]. Another systematic review and meta-analysis detected that ESR positively correlated with COVID-19 severity [23]. CRP is an exquisitely sensitive systemic marker of acute-phase response in inflammation, infection, and tissue damage [24]. Elevation in serum CRP levels has been suggested in several studies as a reliable indicator of the presence and severity of SARS-CoV-2 infection [25–27]. D-dimer arises from the lysis of cross-linked fibrin and indicates the activation of coagulation and fibrinolysis [25,28]. Although the tPA lysis time (tPA LT) tended to be increased among aspirin 'non-responder' COVID-19 patients ($p = 0.06$), the D-dimer concentration was not different between 'responder' and 'non-responder' subgroups. There was no difference in D-dimer concentrations between survival and non-survival subgroups in our cohort as well (also see limitations of our study). In contrast, larger studies reported D-dimer level as a predictor for mortality. Zhang et al. stated that it is an independent factor of all-cause death in hospitalized patients with COVID-19 [29]. Regarding the kinetics, Corrado et al. found that non-survivals had rapidly increasing D-dimer levels [30]. Due to the very low survival rate of our cohort, independent predictors of mortality could not be analyzed here.

Despite the fact that we detected reduced activated partial thromboplastin time (APTT) in non-survivors, in a recent Dutch study evaluating ICU patients with COVID-19, prolongation of the prothrombin time >3 s and activated partial thromboplastin time >5 s were found to be independent predictors of thrombotic complications [31]. In our cohort, the thromboembolic complication was only associated with a reduced clotting time in the FIB test.

In fact, a high number of patients with COVID-19 die due to thromboembolic complications. Della-Morte et al. hypothesized plasminogen as the precursor for fibrinolysis [32]. Our finding was supported by them, because they found that low levels of plasminogen strongly correlated with mortality. Recently, plasminogen was suggested to play a pivotal role in controlling the complex mechanisms beyond COVID-19 complications, so it could be a useful prognostic marker and a potential therapeutic target [33].

Elevated vWF levels, as we also observed in our own cohort, imply activated or damaged endothelium [34]. It would be anticipated that damaged endothelium would

result in the release of ultra-large vWF multimers capable of interacting with platelets, leading to platelet activation, microthrombi, and platelet consumption [5]. In accordance, we also found positive correlations between vWF antigen and activity and H-IPF (%) among patients with high on-aspirin platelet reactivity. Studies have shown that patients with COVID-19 have significantly elevated levels of vWF antigen and activity, likely contributing to an increased risk of thrombosis [35].

In summary, a faster-developing, larger and more solid clot formation was observed in aspirin 'non-responder' COVID-19 patients than 'responders' here. Based on ClotPro analysis, the clot seemed to be resistant to lysis in the 'non-responders' (longer lysis), suggesting that this cluster of patients belong to the 'hypofibrinolysis or fibrinolysis shut-down' group, but this requires further validation. Nevertheless, several physiological aspects should also be considered in viscoelastic studies because activation of the vessel wall, the endothelium, and platelets entering the clot is not present in vitro. Nevertheless, our results suggest the necessity of an individual approach regarding antiplatelet therapy, as was recently confirmed in other vascular diseases [36,37]. Our observations deserve further validation in a larger prospective cohort, as there is an urgent need for individually tailored thromboprophylaxis to prevent fatal complications such as symptomatic thrombosis in severe COVID-19 patients.

4. Limitations

First, this is a small single-center study. Results need to be confirmed on a larger sample size of COVID patients with different severity clusters. Secondly, sampling at multiple time points instead of a single time could clarify whether the kinetics of such variables differ in various outcome subgroups. Thirdly, the rigid inclusion/exclusion criteria limit the generalizability of this study.

5. Methods

This pilot study was approved by the Hungarian Medical Research Council (20783-5/2020/EÜIG). All procedures were performed in accordance with the ethical guidelines of the 1975 Declaration of Helsinki. Written informed consent was provided by all participants or relatives before enrolment in the present study. A total of 21 patients with severe SARS-CoV-2 infection (with the inclusion criteria: requirement of O_2 supplementation and signed informed consent) were retrospectively analyzed from a prospective database at the Coronavirus Crisis Centre of the Clinical Centre at the University of Pécs, Pécs, Hungary. Patients under 18 years old, with congenital hemostatic abnormalities, anamnestic/current malignancy, and pregnant women were excluded from the study. Patients hospitalized in the ICU were on 100 mg/d aspirin and prophylactic anticoagulation (with enoxaparin uniformly, 1x/d) based on our local therapeutic protocol. Patients who were enrolled into the study did not receive non-steroid anti-inflammatory drugs; only paracetamol was given occasionally (e.g., in case of fever) and basic analgosedation was conducted with opioids (sufentanil uniformly). Twenty-one SARS-CoV-2 PCR negative health care workers served as healthy controls. Blood samples for measurements were drawn into a closed system blood sampling tube with 3.2% Na_3-citrate (Becton Dickinson, Diagon Ltd., Budapest, Hungary), hirudin (Sarstedt S-Monovette® 1.6 mL Hirudin), and K_3-EDTA (Becton Dickinson, Diagon Ltd., Budapest, Hungary) as anticoagulant and serum separator tubes without anticoagulant. Samples were processed within a maximum of 1 h after collection. The blood collection from volunteers was carried out through vein puncture with a 21-gauge needle into a closed system.

5.1. Blood Count, Platelet Count, High Immature Platelet Fraction (H-IPF) Measurement

The total blood cell count from the whole blood and the absolute neutrophil count after 1 h of sedimentation from the upper and lower part of the blood were measured on a Sysmex XN 9000 integrated automated hematology analyzer (Sysmex Co., Kobe, Japan, 2017). The platelet number (PLT-F) was measured using the fluorescent platelet channel

of the analyzer. In this channel, the platelets were specifically stained intracellularly with fluorescent dye and measured on the principle of flow cytometry, analyzing the forward scattered light (FSC), side scattered light (SSC) and side fluorescent light (SFL). The platelets were counted and additionally, the plots in the area with high fluorescence intensities were separated into the immature platelet fraction and the research parameter, the high immature platelet fraction (H-IPF).

5.2. The Erythrocyte Sedimentation Rate (ESR)

The ESR test measures how quickly red blood cells sedimentate in the test tube. The rate at which red blood cells settle is measured as the number of millimeters of clear plasma present at the top of the column after one hour (mm/h). For the manual determination of ESR according to Westergren, we used a BD seditainer stand with an adjustable zero mark. After swiveling the tube to mix the blood sample and preparation, the tubes were immediately placed in the stand to start the measurement. After 1 h of sedimentation, the results were read.

5.3. Hemostasis

Fibrinogen (quantitatively determined based on the Clauss method) and Activated Partial Thromboplastin Time (APTT) were measured as part of the routine hemostasis parameters on an ACL-TOP-750 analyzer (Werfen, Hungary) with Q.F.A. Thrombin (Bovine; HemosIL®, Werfen, Hungary) and APTT-SP (liquid; HemosIL®, Werfen, Hungary) reagent, respectively.

The special hemostasis tests were measured on an ACL-TOP-500 analyzer (Werfen, Hungary). The quantitative determination of von Willebrand factor antigen (vWF:Ag) and von Willebrand factor ristocetin cofactor activity (vWF:RCo) was performed with an automated latex enhanced immunoassay, both with HemosIL® reagent. For quantitative measurement of plasminogen we used an automated chromogenic assay (Plasminogen; HemosIL®). The quantitative determination of alpha2-antiplasmin as an important regulator of the fibrinolytic system was carried out using an automated chromogenic assay (Plasmin Inhibitor, HemosIL®).

To monitor the aspirin therapy, we performed a platelet function test from hirudin anticoagulated whole blood within 1 h after blood sampling on a Cobas® Multiplate® Analyzer (Roche Diagnostics, Mannheim, Germany) using the ASPI test (using arachidonic acid as an activator). The aggregation level was expressed as the area under the curve (AUC). The AUC was calculated by the analyzer using the product of aggregation unit (AU) $\times$ time (minutes). Given the lack of universal cut-off values, the normal aggregation range for the ASPI test was expected as AUC: 71-115U according to the manufacturer (laboratory cut off value). However, previous studies suggest that patients were considered as 'responders' to aspirin therapy with an AUC < 40; and 'non-responders' with an AUC $\geq$ 40. In our data set, (n = 13) were defined as 'responders' and (n = 8) 'non-responders', showing high on-aspirin platelet reactivity.

Viscoelastrometric testing was carried out on a ClotPro (DiaCare Solutions, Mumbai, India) in vitro *POCT* coagulation analyzer. It uses pipettes prefilled with starting reagents and 340 µL of citrated whole blood to initiate measurement. For measurement, it uses a stationary pin placed in a moving cup, from which the reduction in movement is detected and charted as the amplitude resulting in thrombelastometry curves. As standard tests in COVID-19 and control patients, we used the EX test (tissue factor-activated assay with polibrene), IN test (ellagic acid-activated assay), FIB test (tissue factor activated assay, without functional platelet), ECA test (ecarin-based assay), and tPA test (r-tPA within an extrinsic pathway-based assay). Of note, the EX test, tPA test, and FIB test contain polybrene to neutralize heparin. In each test, we recorded the next parameters which characterized 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 (n = 9) was defined as LT > 393 s [18,38].

5.4. Statistical Analysis

Statistical analysis of the collected data was evaluated by IBM SPSS Statistics® 27.0. To analyze demographic and clinical factors, the chi-square test was used for categorical data. The Kolmogorov–Smirnov test was applied to test for normality of continuous variables distribution. Comparisons of continuous non-normally distributed data between COVID vs. control groups were carried out using the Mann–Whitney U-test, while COVID vs. controls with or without ASA subgroups were tested using a one-way ANOVA test. A Student's *T*-test was used for the analysis of normally distributed continuous data. Continuous variables are reported as median and interquartile range or mean and standard error of 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. A p value <0.05 was considered statistically significant.

Author Contributions: Conceptualization, T.M.; project administration, T.M.; methodology, M.T.-F. and B.R.; statistical analysis, T.M.; investigation, D.S.; data curation, D.S.; writing—original draft preparation, D.S.; visualization, D.S.; review and editing, D.S., M.T.-F., B.R., and T.M. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: This study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Hungarian Medical Research Council (20783-5/2020/EÜIG).

Informed Consent Statement: Informed consent was obtained from all subjects (or from their relatives due to their critical medical conditions) involved in the study.

Data Availability Statement: The data that support the findings of this study are available on request from the corresponding author, due to high amount of raw data.

Acknowledgments: Our thanks go to all the patients and staff who participated in this research.

Conflicts of Interest: The authors declare no conflict of interest.

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