Aus der Medizinischen Universitätsklinik und Poliklinik Tübingen

Abteilung Innere Medizin III

(Schwerpunkt: Kardiologie und Angiologie)

Impact of dual antiplatelet therapy on thrombus architecture in patients with acute myocardial infarction

Inaugural-Dissertation
zur Erlangung des Doktorgrades
der Medizin

der Medizinischen Fakultät
der Eberhard Karls Universität
zu Tübingen

vorgelegt von

Rogers, geb. Chakkalakal, Claire

2020

Dekan:	Professor Dr. B. Pichler
1. Berichterstatter:	Privatdozentin Dr. I. Müller
2. Berichterstatter:	Professorin Dr. V. Mirakaj

Tag der Disputation: 06.08.2020

Table of Contents

Abbreviations

%: percent

°C: degree Celsius

µl: microliter

µm: micrometer

ACE-I: Angiotensin converting enzyme inhibitor

ACS: Acute Coronary Syndrome

ADP: Adenosine diphosphate

AFib: Atrial fibrillation

AP: Angina Pectoris

Aqua dest.: Aqua destillata

ARB: Angiotensin receptor blocker

ASA: Acetylsalicylic acid

BMI: Body mass index

BMP: Bone Morphogenetic Protein

BP: Blood pressure

Ca^{2+}: Calcium

CAD: Coronary artery disease

CD: Cluster of differentiation

CHD: Coronary heart disease

CK: Creatine kinase

COPD: Chronic obstructive pulmonary disease

COX-1: Cyclooxygenase - 1

CRP: C-reactive protein

CVD: Cardiovascular disease

CVP: Central venous pressure

DAB: Diaminobenzidine

DAPT: dual antiplatelet therapy

dl: deciliters

DM II: Diabetes Mellitus Type 2

DNA: Deoxyribonucleic acid

ECG: Electrocardiogram

EF: Ejection fraction

ESC: European Society of Cardiology

f.ex.: for example

FBS: Fetal bovine serum

FCS: Fetal calf serum

Fig.: Figure

g: gram

GFR-MDRD: Glomerular filtration rate-modification of diet in renal disease equation

GP: Glycoprotein

H/DIC: H/ Differential interference contrast

H2O2: Hydrogen peroxide

Hb: Hemoglobin

HbA1c: HemoglobinA1c

HDL: High-density lipoprotein

HE-Stain: Hematoxylin and Eosin stain

HIER: Heat induced epitope retrieval

Hrs: Hours

i.e.: id est

IAP: Instable Angina Pectoris

IgG: Immunoglobulin G

IHD: Ischemic heart disease

kg: kilogram

LD: Loading dose

LDH: Lactate dehydrogenase

LDL: Low-density lipoprotein

LPS: Lipopolysaccharide

LVEF: Left ventricular ejection fraction

m^2: square meters

mg: Milligram

MI: Myocardial Infarction

MIF: Macrophage Migration Inhibitory Factor

Min (s): Minute (s)

ml: Milliliter

mmHg: Millimeter of mercury

MPO: Myeloperoxidase

MRA: Mineralocorticoid receptor antagonist

NOAC: Novel oral anticoagulant(s)

NSTEMI: Non-ST-Segment-Elevation Myocardial Infarction

NYHA: New York Heart Association

OAC: oral anticoagulant(s)

PAD: Peripheral arterial disease

PAP: Peroxidase- anti-peroxidase

PAP: pulmonary artery pressure

PBS: Phosphate-buffered saline

PCI: Percutaneous Coronary Intervention

PE: Phycoerythrin

PIER: Proteolytic-induced epitope retrieval

PPI: Proton-pump inhibitor

RNA: Ribonucleic acid

rpm: Rounds per minute

s.: see

SAB: Streptavidin-Biotin

SCD: Sudden cardiac death

SD: Standard deviation

sec: Second(s)

SGOT: Serum glutamic oxaloacetic transaminase

STEMI: ST-Segment-Elevation Myocardial Infarction

TGFß: Transforming Growth Factor beta

TLR: Toll-Like receptor

TnI: troponin I

U: units

vWF: von Willebrand factor

WHO: World Health Organization

y: years

1. Introduction

During the past two decades Cardiovascular Disease (CVD) has evolved into the leading cause of death worldwide.[1]

According to the World Health Organization WHO it accounted for over 17 million deaths in 2015, 8.76 million of which were due to Ischemic Heart Disease (IHD), followed by strokes with 6.24 million. The total amount represents a percentage of 31 of all deaths worldwide.[2]

1.1. Coronary Artery Disease

The medical entity Ischemic Heart Disease (IHD) or Coronary Artery Disease (CAD) comprises a group of cardiac conditions including Angina Pectoris (stable and instable), Myocardial Infarction (STEMI and NSTEMI) as well as Sudden Cardiac Death (SCD).[1,3]

Ischemia signifies insufficient blood supply to tissues (partial or total), resulting in lack of oxygen, consecutive disturbance of cell metabolism and cell damage and thus dysfunction of the tissue and organ. Restriction in blood supply can have several reasons such as vasoconstriction, thrombosis or embolism, in IHD it is primarily the result of atherosclerosis of the coronary arteries.[4-6]

Atherosclerosis, a condition describing the hardening and narrowing of arteries, occurs due to plaque build-up in the artery walls and progresses over a long period of time. The mechanisms of plaque formation are not entirely understood, although it is known that plaques are generally lipid-rich.[6] Acute plaque rupture can lead to thrombosis and total occlusion of the vessel. The Framingham Study established a variety of risk factors for CAD [7] most of which are congruent with the risk factors for atherosclerosis. These are still in clinical practice and are part of the baseline patient characteristics involved in this study.

Clinically CAD may present itself as either a chronic condition, which includes stable CAD or as an Acute Coronary Syndrome (ACS). The latter is further subcategorized in IAP, NSTEMI and STEMI.[3]

1.1.1. Stable Coronary Artery Disease (CAD)

Angina Pectoris derives from the Latin words “angere” and “pectus” [8] and describes the classical symptom- chest pain in form of a strangling feeling usually in the center of the chest.[9] Pain or discomfort may also occur or project into the jaw, arms, back or neck, in less frequent cases into the epigastrium.

Those symptoms are signs of myocardial ischemia and are usually a manifestation of atherosclerosis in the coronary vessels.[10] Two pathophysiological factors play a main role in causing ischemia. For one, due to atherosclerosis the diameter of the vessels is reduced to such an extent that blood perfusion and therefore oxygen supply to the myocardium does not meet the metabolic demands of the heart muscle anymore.[11] The second factor is the hardening of the artery walls leading to a loss in elasticity.[12] In case of increased demands the human blood vessels physiologically expand to increase blood perfusion and to provide higher amounts of oxygen to the recipient organs by doing so. Hence loss of elasticity of the arteries’ walls signifies less adaptive, dysfunctional vessels.

In stable CAD symptoms generally occur if oxygen consumption exceeds the supply due to increased physical activity or stress. At rest blood perfusion through the narrowed arteries might still be sufficient for the heart muscle to function normally. This explains why generally an artery obstruction of up to 75% remains without symptoms. In case of increased metabolic demands the second pathomechanism described above comes into play. In stable CAD terminating physical activity or administration of nitroglycerin as vasodilating agent usually leads to an abatement of the typical symptoms and recurrence thereof as soon as physical activity resumes.

1.1.2. Acute Coronary Syndrome (ACS)

ACS is a potentially lethal manifestation of CAD. ACS embraces the following subcategories:

1.1.2.1. Instable Angina Pectoris (IAP)

As opposed to stable CAD in IAP symptoms may also occur at rest. Nitroglycerin might have lost its palliating effect since the disease is too far progressed. Instable Angina Pectoris can be regarded as an indicator of an imminent myocardial infarction. Additionally every de-novo angina is by definition considered an IAP.[3]

1.1.2.2. Non-ST-elevation-Myocardial Infarction (NSTEMI)

A Non-ST-Elevation-Myocardial Infarction signifies ischemia of the myocardium resulting in loss of functional heart muscle tissue. This is indicated by elevated heart enzyme levels, i.e. Troponin, in lab results. The electrocardiogram might show signs of an acute heart attack such as ST-Segment depression, arrhythmias, ventricular extrasystole etc. but never an ST-Segment elevation as also implied in the name.

1.1.2.3. ST-Elevation Myocardial Infarction (STEMI)

An ST-Elevation Myocardial Infarction describes a type of severe myocardial ischemia that often results in significant tissue damage. The resulting loss of function and disturbed electrical repolarization show as elevated ST-segments in the ECG or as first diagnosed left bundle branch block. Troponin levels will also be elevated. The severity of the condition and coherent technical findings express a usually total occlusion of the coronary artery through thrombosis, either due to plaque rupture or thromboembolism. The tissue damage in this case is usually transmural, meaning it affects all layers of the myocardium wall as opposed to NSTEMI where only the most inward layer, which is naturally the one furthest away from blood and oxygen supply, is affected.

Hence a main cause for acute myocardial infarction, STEMI in particular, in patients with coronary artery disease is the formation of intracoronary thrombi. The impact of thrombus formation on size of infarction, reperfusion injury as well as clinical outcome and thus prognosis for the patient varies. It is determined by the morphological structure, composition and differentiation of intracoronary thrombi. Therefore these are subject to different pharmacological and invasive treatment strategies and form the investigative background of this research.

1.2. Thrombus formation

In the mid 19th century the German pathologist Rudolf Virchow described the causal factors that according to his findings lead to thrombosis.[13, 14] Although he referred to phlebothrombosis, a thrombosis of the veins, at the core the same factors are causal for arterial thrombosis. They became to be known as Virchow's triad. These are:

1. Endothelial damage or alterations: damage or alterations of the inner vessel wall leads to inflammatory processes, attracting platelets and other inflammatory cells to the site of injury. As a natural mechanism and as part of the healing process these cells will clot. Additionally, the damage and/or the resulting clot may lead to a change in blood flow, which is the second causal factor for thrombosis according to Virchow.

2. Hemodynamic changes/Alterations of blood flow velocity: In the venous system this is usually due to speed decrease which is known as stasis. This makes it easier for blood particles to pile up and stick to the vessel walls. In the arterial system thrombosis preferably develops where turbulences occur. This is either the case at division sites within the arterial tree, where larger arteries branch out into smaller ones or at sites of damage as described in the aforementioned 1.

3. Changes in blood viscosity/Hypercoagulability: Virchow found that the thicker the blood the more likely it is to clot. The reason for a higher viscosity can either be obtained, f.ex. through exsiccosis or thrombocytosis or genetic f.ex. due to deficiency of protein C/S or antithrombin III.[13]

Although there are differences in venous and arterial thrombosis, the above factors also apply for the arterial system, the two former ones in particular. In acute myocardial infarction a thrombus forms either as a result of atherosclerotic plaque rupture that leads to endothelial damage and exposes thrombogenic substances that were previously covered by a thin fibrous cap to

the blood stream.[11] Less frequently, arterial thrombi also form at so-called plaque erosion sites that combine endothelial lesions with thickening of the intima.[11] In the case of disrupted atherosclerotic plaque rupture the now exposed material triggers natural hemostasis, including platelet adhesion, aggregation and activation and the formation of a fibrin mesh, together forming a thrombus that may lead to transient or persistent occlusion of the vessel. Although both, arterial and venous thrombi contain platelets and fibrin, arterial thrombi are typically platelet-rich due to the high shear conditions under which they form, whereas the fibrin content of venous thrombi is higher and the platelet count low.[1]

1.2.1. Role of platelets

Platelets stem from megacaryocytes.[15] They are anucleate cells and have an approximate life span of 7-10 days. They circulate in the blood stream and play an important role in primary hemostasis and thrombus formation.[16] Injury to the endothelium leads to exposure of von Willebrand factor (vWF) and subendothelial collagen to which platelets adhere via their surface receptors GP Iα and GP IIb/IIIa for vWF and $\alpha_2ß_1$ as well as GP IV for collagen.[17-20]. The GP IIb/IIIa not only plays an important role in primary adhesion and activation, but also in sustained platelet aggregation and thrombus stabilization. It is particularly susceptible for vWF under high shear conditions. [21] Another receptor ligand is fibrinogen. Both ligands amplify the induced signaling and activation pathway as part of a positive feedback mechanism.[20]

Following adhesion, platelets become activated and release ADP from intracellular granules in addition to Thromboxane A_2, which is synthesized by their intrinsic cyclooxygenase-1 (COX-1). Both substances amplify the signaling pathway even further by recruiting more platelets to the site of lesion and activating them. Thromboxane A_2 also functions as vasoconstrictor.[20, 22]

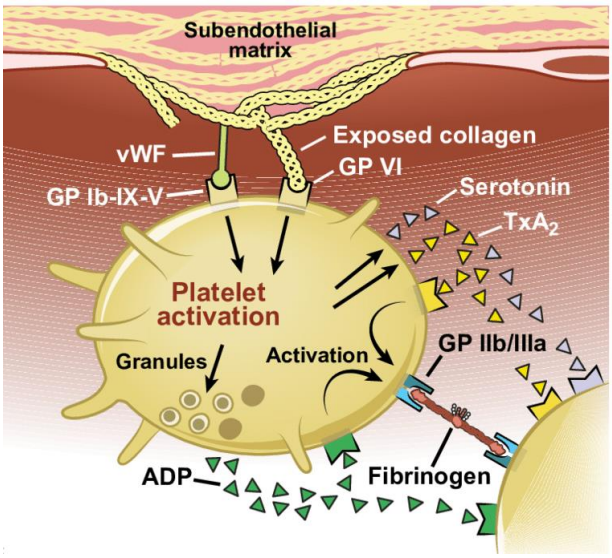
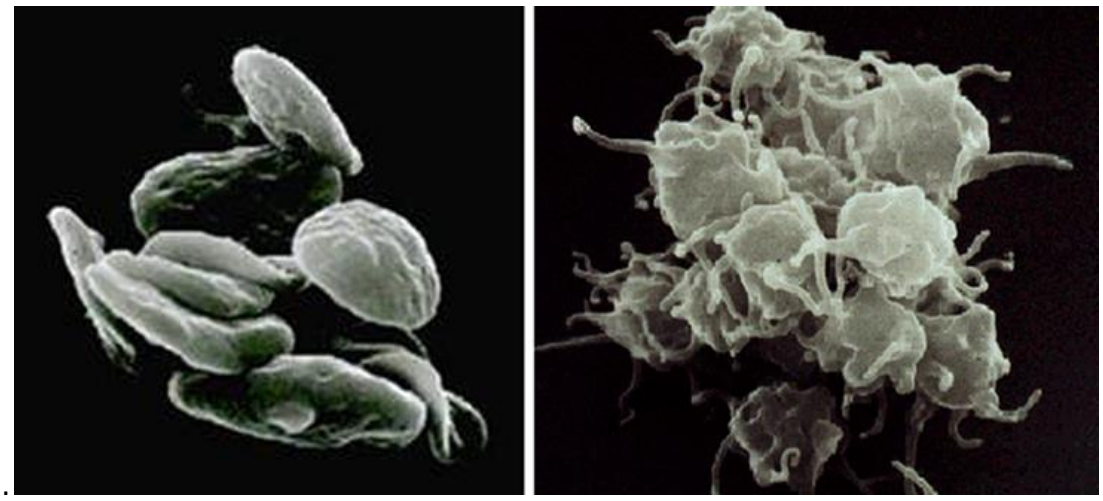


Fig. 1 taken from Halkar M, Lincoff AM, Dual antiplatelet therapy for acute coronary syndromes: How long to continue? Clinic Cleveland Journal of Medicine, 2016 [23]

In order to effectively activate further platelets ADP binds to the platelets membrane receptors $P2Y_{12}$ and $P2Y_1$ and Thromboxane A_2 to its TP receptor that is found on platelets as well as on endothelial cells. Increased intracellular calcium levels after receptor activation lead to conformational change of the platelets cytoskeleton and mobilization and release of further granules, thus amplifying aggregation and activation.[24-27]



Resting platelets *Activated Platelets*

Fig.2 Credit: Department of Pathology College of Veterinary Medicine the University of Georgia Athens [28]

Furthermore, activated platelets support the coagulation system not only by releasing factors V, XI, XIII and fibrinogen but also by providing coagulatory factors with anionic surface phospholipids which are required for the intrinsic pathway of the coagulation cascade as a binding site. The coagulation cascade ultimately leads to formation of thrombin and consequently to fibrin. In the sense of a positive feedback loop thrombin also amplifies platelet attraction and activation, thus supporting the growth of the platelet-fibrin thrombus.[29, 30]

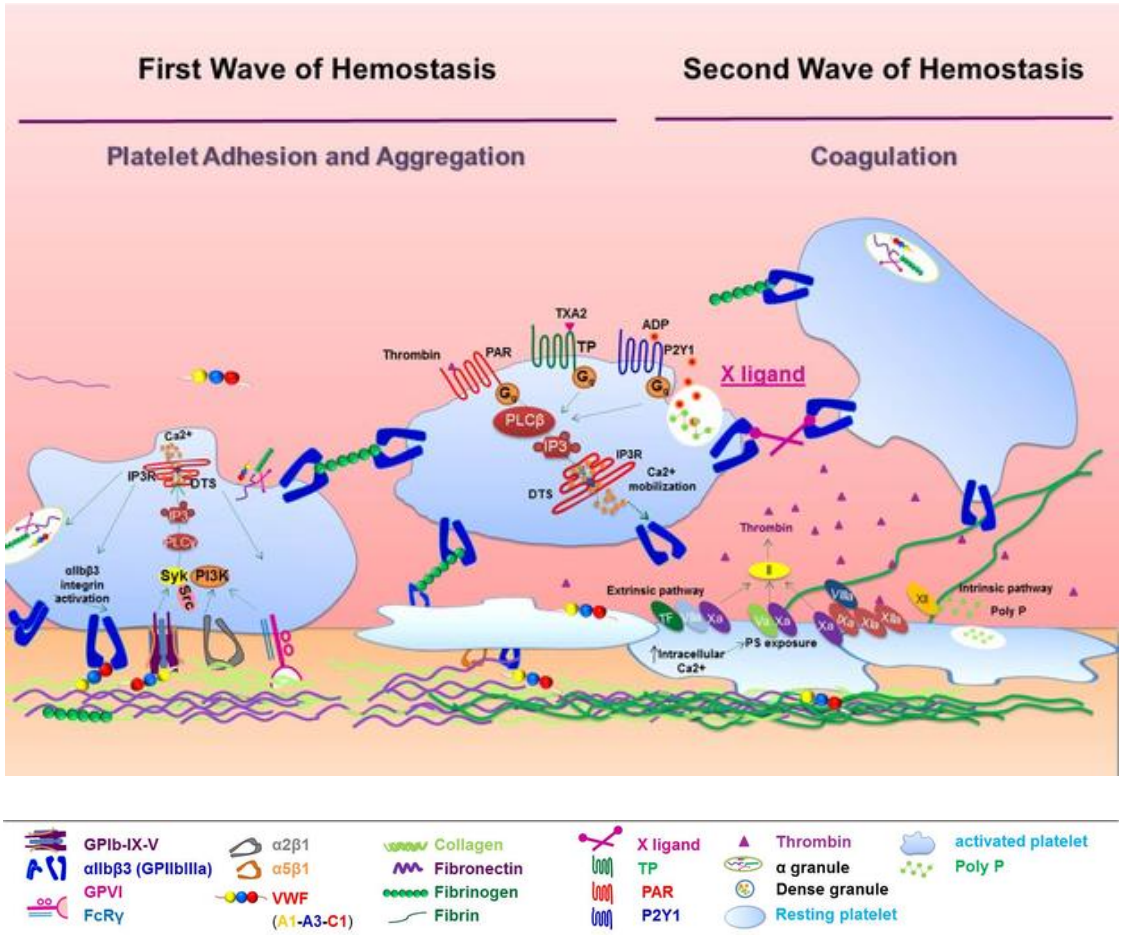


Fig. 3 taken and modified from Xu XR, Zhang D, Oswald BE, Carrim N, Wang X, Hou Y et al., Platelets are versatile cells: New discoveries in hemostasis, thrombosis, immune responses, tumor metastasis and beyond. [31]

Fibrin is rendered into its active form by thrombin which cleaves fibrinogen, a soluble plasma protein, to build fibrin monomers. These insoluble monomers interlink and form fibrin strands that are stabilized by factor XIII. The resulting

fibrin mesh helps anchoring the platelets and so stabilizes the platelet plug.[1, 32-33]

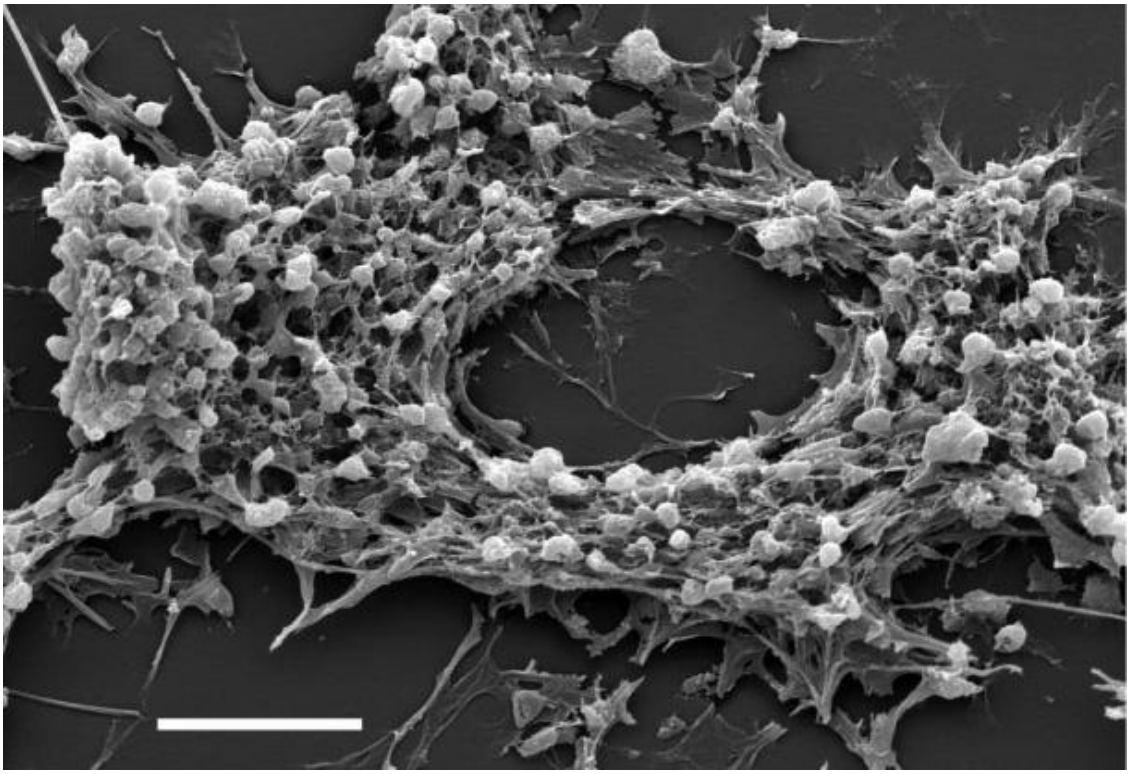


Fig. 4: Three-dimensional platelet-fibrin mesh formed on collagen under high shear conditions. Credit: Perelman School of Medicine, University of Pennsylvania

1.2.2. Intracoronary thrombi

Intracoronary thrombi that cause acute vessel obstruction or in-stent thrombosis consist of platelets, inflammatory cells such as monocytes, macrophages and neutrophils and other blood cells like fibroblasts. Previous studies have shown a high variability in thrombus composition in terms of the relative amounts of different cell types and fibrin content.[34]

The underlying pathophysiological processes of cell aggregation and interaction, thrombus formation and differentiation and the influencing factors of these processes in acute myocardial infarction are not yet fully understood.[35]

1.3. Antiplatelet therapies

The current clinical concept of preventing and treating coronary thrombosis is based on a dual antiaggregatory therapy. The common drug combination in clinical use is ASA plus a drug of the thienopyridine group, such as Clopidogrel, Ticlopidine or Prasugrel.

1.3.1. Aspirin

The standard antiplatelet drug is ASA, which inhibits the platelets' intrinsic enzyme COX-1 irreversibly. The result is a decrease in Thromboxane A_2 synthesis and secretion, thus disrupting the pathway of an amplified adhesion and activation of platelets as well as preventing vasoconstriction.

1.3.2. Clopidogrel, Prasugrel, Ticlopidine

Those drugs target the platelets' $P2Y_{12}$ receptor, inhibiting it irreversibly and thus preventing ADP-mediated platelet activation and recruitment. Drugs of the thienopyridine group are pro-drugs and need to be metabolically activated. Alternatives are non-thienopyridine derivatives such as Ticagrelor or Cangrelor. Since they are not pro-drugs there is no need for metabolic activation. Another difference to the thienopyridine group is that receptor-inhibition is reversible. This study focuses on the two main drugs of the thienopyridine-group Clopidogrel and Prasugrel.

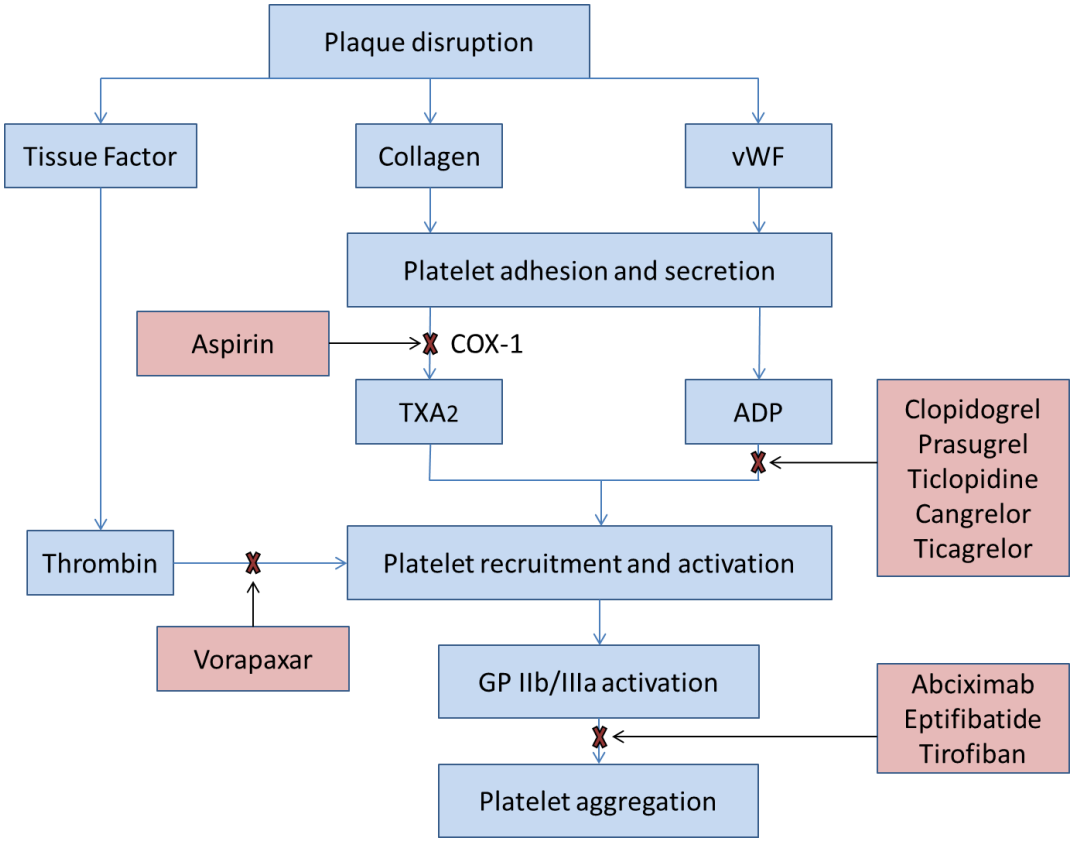


Fig. 5 taken and modified from Braunwald's Heart Disease: A Textbook of Cardiovascular Medicine, 10th ed. 2015 [1]: Antiplatelet drugs and their sites of action

There are preliminary studies suggesting that Prasugrel reduces thrombus size and formation.[36]

Nevertheless the effects of the different antithrombotic premedication on thrombus composition and architecture remain unclear. In particular, the specific antiplatelet regimens such as Prasugrel and Clopidogrel have not been compared yet in regard to their influence on thrombus architecture during or after acute myocardial infarction.

	Clopidogrel	Prasugrel
Dose	LD 600 mg, 75 mg/day	LD 60 mg, 10 mg/day
Receptor Inhibition	Irreversible	Irreversible
Activation	Prodrug	Prodrug
Onset of action	2-6 hrs	30 mins
Duration of effect	3-10 days	7-10 days

Table 1: Main features of P2Y12 inhibitors of the Thienopyridine class, taken and modified from Eurasian Journal of Emergency Medicine [37]

In comparison to Clopidogrel Prasugrel shows more stable pharmacokinetics and –dynamics, an overall more potent platelet inhibition and the most rapid onset of effect. It is therefore the antiplatelet drug of choice in acute ST-elevation myocardial infarction.[38, 39] These beneficial characteristics of Prasugrel in comparison to Clopidogrel and their potential effects on the formation, differentiation and morphological structure of intracoronary thrombi in patients with acute ST-elevated myocardial infarction had not been investigated to date.

1.4. Molecular Biomarkers

The following molecular biomarkers were used in this study for immunohistochemical staining in order to analyze composition and differentiation of intracoronary thrombi.

1.4.1. Cluster of differentiation 14 (CD14)

CD14 is a protein expressed by monocytes, macrophages and to a lesser extent by neutrophils. It is known to function as a co-receptor to Toll-Like receptor 4 (TLR4) and therefore plays an important part in the immune defense of bacterial lipopolysaccharide (LPS) but also other molecular pathogens i.e. lipoteichoic acid.[40] In this study it was used to detect the inflammatory cells named above.

1.4.2. Cluster of differentiation 42b (CD42b)

CD42b is part of the Glycoprotein complex (GP) Ib/V/IX and is used by platelets to bind von Willebrand factor.[41] It is a platelets-specific surface marker. Hence it functioned as an immunohistochemical marker for platelet staining of thrombotic material obtained by aspiration during PCI.

1.4.3. Cluster of differentiation 68 (CD68)

CD68 is a glycoprotein primarily expressed by macrophages and other cells deriving from monocytes. It has been used as an immunohistochemical surface

marker to detect the presence of macrophages in intracoronary thrombi which is an indicator for an underlying inflammatory process.[42]

1.4.4. Cluster of differentiation 105 (CD105) / Transforming Growth Factor beta (TGFß)

CD105 is a cell surface glycoprotein and forms part of the TGFß receptor complex. It is also known as Endoglin. It appears to play an important part in angiogenesis, the development of new vessels from pre-existing ones as part of physiological healing processes and tissue granulation. Angiogenesis also occurs in tumor transformation and growth.[43] CD105 is furthermore expressed in monocytes and macrophages, as well as smooth muscle cells, particularly in vascular ones. The TGFß receptor complex is a cytokine that exists in 4 different isoforms as part of the transforming growth factor superfamily.[44] When activated by a ligand, it will initiate transcriptions of genes that play a crucial role in regulating a variety of functions primarily of immune cells. These functions include cell differentiation and proliferation as well as chemotaxis and activation of immune cells. TGFß-1 is one of the isoforms that was first found in platelets and assumed to play an important role in physiological wound healing processes. Thus TGFß is a key indicator for inflammatory processes and responses.[44-46]

1.4.5. Macrophage Migration Inhibitory Factor (MIF)

MIF is an inflammatory cytokine that, as in vitro tests have shown, regulates monocyte adhesion and migration, regulates macrophage function and induces foam cell formation. Its function is pro-inflammatory.[47] As we have shown in previous clinical studies blood levels of MIF are significantly elevated in patients with symptomatic CAD.[48]

1.4.6. Gremlin 1 (Grem1)

Grem 1 is a protein that acts as inhibitor of BMPs, cytokines which are involved in multiple processes during embryonic development, inflammation and chronic fibrosis. It also acts as endogenous antagonist of MIF.[49] In patients with ACS and acute plaque rupture the Grem1/MIF ratio in blood plasma proved to be

significantly lower than in patients with stable CAD.[48] Whether MIF and Grem1 are up-regulated in intracoronary thrombi was subject of this research study.

1.4.8. H&E-Stain

H&E stain can be seen as the gold standard in histological staining. It is supposed to give an overview of the constituents of a histological section, clearly distinguishing cell nuclei from extracellular proteins. It was therefore used to detect and quantify fibrin content and classify intracoronary thrombi according to their morphological structure.

1.5. Aims

The process of thrombus formation is complex and the influencing factors on thrombus composition and thus on its architecture are diverse. In this study we focused on the effects of different antithrombotic premedication such as Clopidogrel and Prasugrel on thrombus morphology in patients with acute or recent ST-segment elevation myocardial infarction.

2. Material and Methods

For the analysis intracoronary thrombi of a total of 102 patients with acute (< 24 hrs) or recent (24-72 hrs) ST-elevated-myocardial infarction were acquired from 2 cardiological centers. The PCI and thrombectomy were performed at the University of Tübingen, Department of Cardiology, University Hospital of Tübingen and the Department of Cardiology, Klinik am Eichert Göppingen. 51 of the 102 patients were pretreated with 500 mg ASA plus Clopidogrel, the other half with 500 mg ASA plus Prasugrel either in the ambulance or on hospital admission. According to the common local clinical practice the Loading dose was 600 mg for Clopidogrel and 60 mg for Prasugrel. Except for the routine Heparin bolus no other antithrombotic loading was given.

2.1. Patients' baseline characteristics

The gathered clinical data included patients' baseline characteristics such as age at the time of the event, BMI, atrial fibrillation and additional relevant medication. Furthermore recorded were common cardiovascular risk factors such as tobacco use, hypercholesterinemia, hypertension, positive family history, diabetes and renal function.

Transthoracic ultrasound gave an overview of the left ventricular ejection fraction. The PCI gave information to the degree of coronary artery disease.

The study complies with the declaration of Helsinki and the guidelines of good clinical practice and was approved by the institutional ethics committee of the University of Tübingen.

The following table 2 gives an overview of patients' baseline characteristics, the statistical analysis and interpretation of the data will be found in the result section (section 3) of this paper:

Table 2:

Parameters	
Clinical characteristics	***Further Clinical parameters***
Age, y	Gender
BMI [kg/m^2]	Previous MI
BP systolic	In-Stent thrombosis
BP diastolic	PAD
Door-to-balloon time [mins]	AFib
PAP [mmHg]	COPD
Concomitant medication at study entry	***Biomarkers***
ASA	Leukocytes [1/µl]
Marcumar	Hb [g/dl]
Bivalirudin	Platelets [1000/µl]
Heparin 0-3hrs	CRP [mg/dl]
ß-blockers	SGOT [U/l]
ACE-I	CK [U/l]
ARB	LDH [U/l]
MRA	TnI [µg/dl]
Ca^{2+}-Antagonist	HbA1c [%]
Statins	Triglycerides [mg/dl]
PPI	Cholesterol [mg/dl]
	LDL-Cholesterol [mg/dl]
Cardiovascular Risk Factors	HDL-Cholesterol [mg/dl]
Hypertension	Creatinine [mg/dl]
Dyslipidemia	GFR-MDRD [ml/min/1.73m^2]

DM II	
Family History	
Smoker / ex	
LVEF	***Coronary Artery Disease***
>55%	None
50-54%	1 Vessel
36-49%	2 Vessel
<35%	3 Vessel

ACE-I – angiotensin converting enzyme inhibitor, AFib – atrial fibrillation, ARB – angiotensin receptor blocker, ASA – acetylsalicylic acid, BMI – body mass index, BP – blood pressure, Ca^{2+} – Calcium, CK – creatine kinase, COPD – chronic obstructive pulmonary disease, CRP – C-reactive protein, DM II – Diabetes Mellitus Type 2, GFR-MDRD – glomerular filtration rate-modification of diet in renal disease equation, Hb – Hemoglobin, HbA1c – HemoglobinA1c, HDL – high-density lipoprotein, hrs – hours, kg – kilograms, LDH – lactate dehydrogenase, LDL – low-density lipoprotein, LVEF - left ventricular ejection fraction, m^2 – square meters, MI – myocardial infarction, mins – minutes, mmHg – millimetre of mercury, MRA – mineralocorticoid receptor antagonist, NYHA – New York Heart Association, PAD – peripheral arterial disease, PAP – pulmonary artery pressure, PPI – proton-pump inhibitor, SGOT – serum glutamic oxaloacetic transaminase, TnI – troponin I, y – years.

After retrieval of the thrombi the following study was done:

2.2. Immunohistochemical Staining

Parts of the obtained thrombus material were embedded in paraffin and cut into series of 5 µm thick sections on the microtome. For staining Gremlin 1, MIF, CD105, CD68, CD14, CD42b and TGFß were used. Additionally, H&E staining was produced to gain a general overview of the thrombus composition and the fibrin content.

2.3. Material and Lab supplies

2.3.1 Machines and Devices

Scales Denver Instrument

Floating bath	Medax Model Nr.: 25900
Nikon Microscope	Nikon Optiphot-2
Nikon Camera	Nikon Digital Sight DS-U1
Microtome	Leica/Reichert-Jung Model 2045 Multicut
Clean bench	Thermo Electron Corporation HS18 40617682
Exhaust/vent	Waldner Airflow Controller AC3
Magnetic stirrer	IKAMAG®RET-GS
Vortexer	IKA®MS 3 basic Ident-Nr. 0003617000
Microwave	Severin Art.MW 7809
pH – meter	HANNA instruments model nr.: AHI-9124
Timer	Carl Roth GmbH+Co. KG model nr. TR118
Water purification system	Thermo Scientific TKA Genpure

2.3.2. Solutions, agents

Rotihistol	Roth Art.-Nr. 6640.1
Roti-Histokitt	Roth Art.-Nr. 6638.2
Paraplast Plus®	Roth Art.-Nr. X881.2
Citric Acid	SIGMA-ALDRICH 251275
Tri-Natrium-Dihydrate	AppliChem APA3901.1000
Hydrogen peroxide 30%	EMSURE® 1072092500
Methanol	VWR Chemicals 20.847.307
Ethanol 99%	UN 1170 SAV liquid production GmbH
Tween®20 for synthesis	Merck Art.Nr. 8221840500
Eosin G 50 mg	Roth Art.-Nr. 7089.1
Mayers Hämalaun	Merck Art.-Nr. 1092490500
L-Glutamine solution 200 mM	BioXtra Sigma-Aldrich G7513
Paraformaldehyde	Merck Art.Nr. 8187151000

Acetic Acid (glacial) 100%	Merck Art.-Nr. 100063
PBS Tablets	Gibco 1571002
Liquid DAB + Substrate Chromogen System	Dako K3468
Streptavidin-HRP	Dako P0397
Albumin Fraction V (pH 7.0)	AppliChem APA1391.0250

2.3.3. Antibodies, Serum

Primary antibody		**Secondary antibody**	
CD 68	Santa Cruz SC 20060	Biotinylated link universal	Dako K0690
CD14	Biorbyt orb19177	Biotinylated link universal	Dako K0690
CD42b	Santa Cruz SC7070 (discontinued)	Biotinylated link universal	Dako K0690
CD105	Santa Cruz SC 20632 (discontinued)	Rabbit prediluted	Abcam ab27422
TGFß1	Santa Cruz SC146-G	Biotinylated link universal	Dako K0690
Grem1	Abnova PAB 14845	Rabbit polyclonal	Dako X0936
MIF	R&D Systems AF-289-PB	Goat monoclonal	Vector Laboratories I-5000
Serum			
Anti-rabbit serum	Dako X0909		
Anti-goat serum	Dako E0466		

Table 3: Antibodies, Serum

2.3.4. Lab supplies

Pasteur pipette (sterile)	Ratiolab® Art.Nr. 2655135
Scalpel (sterile)	Pfm medical Art.-Nr.: 200130023
Falcon, 50ml, CELLSTAR®	Greiner bio-one Art.-Nr.: 227261
Pipettes	Eppendorf Research®10;100;1000
Pipette tips 200 µl	SARSTEDT 0109/5089011
Ratiolab® Pipette tips Blue 1000 µl	Ratiolab Art.-Nr. 2100610
Pipette tips, 10µl, colorless	Biozym Scientific GmbH Art.Nr. 720011
SuperFrost®Plus slides	R.Langenbrinck 03-0060

Cover slips 24x50 mm Stärke 0,13-0,16mm	R.Langenbrinck 01-2450/1
Cover slips 24x60mm Stärke 0,13-0,16mm	R.Langenbrinck 01-2460/1
Cover slips 24x40mm	Menzel-Gläser BB024040A1
Duran®Laboratory bottles, 500ml, 1000ml	DURAN Group GmbH Art.Nr. 21 801 44 59; 21 801 54 55
Safe-Lock Tubes 0,5 ml	Eppendorf 0030 121.023
Safe-Lock Tubes 1,5 ml	Eppendorf 0030 120.086
Cellstar®Tubes 50 ml	Greiner bio-one Art.-Nr. 227261

2.4. Methods

2.4.1. H&E staining

First the paraffin sections were deparaffinized and rehydrated in rotihistol and a graded alcohol series and then left in Aqua dest. for 5 minutes. Afterwards the sections were stained with a 1:5 hemalum solution (Aqua dest.1:5), blued for 15 minutes under warm tap water and stained for 3 minutes with 1% Eosin. The sections were then washed again with distilled water and dehydrated in a graded alcohol series. Finally sections were mounted, covered with a cover slip using Rotihistol and left for overnight drying.

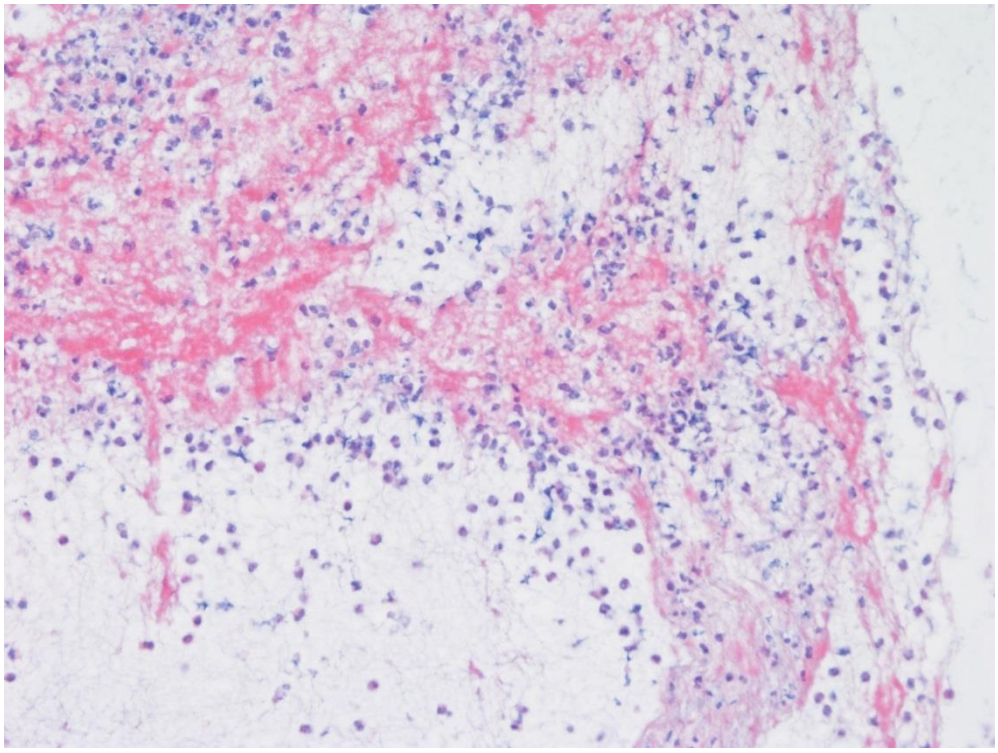


Fig. 6: Cell-rich thrombus in HE Stain

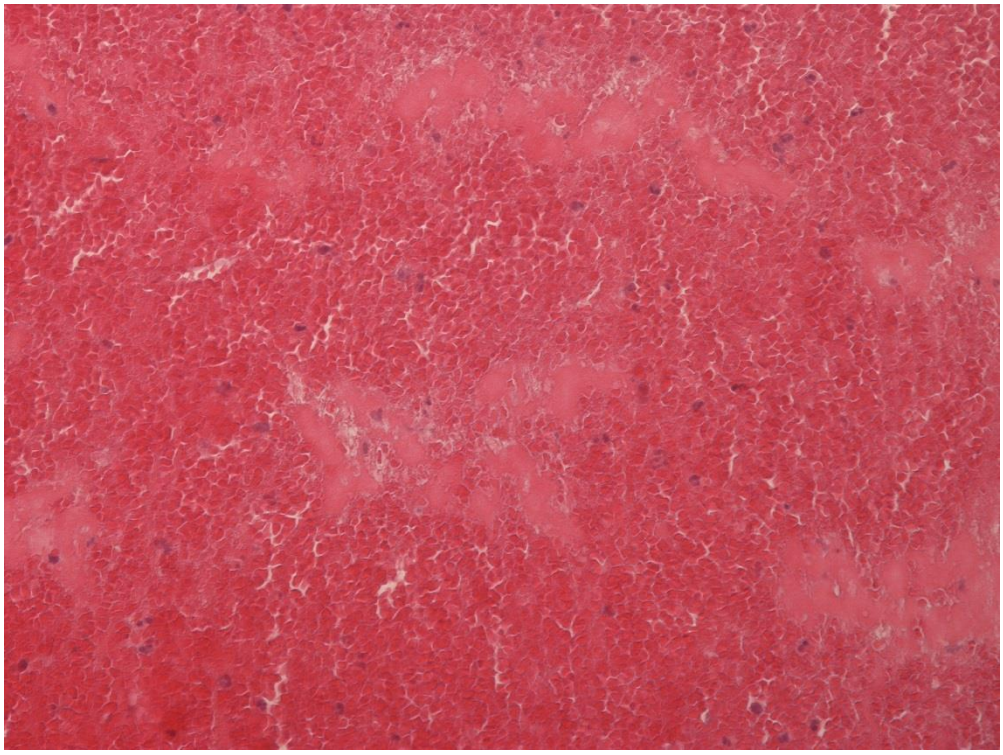


Fig. 7: Thrombus rich in red blood cells in HE Stain

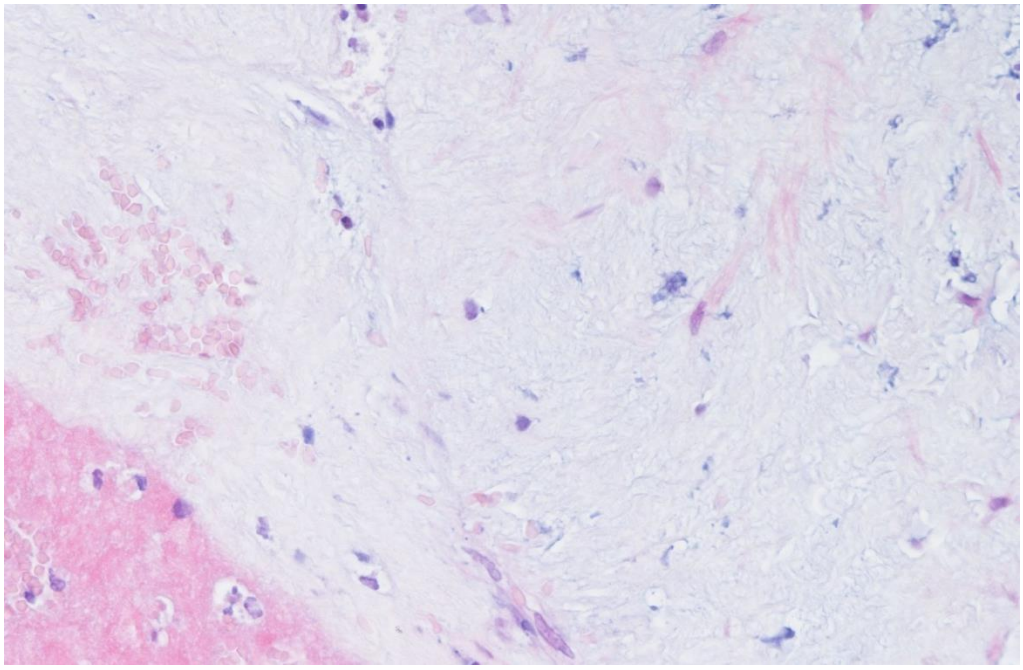


Fig. 8: Fibrotic/ amorphous thrombus in HE Stain

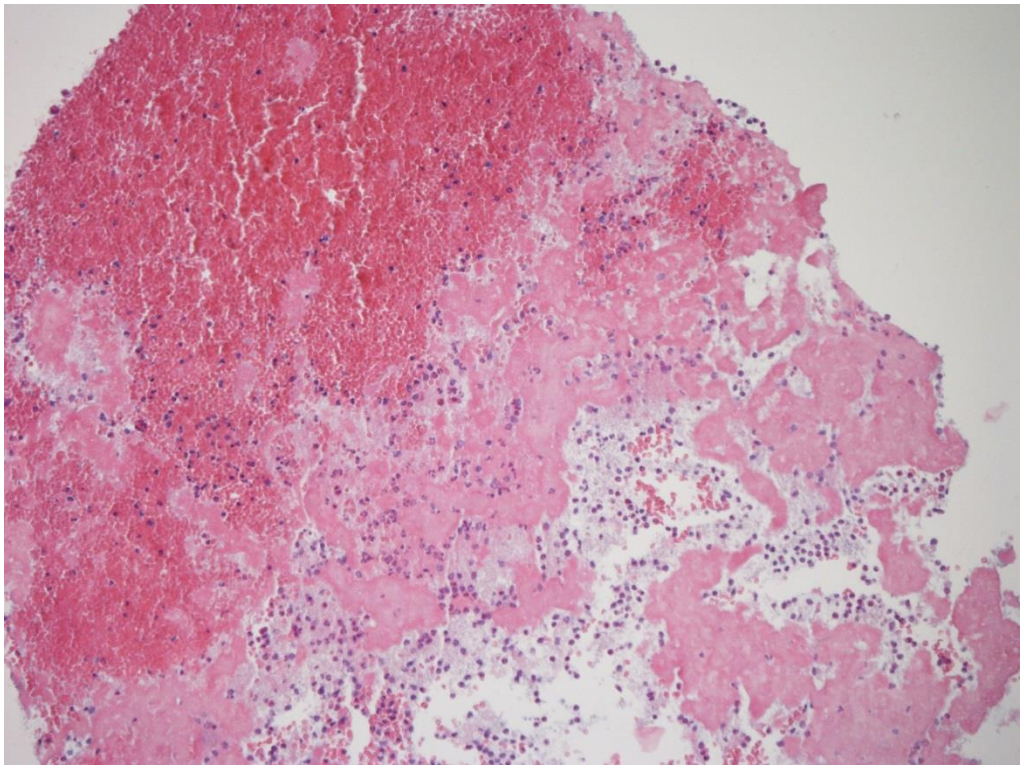


Fig. 9: Thrombus of mixed morphology in HE Stain

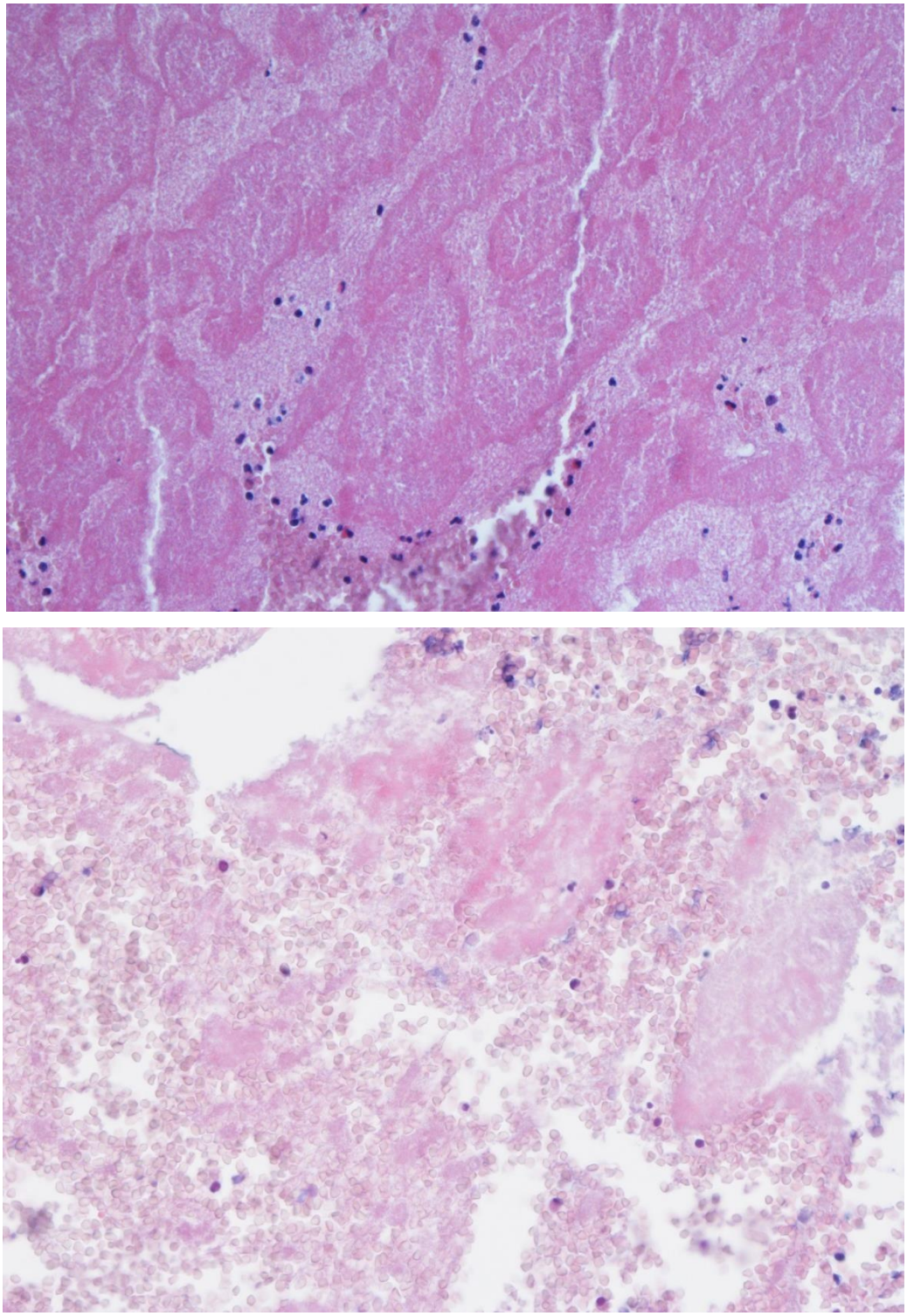


Fig. 10-11: Platelet-rich thrombus in HE Stain

2.4.2. Immunohistochemistry

After deparaffinization and rehydration (s. HE staining) the slides were washed in PBS for 5 minutes and then boiled in citrate buffer (pH 6) for 2 minutes at 600 Watts and consecutively for 3 minutes at 300 Watts. The heat treatment is used

to demask antigen epitopes. Formalin fixation and consecutive paraffin embedding leads to cross-linking of membrane proteins with formaldehyde molecules. The antigen epitopes are thus hardly accessible to primary antibodies and their binding is restricted.

Through HIER (heat induced epitope retrieval) with a HIER-solution like citrate buffer or alternatively PIER (proteolytic-induced epitope retrieval, which uses a proteinase instead of heat) the mentioned cross-links will be dissolved and binding of primary antibodies to the relevant epitope is made possible.

After short cooling time and repeated washings in Tween-PBS 0,05% the slides were put in 3% H2O2. This results in blocking of the endogenous peroxidase, in order to rule out false-positive signals later. After 15 minutes the sections were retransferred into Tween-PBS 0,05% and washed several times. The single objects of each series were then encircled with a Liquid-blocker PAP pen (Peroxidase- anti-Peroxidase) so that the convergence of different (prospectively used) agents (serum, antibodies etc.) was prevented.

Afterwards a 5% serum was used for all objects, which was removed by tilting after 30 minutes. The serum is supposed to block unspecific bindings and should therefore be of the same kind of animal as the secondary antibody. As an unspecific serum was used, it is suited for blocking various secondary antibody types, such as mouse, goat, rat etc.

Onto the first object of a three objects series an unconjugated primary antibody was applied; 30 µl were used per object. Object 2 served as IgG positive control, object 3 as PBS-negative control.

After 1 hour of incubation the sections were washed repeatedly in 0,05% Tween PBS and then incubated for half an hour with a biotinylated secondary antibody.

This method is based on the fact that biotin possesses 4 binding sites for Avidin and therefore the biotinylated antibody will be recognized easily by the SAB complex, rather the Avidin which was added in the following step. Additionally, Avidin is part of the same complex as the enzyme marker, the peroxidase, which will be consequently bound at the antigen at a higher number, and serves as signal amplification in the course of the process.

After another washing session with washing buffer, DAB-solution (1 ml of substrate buffer + 1 drop of stain) was added. The staining period varies between 2 to 10 minutes according to the speed in which brownish staining starts to occur. This is a critical part in the entire process, as there should be sufficient staining of the first object, but no staining of the IgG or PBS control.

The brown stains develop through oxidation of the DAB solution which is linked to the SAB complex and H2O2 by the peroxidase.

The slides were then washed again several times in 0,05% Tween PBS, the nuclei counterstained in 1:5 Hemalum for 3 minutes and consecutively blued for 10 minutes under warm tap water. The following graded alcohol series

(5sec 70% Ethanol, 5 sec 80% Ethanol, 5sec 90% Ethanol, 2x 5mins 100% Ethanol and 3 mins Rotihistol, is employed for dehydration. Finally the slides were mounted and covered with Rotihistol and a glass cover slip and then left to dry for a period of 24 hrs.

2.4.2.1. Immunohistochemistry- Photography

The immunostained sections were photographed using a Nikon microscope with NJS Software. 4 fold, 10 fold and 20 fold magnification were used for each object. In order to establish an ideal contrast and suitable lighting conditions different filter strengths were employed, according to magnification level.

The highest possible filter level (16+ 4+ 2) is needed for a 4 fold enlargement, for a 10 fold magnification, depending on the degree of staining, either the same or a 16+4 filter and for the highest degree of magnification the lowest filter combination of 16+ 2 was used.

2.4.3. Statistical analysis

Continuous variables are given as mean ± standard deviation (mean±-SD), when they were normally distributed. These variables were tested by two-sided t-test. Categorical data are presented as proportions and were analyzed by chi-squared test. Continuous parameters were dichotomized at established cut-off values. Values are given as mean ± standard deviation or standard error of the mean where applicable. Comparisons were considered statistically

significant if the two-sided P value was ≤0.05. Statistical analyses were performed using SPSS software version 22.0 (SPSS Inc., Chicago, IL, USA).

3. Results

The 102 patients of whom the thrombi were attained were either loaded with ASA and Prasugrel (n=51) or ASA and Clopidogrel (n=51) according to standard clinical practice. Loading took place either in the ambulance or on hospital admission.

Additionally, the patients' baseline characteristics were obtained and correlated to the immunohistological findings. The baseline characteristics are shown and evaluated below.

3.1. Background and baseline characteristics

Table 4: Clinical characteristics of patient population (n=102):

Parameters	**Clopidogrel (n = 51)**	**Prasugrel (n = 51)**	**P**
Clinical characteristics			
Age, y	65.25 (±14.83)	57.52 (±10.84)	**0.010**
BMI [kg/m²]	26.59 (±4.19)	27.88 (±4.70)	0.254
BP systolic	126.14 ±20.05)	125.93 (±28.81)	0.986
BP diastolic	70.00 (11.96)	72.67 (±12.16)	0.635
Door-to-balloon time [mins]	1519,32 (±3965,66)	517,2 (±1452,32)	0.151
PAP [mmHg]	26,50 (±6,79)	27,21 (±9,36)	0.822

Values are given as mean ±SD. MI – body mass index, BP – blood pressure, kg – kilograms, m^2 – square meters, mins – minutes, mmHg – millimetre of mercury, PAP – pulmonary artery pressure, y – years.

The clinical characteristics of the patient population embraced standard parameters such as age, BMI and blood pressure measured in the ambulance or on hospital admission. As shown in table 4, there is a significant difference between the Clopidogrel and the Prasugrel group regarding the patients' age. The Prasugrel cohort was with an average of 57.52 years younger than the Clopidogrel pretreated patients with an average age of 65.25 years. This is explained by current ESC guidelines that include certain aspects such as age and patients' medical history in their recommendations. Based on these

guidelines Clopidogrel should be preferred over Prasugrel if there is a high risk for hemorrhage, like for example intracranial or gastrointestinal bleeding. As the risk for severe hemorrhage is elevated in older people Clopidogrel still is the drug of choice for patients above the age of 75 years as well as for patients with under 60 kg of body weight.

Another aspect is the potential need for a so-called triple therapy or dual therapy with a NOAC as is the case in patients with atrial fibrillation undergoing PCI. Clopidogrel is still the only $P2Y_{12}$ inhibitor that is recommended to be used in combination with any oral anticoagulant.[50] Taking into account that the prevalence of atrial fibrillation and therefore the necessity of a combinatory therapy with an oral anticoagulant increases with age, the difference between the two groups regarding that parameter is furthermore explained.

The PAP and the LVEF (s. tables 5 and 8) were measured during PCI. Both parameters were within a close range to each other when comparing the results between the two groups.

Another set of parameters that was analyzed and compared were the biomarkers obtained by blood samples listed in table 2. Blood samples were taken on hospital admission, labeled as “Day 0” and during the following days. The most relevant biomarkers for this study are summarized below.

Table 5: Characteristics of patient population (n=102) - Biomarkers:

Parameters	**Clopidogrel (n = 51)**	**Prasugrel (n = 51)**	**P**
Biomarkers			
Leukocytes Day 0 [1/µl]	14744,29 (±5530,84)	16920,67 (±5562,51)	0.402
Hb Day 0 [g/dl]	12,57 (±1,84)	14,17 (±1,50)	**0.042**
Platelets Day 0 [1000/µl]	238,92 (±91,08)	254,73 (±79,51)	0.461
Platelets Day 1 [1000/µl]	218,42 (±70,53)	245,35 (±73,61)	0.186

CRP Day 0 [mg/dl]	3,06 (±4,56)	1,82 (±3,59)	0.225
CRP Day 1 [mg/dl]	5,11 (±6,47)	7,22 (±14,91)	0.612
CRP max [mg/dl]	9,04 (±8,87)	7,58 (±7,72)	0.701
SGOT Day 0 [U/l]	278,24 (±278,56)	261,17(±333,90)	0.865
SGOT Day 1 [U/l]	316,14 (±317,56)	201,50(±131,22)	0.408
CK Day 0 [U/l]	1005,12(±1677,56)	1428,85(±2099,53)	0.376
CK Day 1 [U/l]	1400,27(±1644,16)	1977,91(±1572,15)	0.196
LDH Day 0 [U/l]	498,86(±391,79)	438,81(±410,42)	0.583
LDH Day 1[U/l]	725,75(±434,83)	839,74(±405,78)	0.464
TnI Day 0 [µg/dl]	99,16(±115,99)	127,27(±121,78)	0.510
TnI max [µg/dl]	129,57(±123,48)	130,73(±119,61)	0.893
HbA1c [%]	6,75(±0,9)	6,22(±1,10)	0.311
Triglycerides [mg/dl]	135,85(±84,51)	119,41(±51,72)	0.563
Cholesterol [mg/dl]	182,33(±50,78)	193,06(±42,2)	0.616
LDL-Cholesterol [mg/dl]	115,17(±43,82)	124,76(±31,5)	0.568
HDL-Cholesterol [mg/dl]	40,5(±8,69)	43(±14,67)	0.701
Creatinine Day 0 [mg/dl]	1,07(±0,66)	1,12(±1,64)	0.906
Creatinine Day 1 [mg/dl]	1,03(±0,60)	1,21(±1,82)	0.654
GFR-MDRD Day 0 [ml/min/1.73m^2]	80,93(±32,81)	87,93(±29,81)	0.387
GFR-MDRD Day 1 [ml/min/1.73m^2]	80(±38,98)	86,28(±28,70)	0.491

Values are given as mean ±SD. CK – creative kinase, CRP – C-reactive protein, GFR-MDRD – glomerular filtration rate-modification of diet in renal disease equation, Hb – Hemoglobin, HbA1c – HemoglobinA1c, HDL – high-density lipoprotein, LDH – lactate dehydrogenase, LDL – low-density lipoprotein, SGOT – serum glutamic oxaloacetic transaminase, TnI – troponin I.

As shown in table 5 there was a notable difference in Hemoglobin levels, which was with an average of 12,57 in the Clopidogrel group significantly lower than the Hb levels of the Prasugrel group with 14,17. This corresponds on one hand with the difference in age range, since Hemoglobin levels are often naturally lower in the older population. On the other hand, as mentioned above, a high risk for internal bleeding is a contraindication for Prasugrel administration. This

risk is usually determined by the patient's medical history, which together with the lower Hb levels might be an indicator for more frequent bleeding incidences in the past in the Clopidogrel group, so that Prasugrel could not be administered in respect of current ESC guidelines.

Leukocyte and CRP levels as marker for inflammatory processes did not differ significantly, neither did the platelets levels. However it has to be mentioned at this point that platelet cell count does not give any evidence about platelets' function or activity. This marker was merely used to rule out any significant differences between the two groups that might have influenced the result of immunohistochemical staining and further statistical analysis.

Further biomarkers were those indicating cell damage in general and myocardial cells in particular, such as troponin, creatine kinase (CK), lactate dehydrogenase (LDH), and serum glutamic oxaloacetic transaminase (SGOT).

Although there was no significant difference between the two groups, the troponin levels in the Prasugrel group were initially distinctly higher, whereas the Clopidogrel group showed lower levels on admission, but reached nearly exactly the same maximum levels within the course of a few days as the Prasugrel group.

Other cardiovascular risk markers such as HbAc1 as a diabetes marker or Triglycerides and Cholesterol levels as markers for dyslipidemia did not show any significant differences between the Clopidogrel and the Prasugrel arm. Nevertheless it has to be pointed out that the standard HbAc1 cut-off value for the diagnosis of Diabetes lies at 6.5%. The Clopidogrel group showed a median value of 6.75%, while the Prasugrel group had an average level of 6.22%. This signifies that the prevalence of Diabetes was more frequent in the Clopidogrel group than in the Prasugrel group. Both groups showed LDL-cholesterol levels above the recommended values of 70-100 mg/dl, with the Prasugrel group leading slightly.

Renal function measured by creatinine levels and GFR did not differ significantly and stayed more or less stable after contrast medium exposure during PCI.

The following table gives an overview of the patient baseline medication with main focus on drugs relevant for CAD treatment or treatment of related conditions.

Table 6: Baseline characteristics of patient population (n=102) - Medication:

Parameters	**Clopidogrel [%]**	**Prasugrel [%]**	**P (Chi square)**
Concomitant medication at study entry			
ASA	80	97,8	**0.010**
Bivalirudin	48	34,1	0.264
Heparin 0-3hrs	92,3	97,6	0.300
ß-blockers	33,3	18,6	0.162
ACE-I	25,9	23,3	0.800
ARB	18,5	16,3	0.809
MRA	14,3	6,7	0.563
Ca^{2+}-Antagonist	25,9	9,3	0.063
Statins	25,9	18,6	0.467
PPI	14,3	26,7	0.519
Marcumar	11,1	2,3	0.123

Values are given in [%] of group total. ACE-I – angiotensin converting enzyme inhibitor, ARB – angiotensin receptor blocker, ASA – acetylsalicylic acid, Ca^{2+} – Calcium, hrs – hours, MRA – mineralocorticoid receptor antagonist, PPI – proton-pump inhibitor.

There is a significant difference between the two pretreatment groups regarding ASA as concomitant medication. In the Prasugrel group 97,8% used ASA as part of their long-term medication as opposed to merely 80% in the Clopidogrel group. The most conceivable reason for this significant difference is again, as mentioned above, the additional presence of other conditions such as atrial fibrillation that requires a different long-term strategy than a dual antiplatelet

therapy (DAPT). Another reason might be an intolerance or allergy to ASA with the simultaneous need for a life-long antiplatelet therapy. In this case patients are usually treated with Clopidogrel instead of ASA. Although not significantly, Phenprocoumon (Marcumar) use was slightly higher in the Clopidogrel group which further supports the additional-coagulant-theory, particularly since the use of other oral anticoagulants was not investigated upon. Regarding the common heart insufficiency/failure and CAD medication including ACE-Inhibitors, beta blockers, angiotensin receptor blockers and mineralocorticoid receptor antagonists there was again no significant difference between the two groups. Also other anticoagulants showed similar usage values for both pretreatment arms. Co-medication such as Ca^{2+}-antagonists that are used to treat underlying causes for CAD, such as arterial hypertension and that are also known to have some antianginal effects, was clearly used more often by the Clopidogrel group, although the difference was not significant.

Further clinical baseline characteristics of the patient population as well as the main cardiovascular risk factors and the type of CAD in terms of the number of vessels affected were obtained and are given in the table below in percentage for each group.

Table 7: Further clinical baseline characteristics of patient population (n=102):

Parameters	**Clopidogrel [%]**	**Prasugrel [%]**	**P (Chi square)**
Further Clinical parameters			
Male	71,9	82,6	0.259
Previous MI	14,3	20	0.746
In-Stent thrombosis	12	4,9	0.289
PAD	7,7	9,5	0.796
AFib	32	4,8	**0.002**
COPD	4,0	4,9	0.868
Cardiovascular Risk Factors			

Hypertension	65,4	63,6	0.883
Dyslipidemia	42,3	45,5	0.798
DM II	23,1	13,6	0.311
Family History	11,5	27,3	0.144
Smoker / ex	36	54,8	0.111
LVEF			**Overall P**
>55%	28,6	12,0	
50-54%	14 ,3	30 ,0	
36-49%	47,6	42 ,0	0,087
<35%	9,5	16 ,0	
Coronary Artery Disease			**Overall P**
None	7,7	11,9	
1 Vessel	42,3	21,4	
2 Vessel	15,4	26,2	0.302
3 Vessel	34,6	40,5	

Values are given in [%] of group total. AFib – atrial fibrillation, COPD – chronic obstructive pulmonary disease, DM II – Diabetes Mellitus Type 2, LVEF - left ventricular ejection fraction, MI – myocardial infarction, NYHA – New York Heart Association, PAD – peripheral arterial disease.

As documented in the above table 7, the patients in both groups were predominantly male. An average of 18% of the study population had a myocardial infarction in the past, with no significant difference between the two groups, although the rate for previous MI was slightly elevated in the Prasugrel group. In-stent-thrombosis occurred in 12% of the Clopidogrel group and in nearly 5% of the Prasugrel group.

There were approximately 1/3 of patients with atrial fibrillation in the Clopidogrel group in contrast to only 4.8 % in the Prasugrel group. This significant difference is explained by the fact that for patients in need of an OAC/NOAC in addition to an antiplatelet therapy Clopidgrel is the drug of choice.[50] The values for patients with peripheral arterial disease and chronic obstructive pulmonary disease did not differ considerably between the two groups.

In regard to cardiovascular risk factors the percentages for patients with hypertension and dyslipidemia were almost identical. Differences between the two groups regarding prediagnosed diabetes, family history for CAD or tobacco consumption were present but statistically not significant. There seems to be a higher prevalence of smokers and familial disposition in the Prasugrel group, whereas the prevalence of diabetes seemed to be higher in the Clopidogrel group.

Left ventricular function was classified in 4 categories ranging from normal left ventricular function with an ejection fraction of >55% to mild (EF = 50-54%), moderate (EF = 36-49%) and severe left ventricular dysfunction with an EF <35%. In both groups the majority of patients had a moderate dysfunction. In the Clopidogrel group nearly 30% had a normal LVEF as opposed to the Prasugrel group where only 12% had a normal LVEF.

In regard to the number of vessels affected in CAD patients, there were no significant differences between the Clopidogrel and the Prasugrel pretreated patients. No relevant CAD was present in 10% of the study population, which speaks for an acute thromboembolic event. In the Prasugrel group these accounted for 12%, in the Clopidogrel group for nearly 8%. In the Clopidgrel group a majority of 42% had 1-vessel-disease, in the Prasugrel group this occurred in only 21%. In the Prasugrel group in the majority of patients all 3 vessels were affected (>40%) in the Clopidogrel group a 3-vessel disease was present in 34%.

The primary aim for the collection of the data above was to rule out any statistically significant differences between the Clopidogrel and the Prasugrel group that might influence the results of immunohistochemical analysis. Although there were a few significant differences as mentioned above, these are partly the reason why one of the pretreatments was chosen over the other. It is therefore impossible to exclude these patients from a research study like this one. Overall the two medication groups did not show relevant and statistically significant differences so that a potential impact of patients' baseline characteristics can be excluded.

3.2. Immunohistochemistry

The aspirated thrombi were stained using the biomarkers CD14, CD42b, CD68, CD105, MIF, Grem1, TGFß and H&E staining for a general overview and to quantify the fibrin content. The results of the stainings were then analyzed comparing the Prasugrel with the Clopidogrel group. Interestingly, the immunohistochemical analysis showed a significant difference between the Clopidogrel and the Prasugrel group regarding the fibrin content. The obtained thrombotic material was categorized by fibrin content in percentage of the overall sample area.

3.2.1. Immunohistochemistry H&E stain

The thrombi of patients that were loaded with Clopidogrel showed significantly more often a fibrin content of over 50% in comparison to Prasugrel-pretreated patients as shown below in diagram 1:

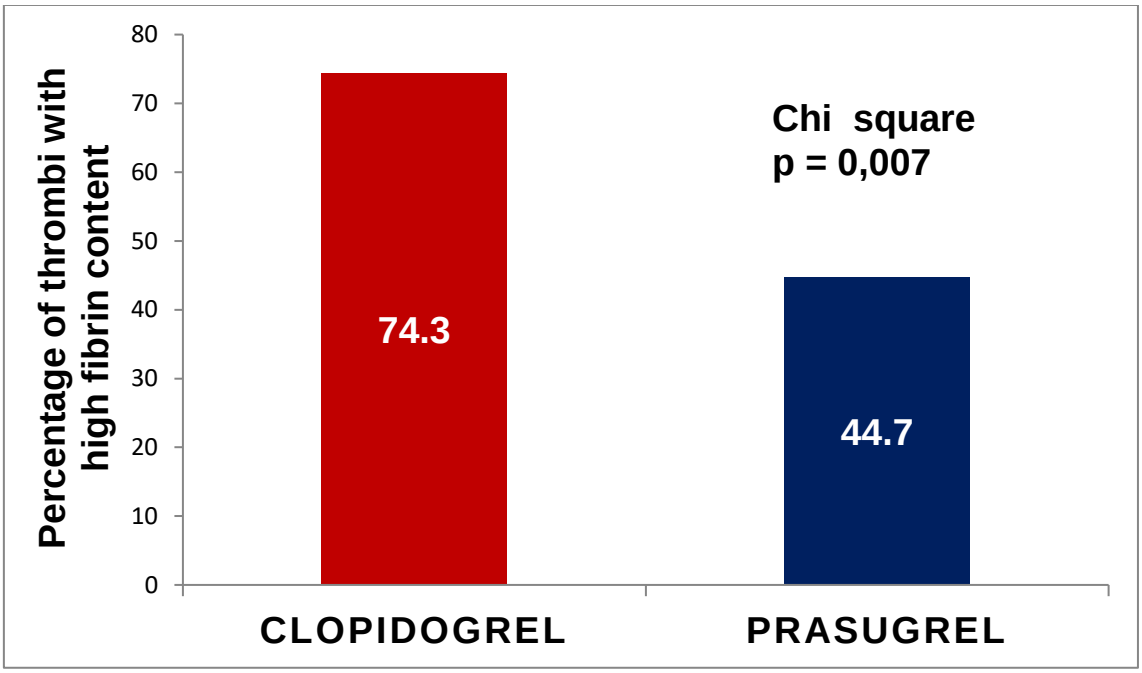


Diagram 1: 74,3% of thrombi showed a fibrin content of >50% in the Clopidogrel group. In the Prasugrel group 44,7% showed a fibrin content of >50%.

Fibrin derives from Fibrinogen and is brought into its active, insoluble form by thrombin. Fibrin molecules form strands which weave into a fibrin mesh. The mesh is then stabilized by factor XIII of the coagulation system. The entire aggregate of blood cells, platelets in particular and fibrin mesh turns the initially instable platelet plug into a solid thrombus. The two constituents interact in several ways at different points in time during the whole process of thrombus formation. Fibrinogen is one of the platelets' GP IIb/IIIa receptor ligand and, early in the process, leads to adherence and activation of platelets. The receptor-ligand complex also acts as positive feedback loop for the signaling and activation pathway, thus not only amplifying the initial reaction but also stabilizing the entire conglomerate. Additionally, platelets, once activated, release several coagulation factors as well as fibrinogen, which is, as mentioned above, not only a ligand to the platelets' own receptor, it is also the precursor of fibrin. This leads to another more indirect way of fibrin-platelet-interaction: Thrombin, the activated factor II, is the end product of the coagulation cascade. On one hand it plays a vital role in fibrin mesh formation since it functions as protease that cleaves soluble fibrinogen in fibrin. On the other hand it catalyzes the entire coagulation process by recruiting and activating more platelets in terms of a positive feedback mechanism.

With these findings, although presumably neither Prasugrel nor Clopidogrel acts directly on fibrin mesh formation, it has to be assumed that the effect of Prasugrel on platelet inhibition is stronger, particularly in regard to preventing the amplification process by positive feedback loops that are mentioned above.

In regard to the molecular biomarkers, 3 subgroups were formed for each individual marker according to the level of expression: no to low expression meaning no or only low staining, fair or medium and high expression level. Diagrams below give an overview of the three expression levels in percentage of the overall amount of stained thrombi. The photographs show examples of the correspondent biomarker stained sections with none/low, medium and high grade of expression. In order to statistically analyze and compare the Prasugrel- with the Clopidogrel- loaded group, stained cells of each section were counted.

The only exception was made for the “platelet marker” CD42b; the areal staining did not permit for cells to be counted. Hence the expression grade was used as a powerful indicator for platelet prevalence.

The results of the statistical analysis are presented consecutively in diagrams for each individual biomarker, followed by exemplary immunohistochemical stains of every marker and correspondent HE stain for each group:

3.2.2. Immunohistochemistry CD14

CD14 expression levels:

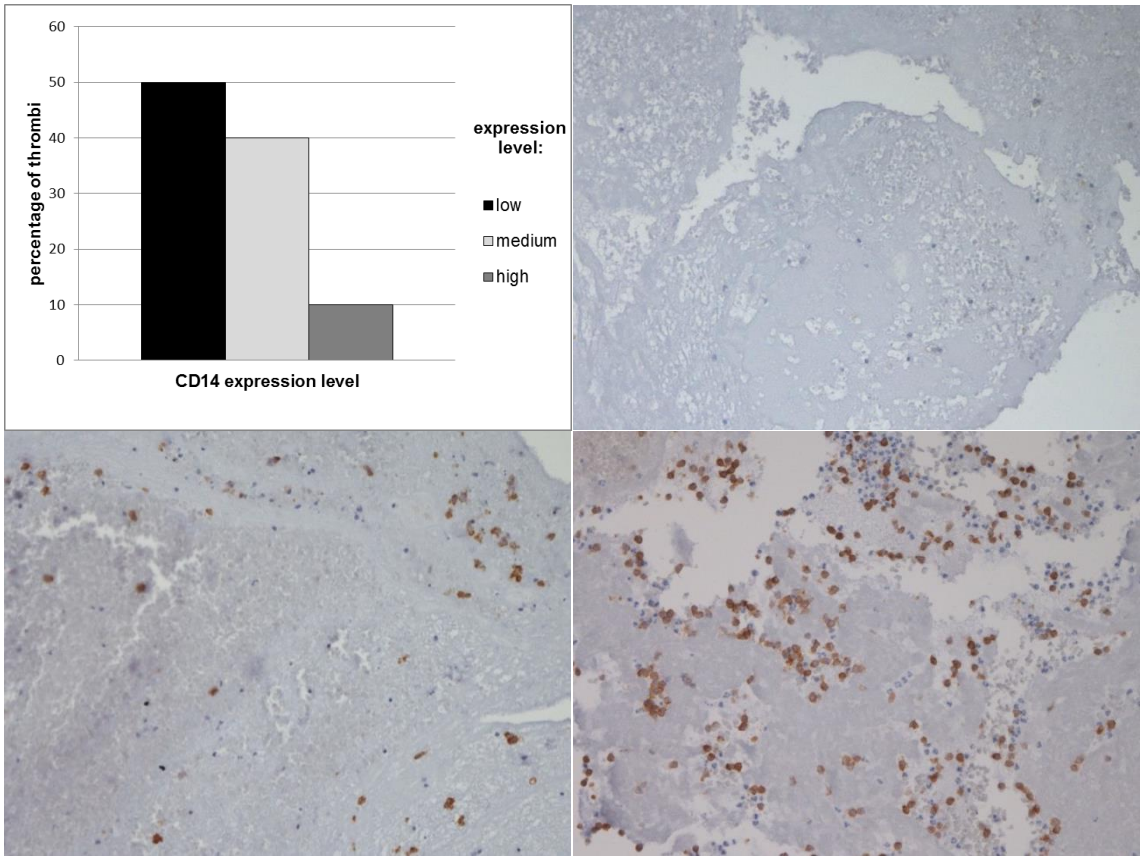


Diagram 2: CD14 expression level in percentage of stained thrombi.

Fig.12-14: Low, medium and strong expression level for CD14.

Diagram 2 above demonstrates that the CD14 stained sections showed to 50% no or low expression of the marker. There were 40% with a medium expression level and 10% showed a high expression grade.

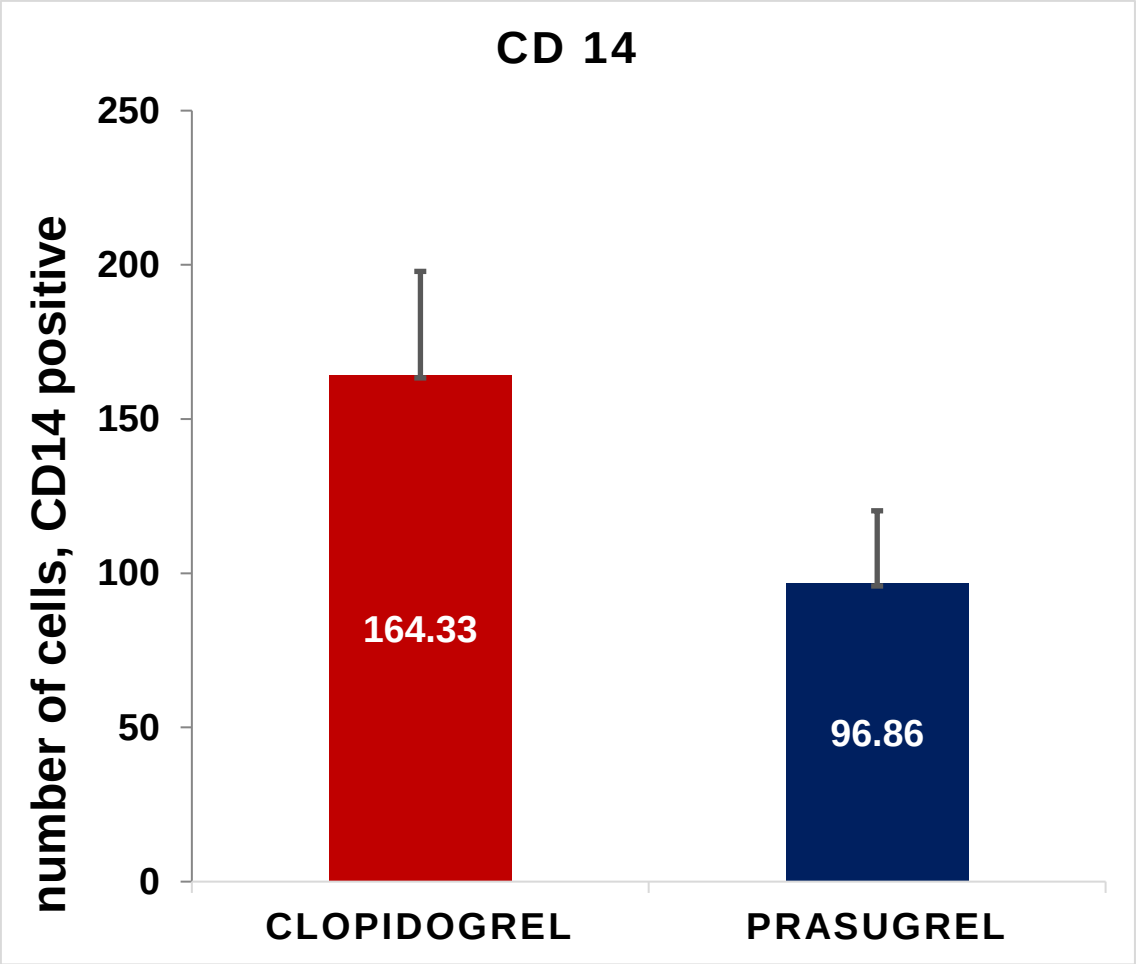


Diagram 3: Values are given as mean ± standard error, number of CD14 positive cells (Monocytes) per high power field (10x)

When comparing the CD14 stained sections of the Prasugrel- with the Clopidogrel- group they showed more often a higher expression level in the Clopidogrel group. Although there was not a significant difference in cell count of CD14 stained cells this clearly signifies a trend towards the Clopidogrel group. CD14 is a marker expressed by monocytes and macrophages, as well as neutrophils. It is therefore a valuable indicator for inflammatory processes.[40] Especially in atherosclerosis, plaque formation, development and eventually plaque rupture monocytes or rather their derivatives have been found to play a significant role. Macrophages derive from monocytes, and in case of lipid accumulation within the arterial vessel walls as part of plaque formation and development, evolve into foam cells.[51] This process is mainly due to the macrophages' scavenger function: Macrophages as part of the immune system

are recruited to the site of damage and take up cholesterol and lipid molecules. These molecules accumulate intracellular and lead to foam cell development with their typical lipid-rich appearance. Foam cells are suspected to play a crucial part in plaque rupture.[52, 53]

The statistical trend towards the Clopidogrel group speaks therefore for an influence of the pretreatment agent on inflammation as well as on monocyte/macrophage and thus foam cell prevalence in acute or recent myocardial infarction.

H&E and CD14 stains, Clopidogrel and Prasugrel:

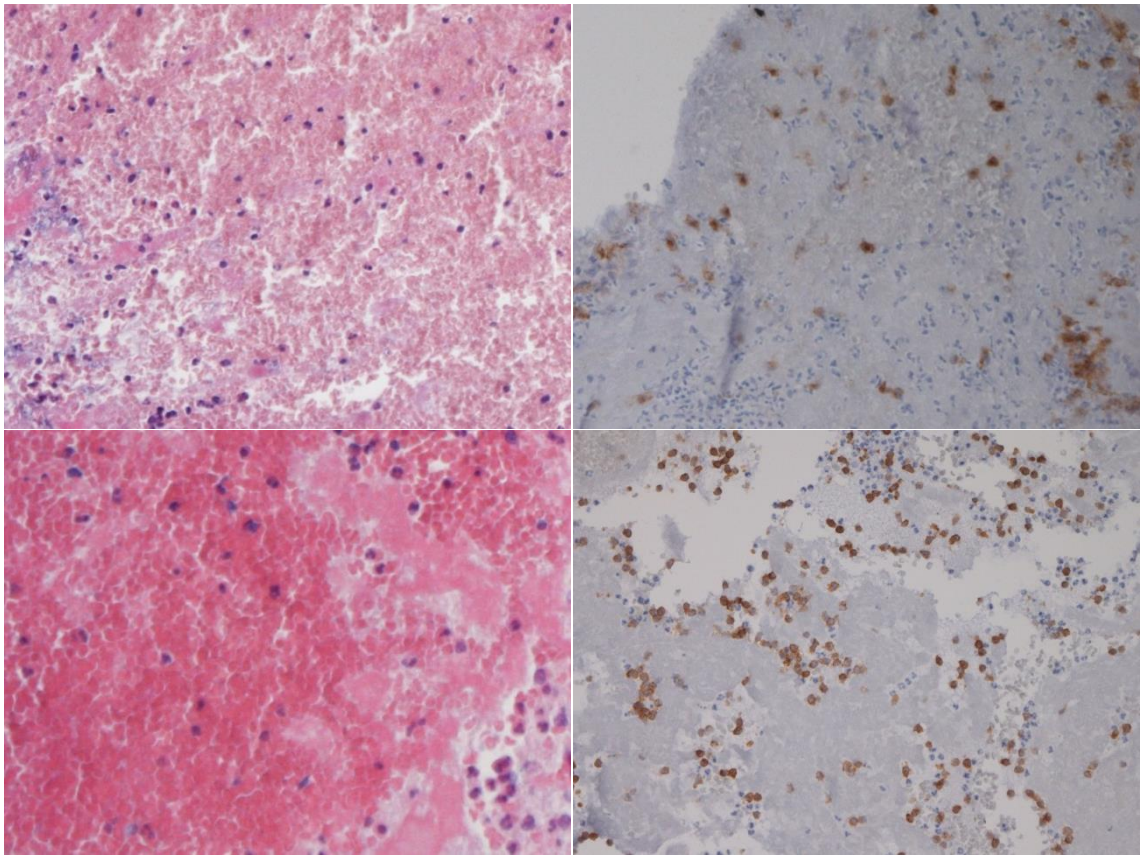


Fig.15-16: Clopidogrel Loading, HE and CD14 stain
Fig.17-18: Prasugrel Loading, HE and CD 14 stain

3.2.3. Immunohistochemistry CD42b

CD42b expression levels:

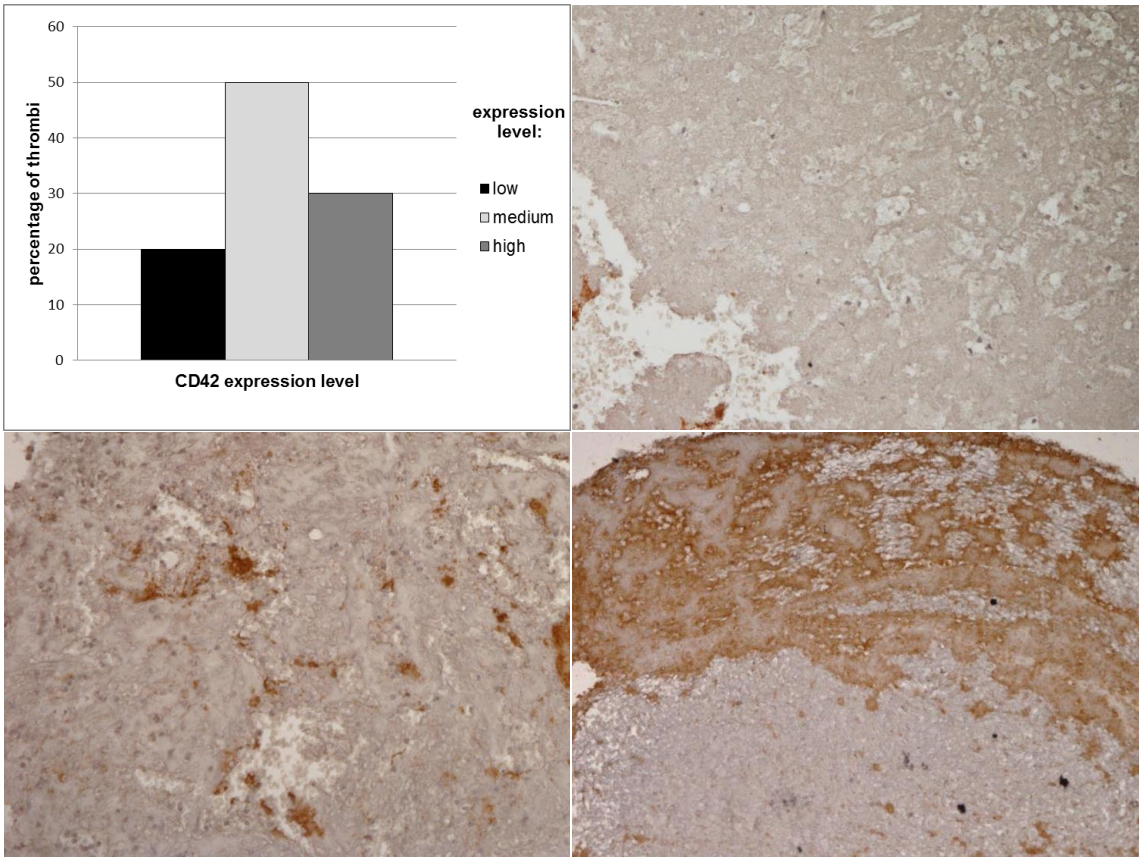


Diagram 4: CD42b expression level in percentage of stained thrombi.

Fig.19-21: Low, medium and strong expression level for CD42b.

CD42b is a “platelet marker”. Overall, 30% of thrombi had a high expression grade of CD42b, an even higher amount with 50% a medium expression grade and a minority of 20% no to low expression. This underlines the fact that there is a great prevalence of platelets in coronary thrombi.

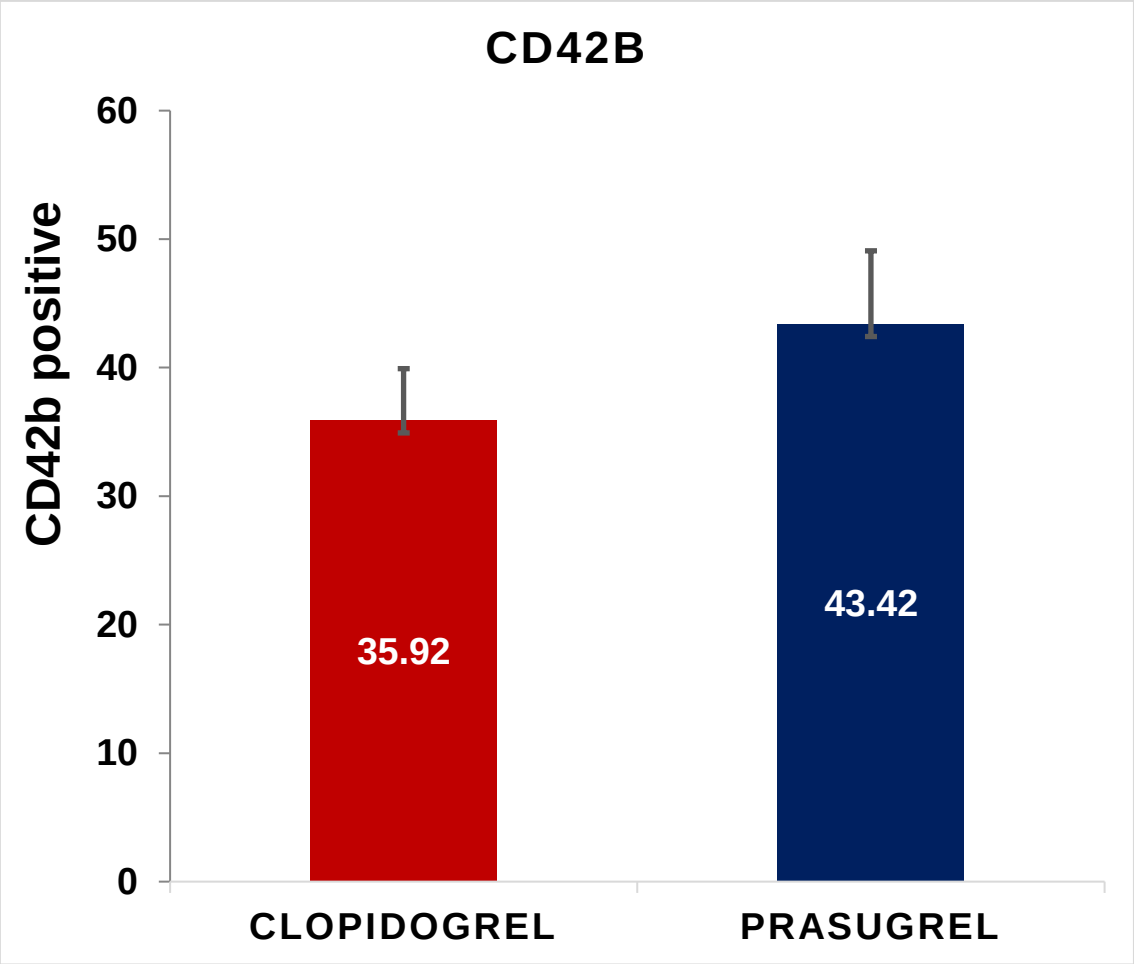


Diagram 5: CD42b positive area (platelets) in percentage of sample area per high power field (10x)

Statistically, there was no significant difference in CD42b expression between the Clopidogrel- and the Prasugrel group as can be seen in diagram 5. The correspondent stains are examples for H&E as well as CD42b stained sections for each group. The latter also show the patchy stain patterns of this particular biomarker and therefore explain why the grading level was a more adequate tool for quantification of platelet prevalence.

H&E and CD42b stains, Clopidogrel and Prasugrel:

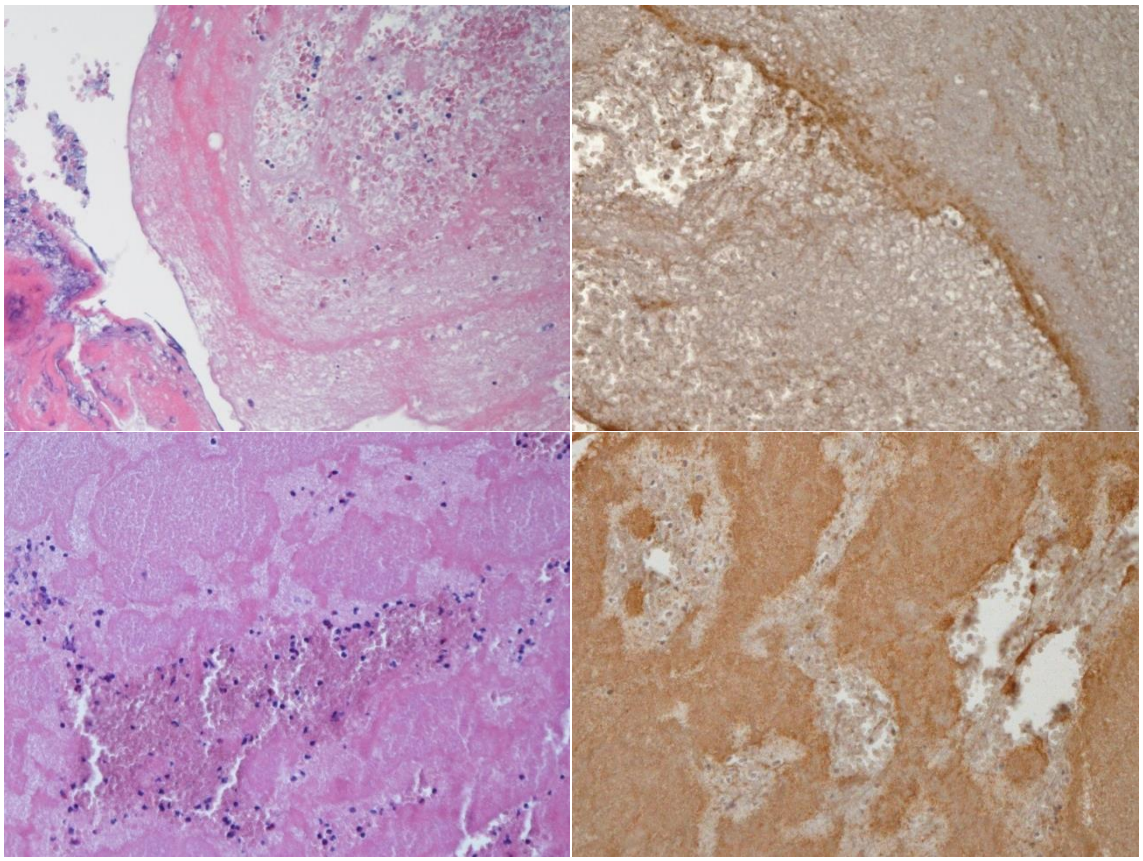


Fig.22-23: Clopidogrel Loading, HE and CD42b stain

Fig.24-25: Prasugrel Loading, HE and CD 42b stain

3.2.3. Immunohistochemistry CD68

CD68 expression levels:

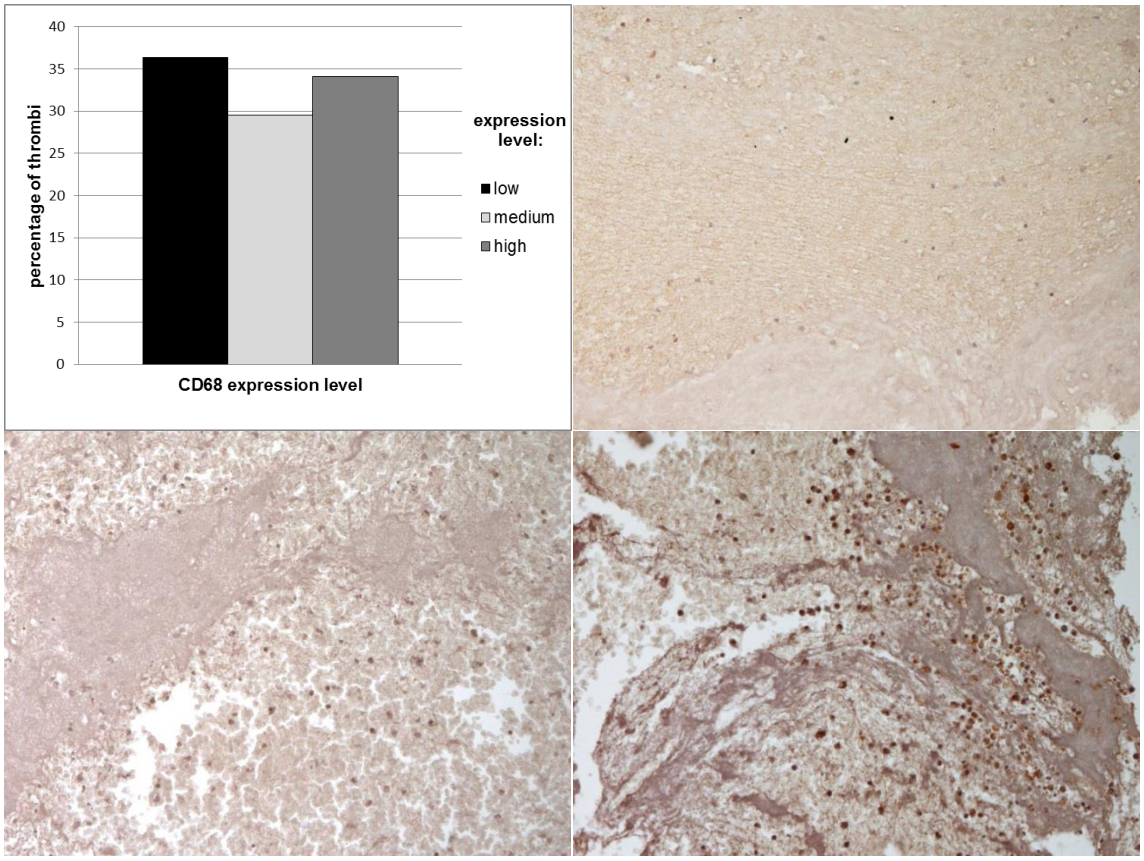


Diagram 6: CD68 expression level in percentage of stained thrombi.

Fig.26-28: Low, medium and strong expression level for CD68.

There were no remarkable differences regarding the expression levels of CD68 stained thrombi. Just above 35% showed low staining, just below 35% high staining and the rest a medium staining grade.

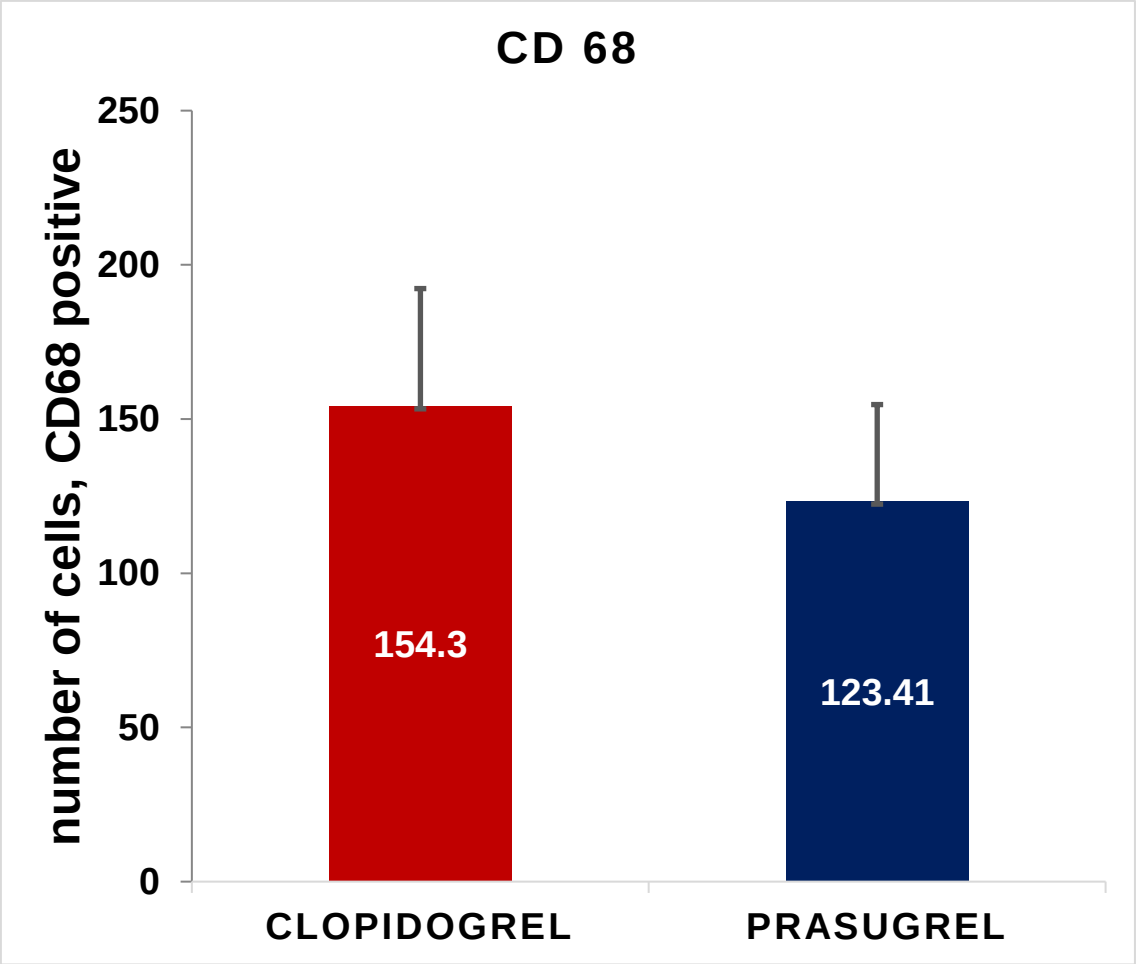


Diagram 7: Values are given as mean ± standard error, number of CD68 positive cells (Monocytes/Macrophages) per high power field (10x)

The cell count of CD68 stained cells was with an average of >150 positive cells higher in the Clopidogrel group, although the difference to the Prasugrel group was not significant. CD68 is a monocyte- and macrophage-specific marker. The findings imply that in approximately 2/3 of the stained thrombi an inflammatory process involving monocytes and macrophages occurred, regardless of the medical pretreatment.

H&E and CD68 stains, Clopidogrel and Prasugrel:

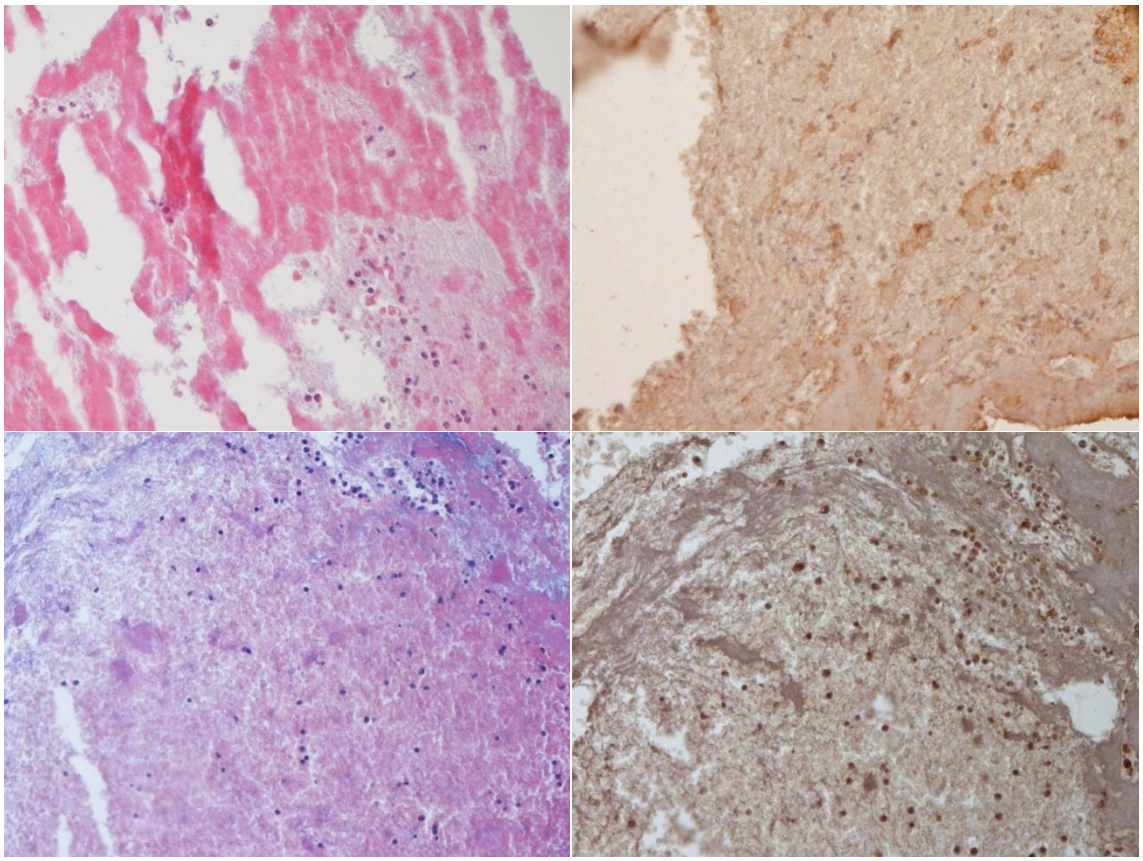


Fig.29-30: Clopidogrel Loading, HE and CD68 stain

Fig. 31-32: Prasugrel Loading, HE and CD68 stain

3.2.4. Immunohistochemistry CD105

CD105 expression levels:

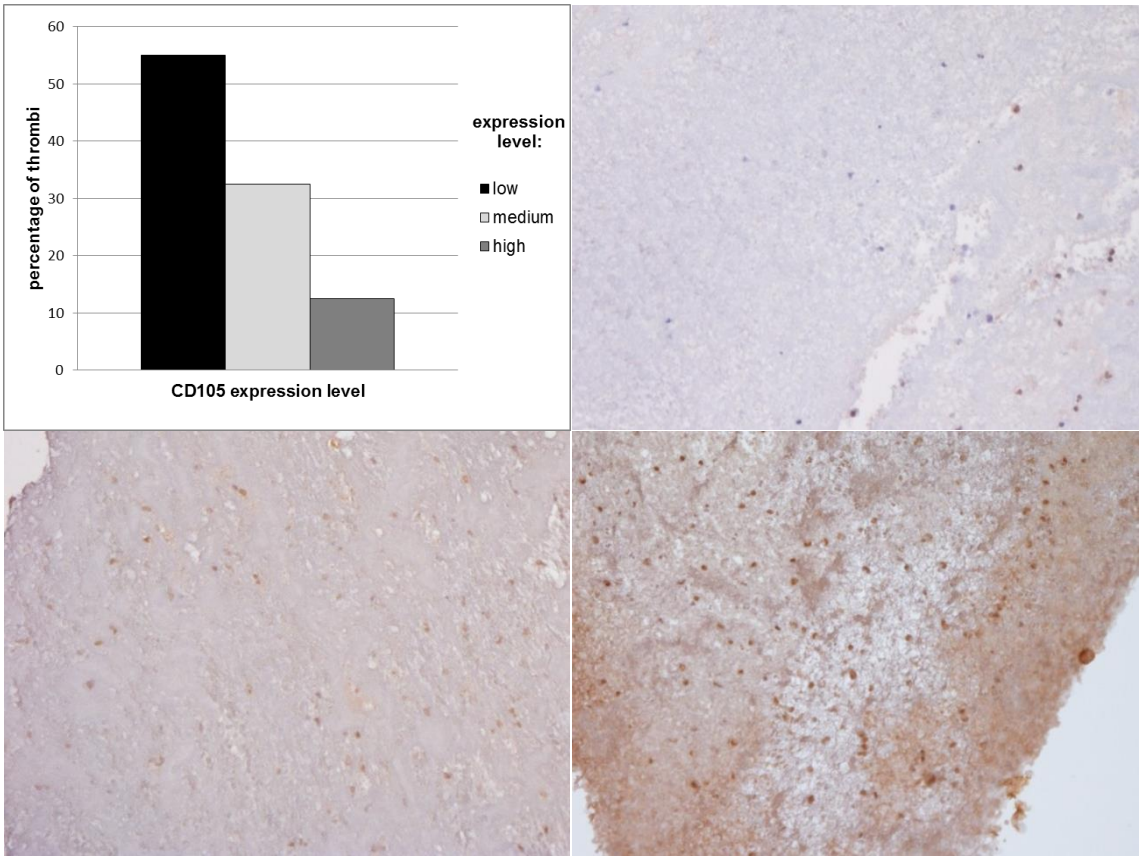


Diagram 8: CD105 expression level in percentage of stained thrombi.
Fig.33-35: Low, medium and strong expression level for CD105.

The analysis of CD105 expression levels clearly indicates a low expression for this marker with over 50% of all stained sections showing no or low expression and only just over 10% a high expression level.

CD105 is part of the TGFß-receptor complex and a marker primarily found in endothelial and smooth muscle cells, but also in monocytes and macrophages. It is known to play an important part in angiogenesis, which occurs in physiological healing processes as well as tumor growth and transformation.[43]

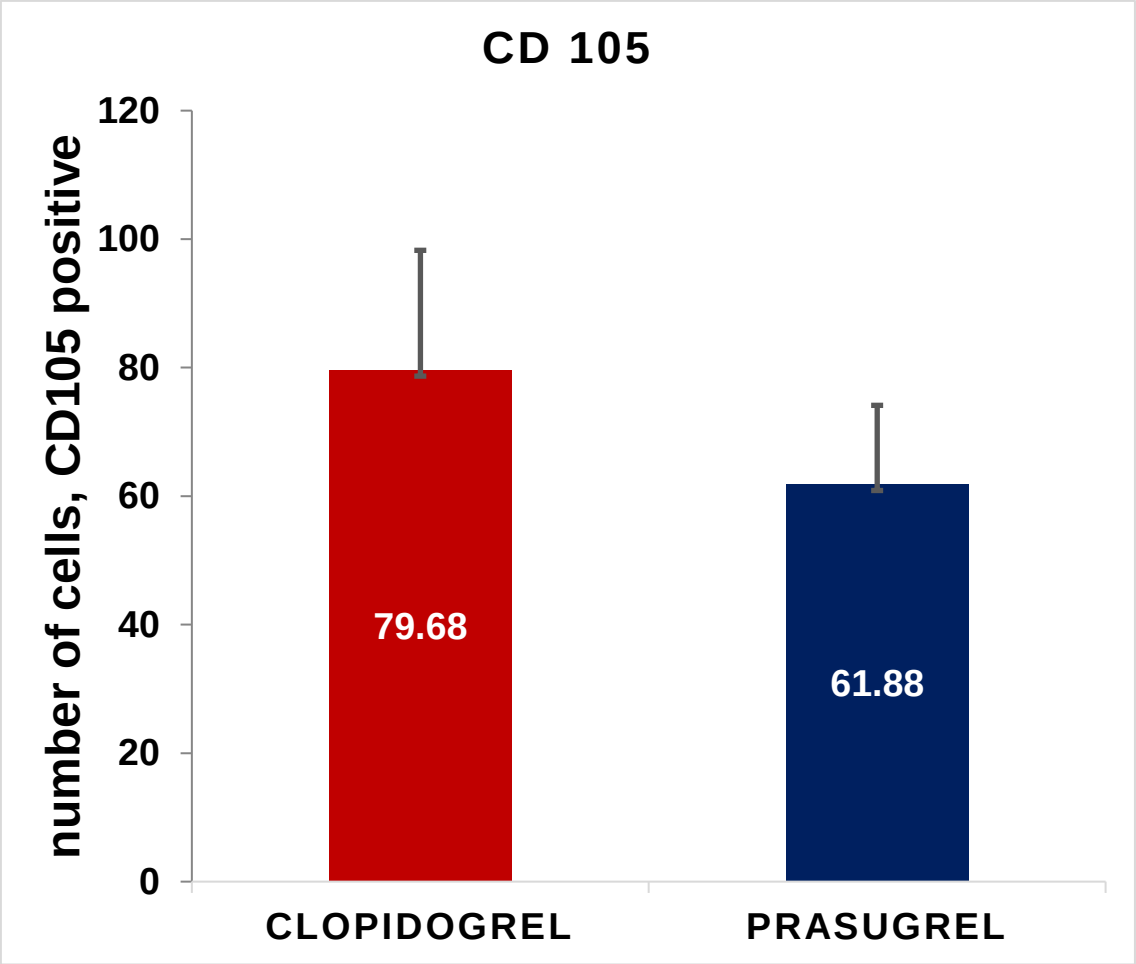


Diagram 9: Values are given as mean ± standard error, number of CD105 positive cells (Endothelial cells, Monocytes/Macrophages, smooth muscle cells) per high power field (10x)

Despite the overall low expression in all thrombi, the cell count of CD105 positive cells in the Clopidogrel group was slightly higher, although the difference was not significant.

H&E and CD105 stains, Clopidogrel and Prasugrel:

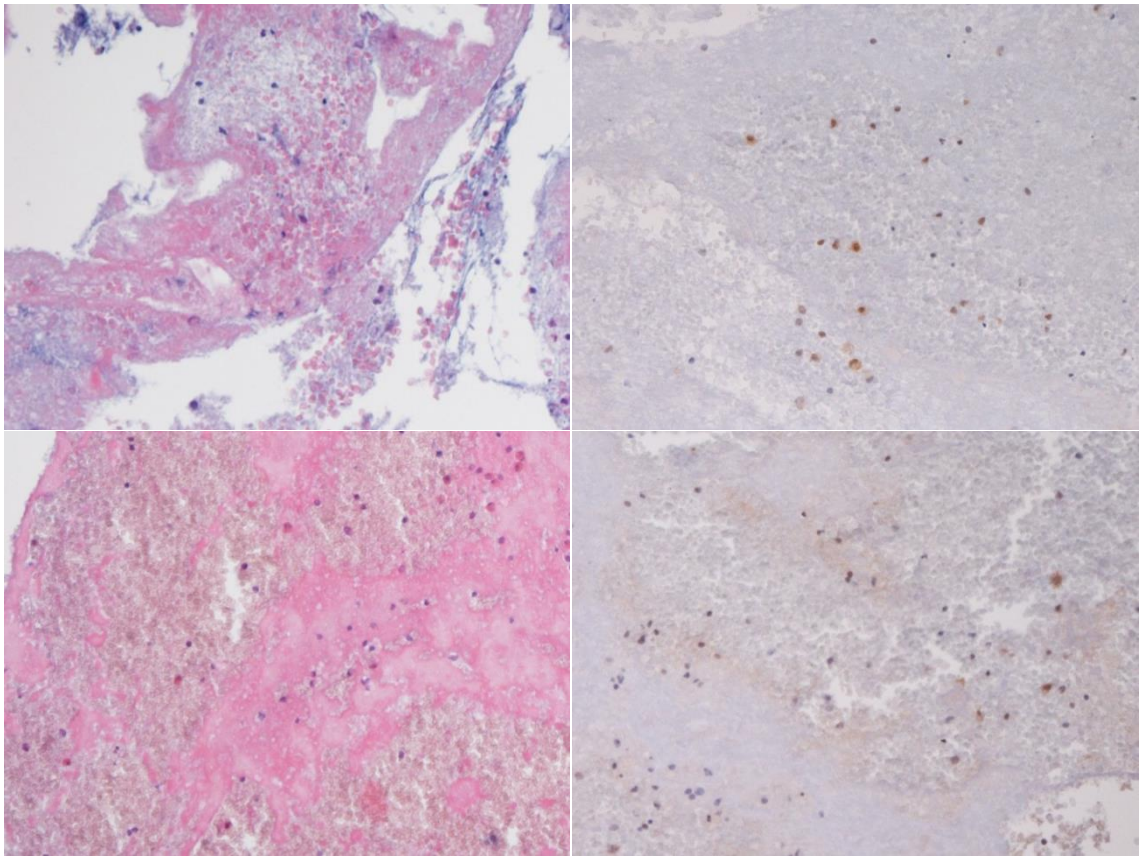


Fig.36-37: Clopidogrel Loading, HE and CD105 stain

Fig. 38-39: Prasugrel Loading, HE and CD105 stain

3.2.5. Immunohistochemistry TGFß

TGFß expression levels:

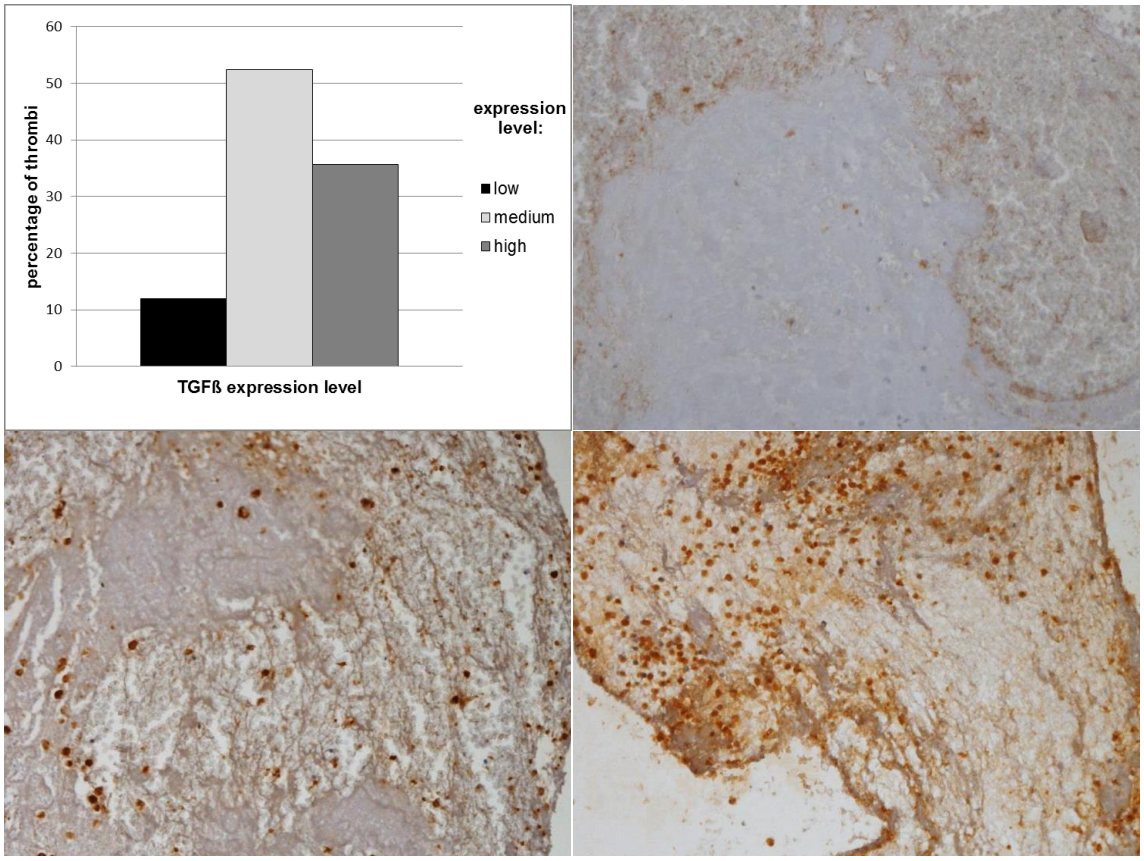


Diagram 10: TGFß expression level in percentage of stained thrombi.

Fig.40-42: Low, medium and strong expression level of TGFß.

As represented in the above diagram 10 TGFß was expressed to a medium or high extent, together accounting for almost 90%. Merely 12% of all stained sections had no or a low expression grade. Interestingly, although CD105 is part of the TGFß-receptor complex its expression levels were almost to the contrary to the TGFß-levels.

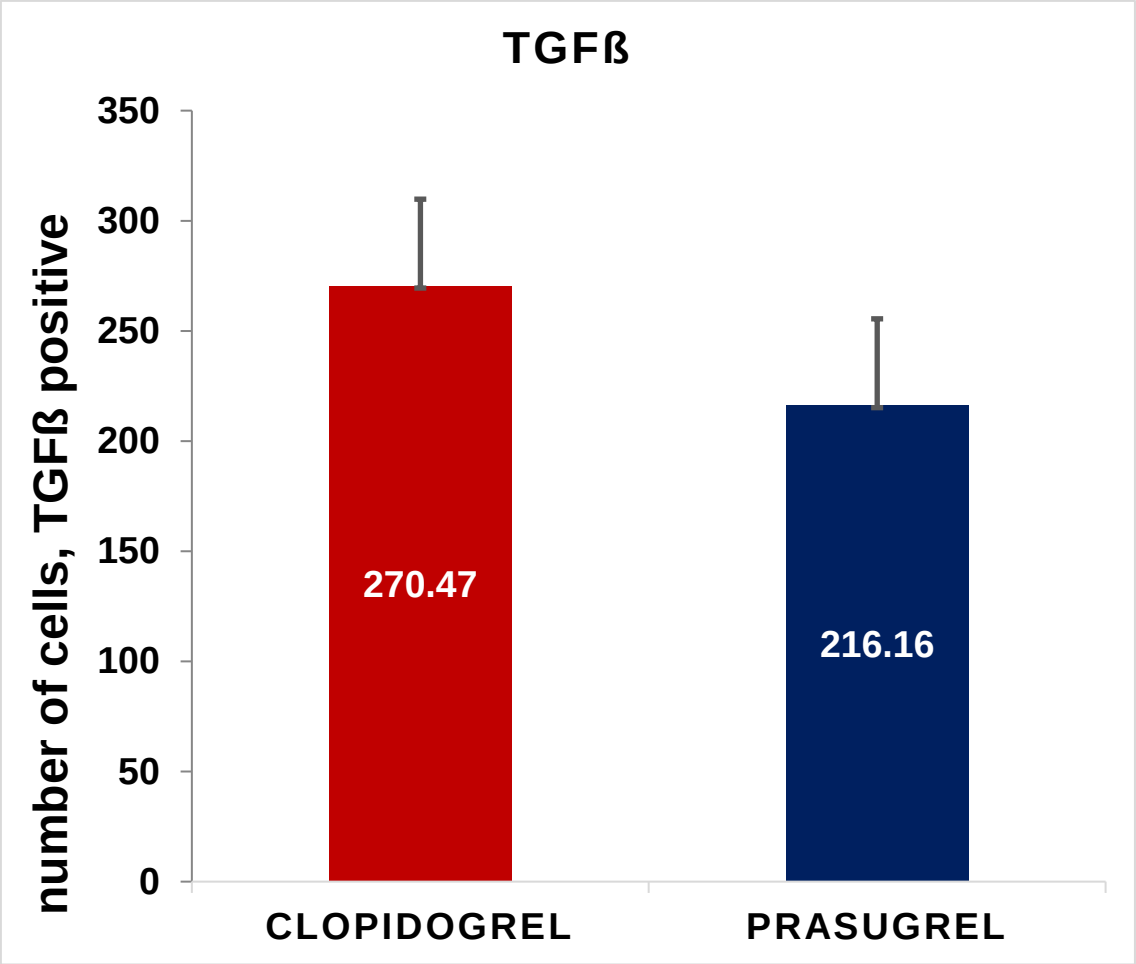


Diagram 11: Values are given as mean ± standard error, number of TGFß positive cells (platelets, monocytes/macrophages) per high power field (10x)

The TGFß-receptor complex has a wide range of functions in immune response such as chemotaxis, proliferation, differentiation, and activation of immune cells. Although the multiple functions of the complex are not yet fully understood, TGFß is known to be secreted by monocytes/macrophages and neutrophils and therefore to play a role in physiological wound healing processes. Furthermore, the TGFß-1 isoform is expressed in platelets. These facts make it a valid indicator for platelet prevalence and activity on one hand and for inflammatory and healing processes on the other.[43-46, 54] This is underlined by the findings shown above. In both groups there was a medium to high expression of TGFß in general, with no significant difference between the Clopidogrel and the

Prasugrel pretreated patients. A discretely higher prevalence was documented for the Clopidogrel group.

H&E and TGFß stains, Clopidogrel and Prasugrel:

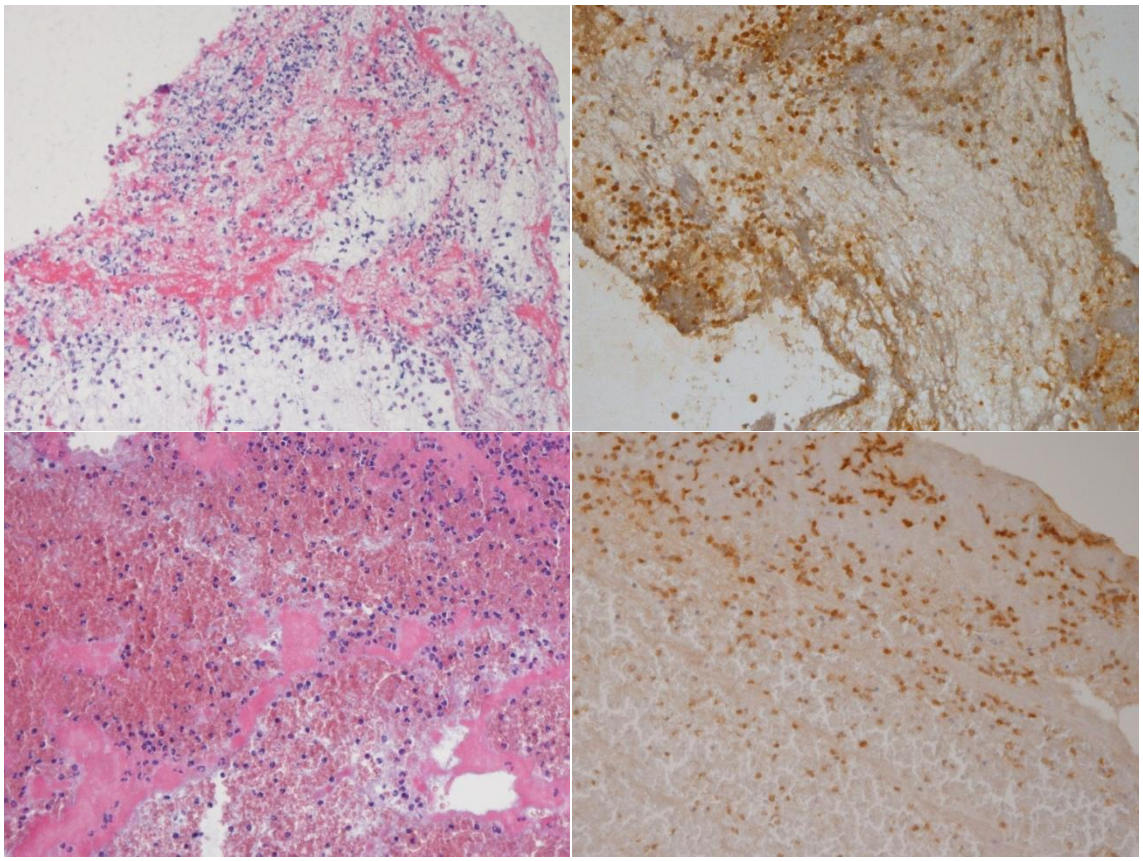


Fig. 43-44: Clopidogrel Loading, HE and TGFß stain

Fig. 45-46: Prasugrel Loading, HE and TGFß stain

3.2.6. Immunohistochemistry MIF

MIF expression levels:

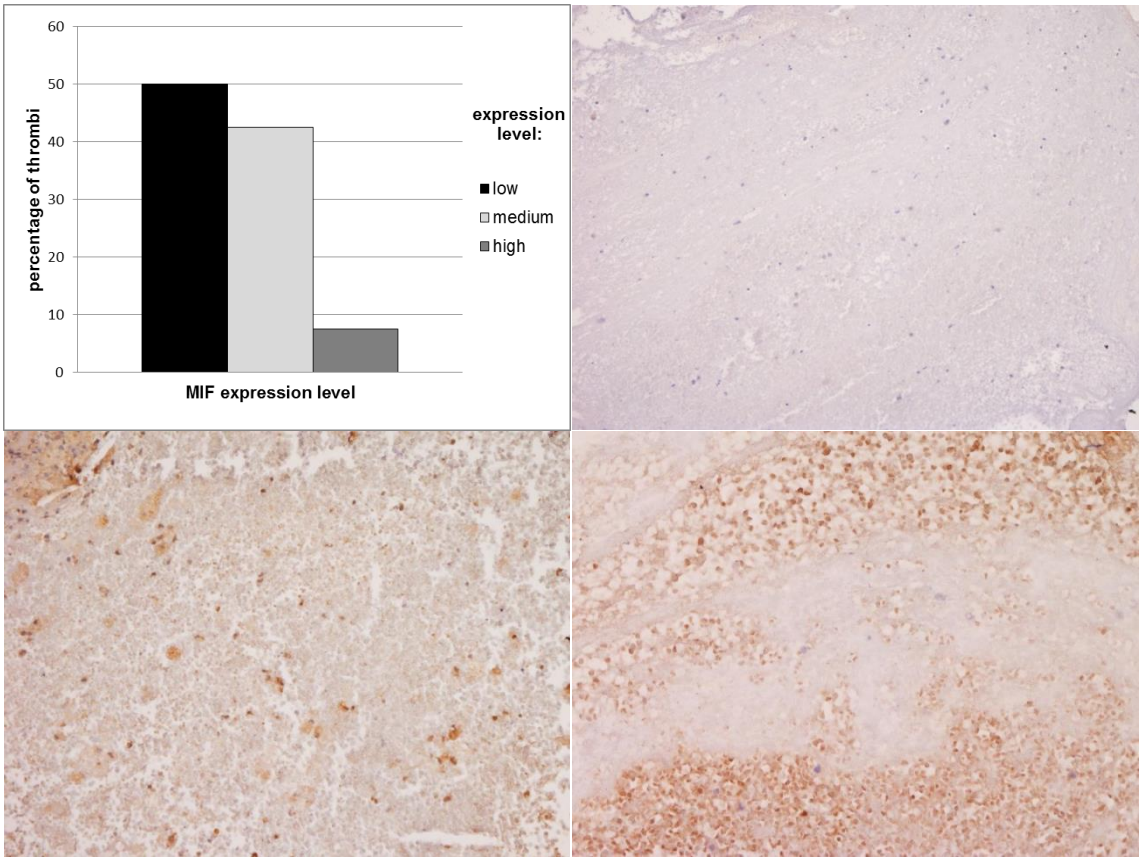


Diagram 12: MIF expression level in percentage of stained thrombi.
Fig.47-49: Low, medium and strong expression level of MIF.

According to the statistical analysis as shown in diagram 12, 50% of all MIF-stained thrombi had no to low, the other half medium to high expression levels of the marker, although it was only a minority of not only 10% that had a high expression of MIF.

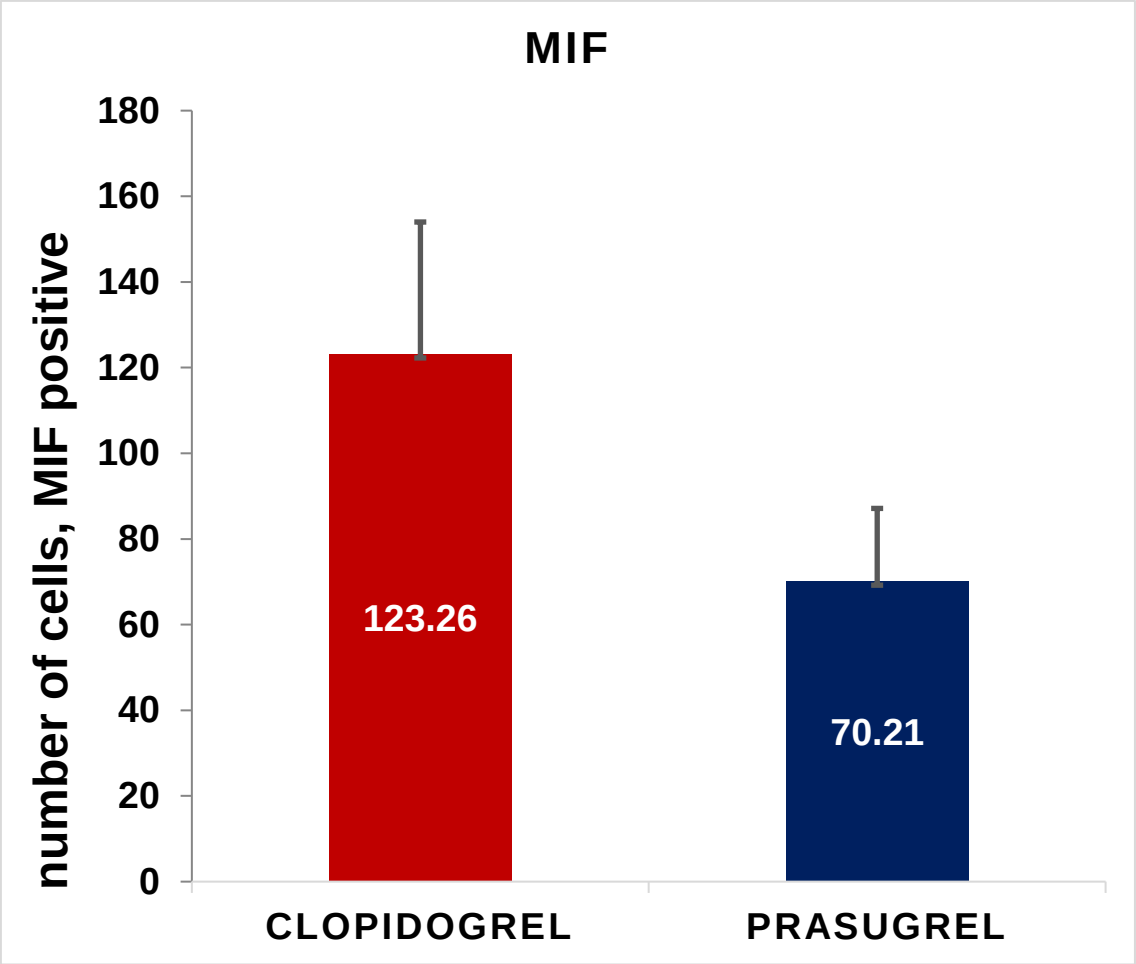


Diagram 13: Values are given as mean ± standard error, number of MIF positive cells (monocytes/macrophages) per high power field (10x)

MIF is a monocyte- and macrophage specific pro-inflammatory cytokine. It is responsible for monocyte adhesion and migration to the site of lesion and regulation of macrophage function. It is known for its involvement in atherosclerosis and plaque progression in CAD.[47] As indicated in diagram 13, there was a visible although not significant difference in the number of MIF positive cells between the two groups with a tendency of more positive cells in the Clopidgrel group.

H&E and MIF stains, Clopidogrel and Prasugrel:

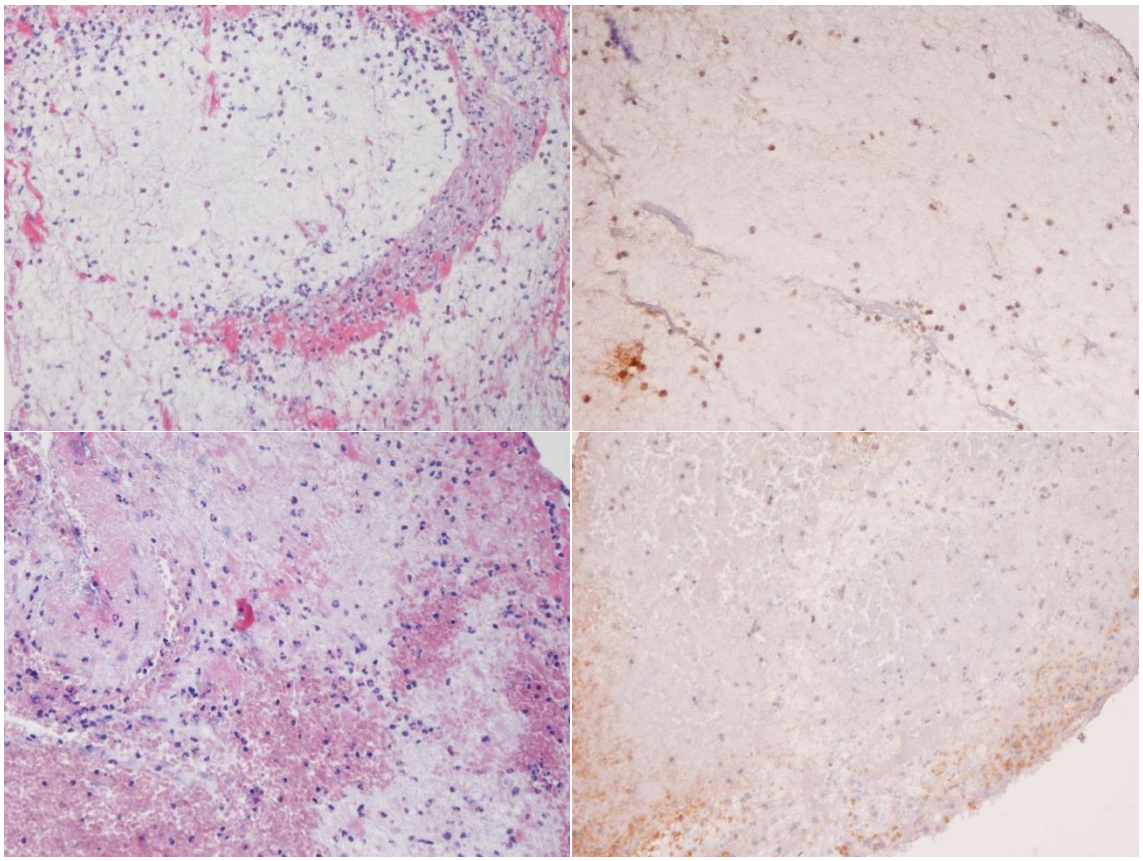


Fig.50-51: Clopidogrel Loading, HE and MIF stain

Fig.52-53: Prasugrel Loading, HE and MIF stain

3.2.7. Immunohistochemistry Grem1

Gremlin1 expression levels:

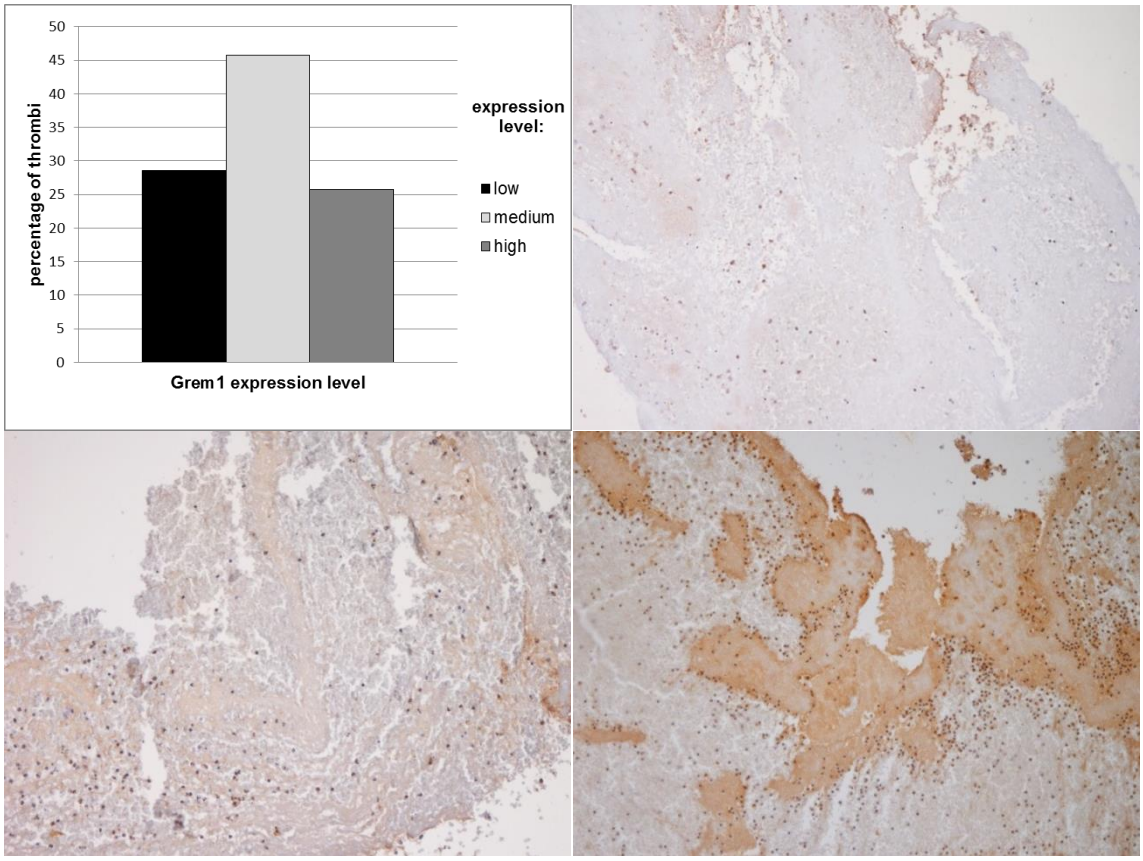


Diagram 14: Grem1 expression level in percentage of thrombi.

Fig.54-56: Low, medium and strong expression level of Grem1.

Medium and strong Grem1 expression accounted for over 2/3 of all stained sections, of which 46% showed a fair staining level. Low expression was present in 28% of all cases.

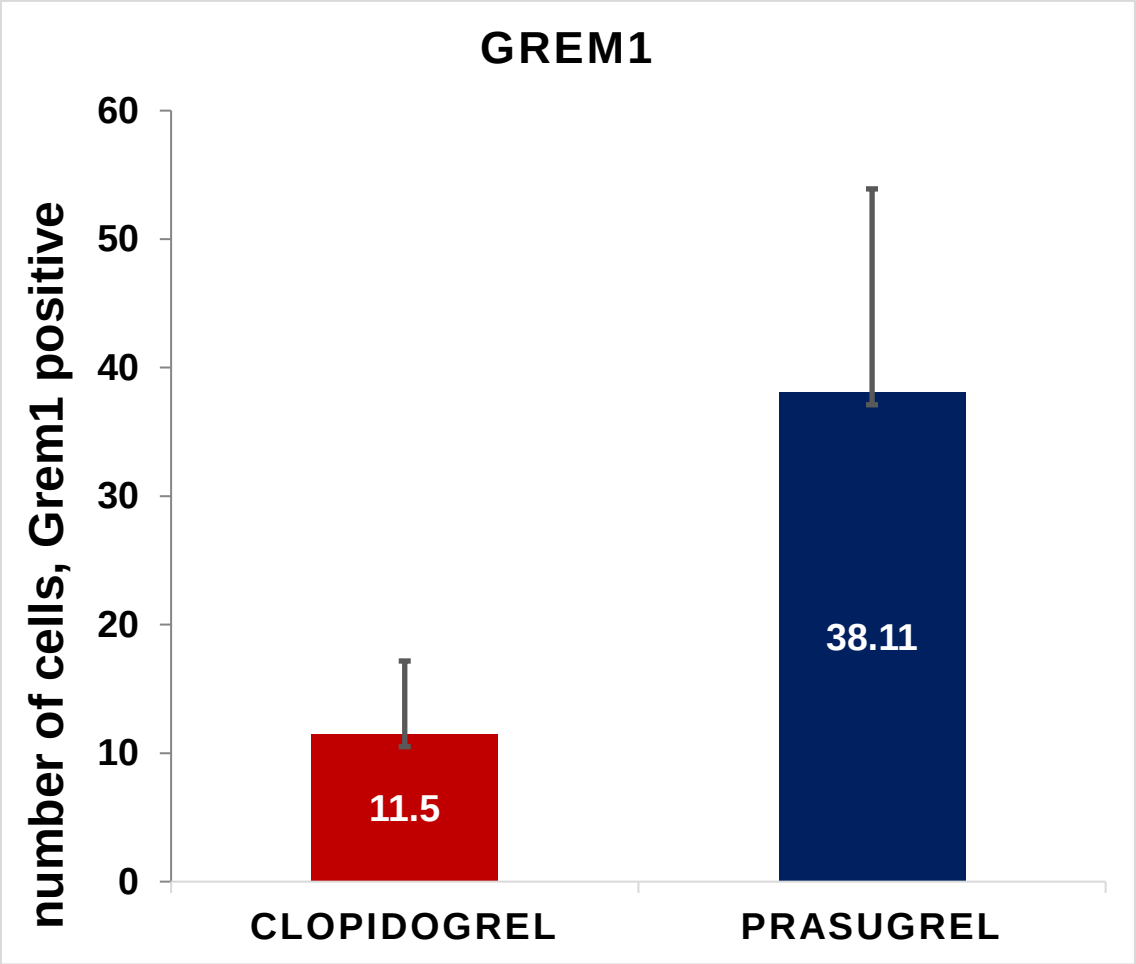


Diagram 15: Values are given as mean ± standard error, number of Grem1 positive cells (monocytes/macrophages) per high power field (10x)

Grem1 in recent years has been found to be an endogenous antagonist of the pro-inflammatory cytokine MIF. Previous studies have shown that the blood plasma Grem1/MIF ratio is significantly lower in patients with ACS in contrast to patients with stable CAD.[48, 49] The statistical analysis as documented in diagram 15 clearly shows a higher amount of Grem1 positive cells in the Prasugrel group. Although the differences between the two groups were not significant, it has to be noted that the expression of MIF was higher in the Clopidgrel group in comparison to the Prasugrel group. Interestingly for Grem1 the contrary is the case. This results in a lower Grem1/MIF ratio in the Clopidogrel group than in the Prasugrel group, suggesting that Clopidogrel

pretreated patients show a higher level of inflammatory processes in intracoronary thrombi.

H&E and Grem1 stains, Clopidogrel and Prasugrel:

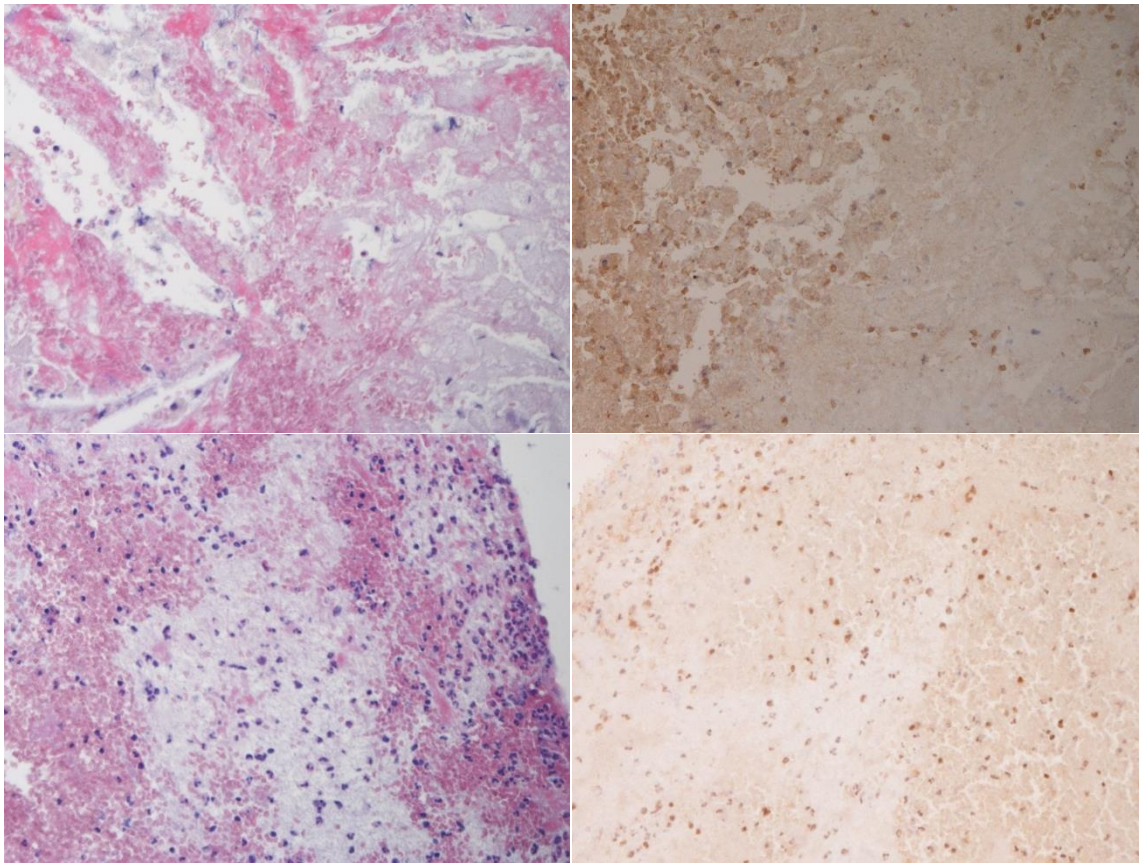


Fig. 57-58: Clopidogrel Loading, HE and Grem1 stain

Fig. 59-60: Prasugrel Loading, HE and Grem1 stain

3.3. Results- Conclusion

In summary, the most significant result of this research study is the difference in fibrin content of intracoronary thrombi when comparing the Clopidogrel with the Prasugrel pretreated group. Statistically the Clopidogrel group showed significantly more often a fibrin content of over 50% in comparison to the Prasugrel group.

Furthermore the CD14 expression was higher in the Clopidogrel group; the number of CD14 positive cells signifying a trend towards the Clopidogrel group.

The CD42b, CD68, CD105, MIF, Grem1 and TGFß stained cells did not show significant differences or trends between the Clopidogrel and the Prasugrel group although there were some interesting findings regarding the relation of CD105 and TGFß as well as MIF and Grem1 expression.

4. Discussion

This study focused on Clopidogrel and Prasugrel, two of the main antiplatelet regimens used worldwide in patients with acute ST-elevation myocardial infarction undergoing PCI and their influence on architecture and composition of intracoronary thrombi.

Although Prasugrel seems to have an effect on thrombus size and formation as suggested by Silvain, J et al. [34] the thrombi components and morphology had not yet been analyzed in regard to the different pretreatment strategies that are in common clinical practice today.

In this study we could demonstrate that intracoronary thrombi of patients that were loaded with ASA and Clopidogrel before PCI and thrombus aspiration showed significantly more often a fibrin content of over 50 percent as opposed to intracoronary thrombi of Prasugrel-loaded patients.

This is remarkable since fibrin, together with factor XIII, is the component at the end of the coagulation cascade that turns the initial platelet plug into a stable, insoluble thrombus. This suggests that Prasugrel's rapid onset of effect and more potent platelet inhibition [38] results in an overall reduced fibrin content in intracoronary thrombi when compared to Clopidogrel and thus makes it less resistant to ongoing antithrombotic treatment. The composition of thrombotic material of patients with acute ST-myocardial infarction has been subject to several research studies before.[34, 55] A significant difference in fibrin content had been observed, but had been correlated to time as the main impact factor on thrombus formation.[34] As documented in the baseline characteristics of our patient cohort, there was no statistically significant difference between the two groups regarding door-to-balloon time. Although the Clopidogrel- group's door-to-balloon time was overall longer, it also has to be taken into account that the onset of action for Clopidogrel ranges from 2 to 6 hours, whereas Prasugrel starts taking effect after 30 minutes.[37] Therefore, in order to be able to correlate the effect of the two antithrombotic agents to our findings and to compare the two groups with each other we selected patients that were treated and the thrombotic material obtained within 24 hours (acute myocardial infarction, majority of patient cohort) or between 24-72 hours (recent myocardial

infarction). Our study therefore differs from the previous one by Silvain et al. primarily in its objective to compare two $P2Y_{12}$-inhibitors regarding their impact on thrombus architecture, an extended time span and the methods used. In respect of our aim to investigate the differences between Clopidogrel and Prasugrel treated patients the time frame had to be adjusted due to the difference in pharmacodynamics and –kinetics. With this premise, we established a basis where time can only be factored in if related to the $P2Y_{12}$-inhibitor that was being analyzed, leading to valid, unbiased results.

In our study we could furthermore show that there is a statistical trend of CD14 positive cells towards the Clopidogrel group. CD14 is a monocyte/macrophage marker. Although it is to a lesser extent also expressed by neutrophils, it has lipopolysaccharide-receptor functions [51,56], a crucial feature for lipid-uptake and thus for the transformation of macrophages into foam cells. Since foam cells are known to play an important role in atherosclerosis, plaque formation and rupture, a higher prevalence of CD14 positive cells implies a higher incidence of these and similar pro-inflammatory processes in the Clopidogrel group. CD14 has so far mainly been investigated in atherosclerotic plaques of the carotids.[56, 57] In the study by Hermansson et al. it was not only shown that CD14 is expressed in thrombotic carotid lesions but also that the receptor was suppressed in macrophages by angiotensin receptor blockers. It has been furthermore suggested that angiotensin receptor blockers have anti-inflammatory effects on patients with atherosclerotic lesions like the ones studied by Hermansson et al.

According to the baseline characteristics of our study population only 17% of the entire patient cohort had an ARB in their concomitant medication, 18.5% in the Clopidogrel and 16.3% in the Prasugrel group. The similar percentage is favorable in order to be able to compare the two treatment arms. The trend towards the Clopidogrel group therefore signifies that CD14 expression and therefore inflammation, lipid-uptake and foam cell formation are higher in the Clopidgrel group. In regard to Hermansson’s study and the observed results of

our analysis of intracoronary thrombi a routine combination of Clopidogrel and ARBs might be considered.

CD42 as a platelet marker was selected to be used in immunohistochemical stainings since platelets and fibrin are the main constituents of arterial thrombi as had been shown before.[1, 34] Again, Silvain et al. could show that there is a proportional shift towards the amount of fibrin at the expense of the amount of platelets in correlation to time. In regard to the different antiplatelet regimens compared in our study and platelet prevalence there was no significant difference between the two groups. It is noteworthy that platelet prevalence is not affected by either Clopidogrel or Prasugrel. In fact a similar prevalence of platelets implies a valid comparability between the two pretreatment arms. In order to additionally investigate the effects of the two drugs on platelets' activity and function corresponding studies such as f.ex. blood plasma multiplate testing would be useful.

The monocyte/macrophage specific marker CD68 is known and has been commonly used as an indicator for inflammatory processes. In our study CD68 was expressed to similar amounts in both pretreatment arms. The statistical results therefore suggest that inflammation is equally high in both groups. Fukijkschot WW et al. have previously shown that CD68 is more strongly expressed in lytic coronary thrombi than in fresh or organized ones implying a correlation to thrombus age.[42] Based on this finding, it can be claimed that thrombus age did not differ significantly between the two groups in our study. Fukijkschot WW et al. also found a significant positive correlation between patient's age and the prevalence of CD68 and thus inflammation in intracoronary thrombi. Interestingly, in our patient cohort there was a statistically significant age difference between the two groups, with the Clopidogrel group's average patient age being higher. This is in accordance with the, although not significantly, higher maximum CRP plasma levels as well as the higher expression of CD68. Further investigation especially regarding differences in genetic expression, as has exemplary been conducted for soluble CD14 by Reiner et al. [58], and correlation to age between the two groups would elucidate these tendencies.

TGFß was highly expressed in both groups, as opposed to CD105 which forms part of the TGFß receptor complex. CD105 in its soluble form has been shown to be decreased in serum of patients with CAD.[59] Saita et al. found a positive correlation between the degree of CAD and a stepwise decrease of Endoglin serum levels with the severity of the disease. In line with these findings Li et al. had also shown that serum levels of CD105 and TGFß1 are decreased in patients with CAD, the amount of CD105/TGFß1 receptor complex though was increased as a probable reason for the decrease of the single complex constituents.[60] Although part of the same complex it has to be mentioned that CD105 is known for playing a part in neovascularization in tumors and atherosclerotic plaques [61] , whereas TGFß plays a significant role in platelet activation and wound healing. There are also preliminary studies suggesting a genetic component in TGFß expression.[54]

In our study we could show that the expression of TGFß is generally high in all patients with acute or recent myocardial infarction suggesting a high level of platelet activation whereas CD105 expression is rather low in all intracoronary thrombi with no significant difference between the Clopidogrel and the Prasugrel group.

MIF, being an inflammatory cytokine has been used in this study to compare and detect potential differences in inflammation present in intracoronary thrombi between the two antithrombotic treatment arms. The MIF prevalence was statistically higher, although not significantly in the Clopidogrel group, suggestive for a higher amount of inflammation and macrophage activity in this group in accordance with the results of CD68 expression. Grem1 is known to be an endogenous antagonist of MIF. Its expression levels were higher in the Prasugrel group. In a previous study where we compared MIF and Grem1 blood plasma levels we could show that the Grem1/MIF ratio was significantly lower in a cohort of ACS patients in contrast to patients with stable.[48] The current study shows that the Grem1/MIF ratio is although not significantly but nonetheless notably lower in the Clopidogrel group. This again suggests a higher level of inflammation in the Clopidogrel group.

In summary we could show in our study that the antithrombotic pretreatment has an impact of thrombus composition and architecture, although not all underlying factors are completely understood.

Further investigations in regard to time, age and genetics of a larger patient cohort and the potential influences of these factors especially on inflammatory processes represented in intracoronary thrombi would be useful to elucidate our findings and to find more individualized treatment strategies of patients with acute or recent myocardial infarction.

5. Summary

With the rapid progress of CAD artery disease becoming the leading cause of mortality and morbidity worldwide a thorough understanding and constant improvement of treatment strategies of the underlying causes has become an ongoing task for researchers and clinicians around the globe.

The standard treatment for patients undergoing PCI is currently a dual antiaggregatory therapy with ASA and another antiplatelet drug either of the thienopyridine group (Clopidogrel, Prasugrel, Ticlopidine) or with a non-thienopyridine derivative such as Ticagrelor or Cangrelor. In this research study we focused on the two main representatives of the thienopyridine group, Clopidogrel and Prasugrel.

It has been demonstrated before that Prasugrel shows more stable pharmacokinetics and –dynamics, an overall more potent platelet inhibition and a more rapid onset of effect.[38]

Based on the hypothesis that Prasugrel in comparison to Clopidogrel not only reduces intracoronary thrombus size but also affects its composition and thus its architecture due to its beneficial effects mentioned above, the aim of this research study was to analyze and compare those features of intracoronary thrombi of a cohort of STEMI – patients.

For the analysis, a total of 102 patients from 2 cardiological centers underwent PCI and thrombectomy. 51 of the entire cohort received Clopidogrel, the other half Prasugrel pretreatment either in the ambulance or on hospital admission with loading doses of ASA 500 mg + Clopidogrel 600 mg or ASA 500 mg + 60 mg Prasugrel according to standard clinical practice. The aspirated thrombi of those two groups were consecutively analyzed further by histological staining. In order to gain a differentiated overview of the thrombi composition the sections were stained using a variety of molecular biomarkers (CD14, CD42b, CD68, CD105, MIF, Grem1, TGFß) besides the standard H&E stain. The obtained results were then compared between the two groups and related to clinical findings and patients' characteristics.

The most significant result was the difference in fibrin content between the two groups. The intracoronary thrombi of patients preloaded with Clopidogrel showed significantly more often a fibrin content of over 50% of the sampled area in comparison to the Prasugrel group. Regarding the clinical parameters, risk factors and other patients' characteristics there were no significant differences between the two groups so that these parameters can be excluded as potential influencing factors. We could also show a trend towards CD14 prevalence in the Clopidogrel group. Although not significantly different, this speaks for an augmented inflammatory reaction with a higher involvement of monocytes and macrophages in the Clopidogrel group. The other biomarkers did not differ significantly in terms of expression between the two treatment arms.

We could therefore show that the type of antithrombotic pretreatment with Prasugrel or Clopidogrel has a direct impact on thrombus composition and thus architecture and that there is a causal relationship between Prasugrel pretreatment and reduced fibrin content in intracoronary thrombi of patients with STEMI.

Further investigation of these effects in a larger patient cohort also in consideration of the individual's predisposition, such as protein and gene expression and additional research regarding different antiplatelet regimens might help us to further understand the underlying pathophysiological processes and thus, develop new, possibly more individualized, treatment strategies for patients with ACS.

6. References

1. Mann DL, Zipes DP, Libby P, Bonow RO et al., *Braunwald's Heart Disease: A Textbook of Cardiovascular Medicine*. 10th ed. 2015, Philadelphia: Elsevier Saunders.
2. WorldHealthOrganization *Cardiovascular diseases (CVDs)*. 2017. **2018**.
3. Herold G, *Innere Medizin: Eine vorlesungsorientierte Darstellung; unter Berücksichtigung des Gegenstandskataloges für die Ärztliche Prüfung; mit ICD 10-Schlüssel im Text und Stichwortverzeichnis*. 2017: Herold.
4. Erling F, Prediman SK, Fuster V, *Coronary Plaque Disruption*, in *Circulation*. 1995. p. 92:657–671.
5. Martin EA, *Concise Medical Dictionary*. 1987: Oxford University Press.
6. Wikipedia. *Ischemia*. 2018 [cited 2018]; Available from: https://en.wikipedia.org/wiki/Ischemia.
7. Lloyd-Jones DM, Wilson PWF, Larson MG, Beiser A, Leip EP, D'Agostino RB, Levy D, *Framingham risk score and prediction of lifetime risk for coronary heart disease.* American Journal of Cardiology, 2004. **94**(1): p. 20-24.
8. Wikipedia. *Angina*. 2018; Available from: https://en.wikipedia.org/wiki/Angina.
9. Warren J, *Remarks on Angina Pectoris.* The New England Journal of Medicine, Surgery and Collateral Branches of Science, 1812. **1**(1): p. 1-11.
10. MayoClinic. *Myocardial Ischemia*. [cited 2018]; Available from: https://www.mayoclinic.org/diseases-conditions/myocardial-ischemia/symptoms-causes/syc-20375417.
11. Bentzon FJ, Fumiyuki O, Renu V, Erling F, *Mechanisms of Plaque Formation and Rupture.* Circulation Research, 2014. **114**(number 12): p. 114:1852–1866.
12. The Editors Of Encyclopaedia Britannica. *Atherosclerosis*. 2018; Available from: https://www.britannica.com/science/atherosclerosis#.
13. Riede UN, Schäfer HE et al., *Allgemeine und spezielle Pathologie*. Vol. 4. 1995: Thieme.
14. Urban & Fischer, *Roche Lexikon Medizin*. Vol. 5. 2003: Urban & Fischer.
15. Machlus KR, Thon JN, Italiano JE Jr., *Interpreting the developmental dance of the megakaryocyte: a review of the cellular and molecular processes mediating platelet formation.* Br J Haematol, 2014. **165**(2): p. 227-36.
16. Laki K, *Our ancient heritage in blood clotting and some of its consequences.* Ann N Y Acad Sci, 1972. **202**: p. 297-307.
17. Yip J, Shen Y, Berndt MC, Andrews RK, *Primary platelet adhesion receptors.* IUBMB Life, 2005. **57**(2): p. 103-8.
18. Dubois C, Panicot-Dubois L, Merrill-Skoloff G, Furie B, Furie BC, *Glycoprotein VI-dependent and -independent pathways of thrombus formation in vivo.* Blood, 2006. **107**(10): p. 3902-6.
19. Calvete JJ, *On the structure and function of platelet integrin alpha IIb beta 3, the fibrinogen receptor.* Proc Soc Exp Biol Med, 1995. **208**(4): p. 346-60.
20. Li Z, Delaney KM, O'Brien KA, Du X, *Signaling during platelet adhesion and activation.* Arteriosclerosis, thrombosis, and vascular biology, 2010. **30**(12): p. 2341-2349.
21. Sadler JE, *Biochemistry and genetics of von Willebrand factor.* Annu Rev Biochem, 1998. **67**: p. 395-424.
22. Offermanns S, *Activation of Platelet Function Through G Protein–Coupled Receptors.* Circulation Research, 2006. **99**(12): p. 1293-1304.
23. Halkar M, Lincoff AM, *Dual antiplatelet therapy for acute coronary syndromes: How long to continue?* Cleve Clin J Med, 2016. **83**(9): p. 675-88.
24. Joo S-J, *Mechanisms of Platelet Activation and Integrin αIIβ3.* Korean circulation journal, 2012. **42**(5): p. 295-301.

25. Damman P, Woudstra P, Kuijt WJ, de Winter RJ, James SK, *P2Y12 platelet inhibition in clinical practice.* Journal of thrombosis and thrombolysis, 2012. **33**(2): p. 143-153.
26. Dorsam RT, Kunapuli SP, *Central role of the P2Y12 receptor in platelet activation.* The Journal of clinical investigation, 2004. **113**(3): p. 340-345.
27. Nicholas RA, *Insights into platelet P2Y₁₂ receptor activation.* Blood, 2015. **125**(6): p. 893.
28. *Department of Pathology College of Veteinary Medicine the University of Georgia Athens, GA 30602-7388*, 2009. Available from: https://vet.uga.edu/ivcvm/courses/VPAT3100/01_circulation/hemostasis/hemostasis03.html
29. Lishko VK, Faust JJ, Ugarova TP, *The Dual Role Of Platelets In Thrombus Formation.* Blood, 2013. **122**(21): p. 1098.
30. Swieringa F, Baaten CCFMJ, Verdoold R, Mastenbroek TG, Rijnveld N, van der Laan KO et al., *Platelet Control of Fibrin Distribution and Microelasticity in Thrombus Formation Under Flow.* Arteriosclerosis, Thrombosis, and Vascular Biology, 2016. **36**(4): p. 692-699.
31. Xu XR, Zhang D, Oswald BE, Carrim N, Wang X, Hou Y et al., *Platelets are versatile cells: New discoveries in hemostasis, thrombosis, immune responses, tumor metastasis and beyond.* Critical Reviews in Clinical Laboratory Sciences, 2016. **53**(6): p. 409-430.
32. Hawiger J, *Formation and regulation of platelet and fibrin hemostatic plug.* Human Pathology, 1987. **18**(2): p. 111-122.
33. de Gaetano G, *Historical overview of the role of platelets in hemostasis and thrombosis.* Haematologica, 2001. **86**(4): p. 349-56.
34. Silvain J, Collet JP, Nagaswami C, Beygui F, Edmondson KE, Bellemain-Appaix A et al., *Composition of coronary thrombus in acute myocardial infarction.* J Am Coll Cardiol, 2011. **57**(12): p. 1359-67.
35. Furie B, Furie BC, *Mechanisms of Thrombus Formation.* New England Journal of Medicine, 2008. **359**(9): p. 938-949.
36. Sugidachi A, Asai F, Ogawa T, Inoue T, Koike H, *The in vivo pharmacological profile of CS-747, a novel antiplatelet agent with platelet ADP receptor antagonist properties.* Br J Pharmacol, 2000. **129**(7): p. 1439-46.
37. Sarıtaş A, Güneş H, *Comparison of ESC and ACCF/AHA Guidelines for Oral Antiplatelet Treatment in the Management of Patients with Acute Coronary Syndrome.* Eurasian J Emerg Med, 2016(15): p. 150-152.
38. Brandt JT, Payne CD, Wiviott SD, Weerakkody G, Farid NA, Small DS et al., *A comparison of prasugrel and clopidogrel loading doses on platelet function: magnitude of platelet inhibition is related to active metabolite formation.* Am Heart J, 2007. **153**(1): p. 66.e9-16.
39. Geisler T, Gawaz M, Steinhubl SR, Bhatt DL, Storey RF, Flather M, *Current strategies in antiplatelet therapy--does identification of risk and adjustment of therapy contribute to more effective, personalized medicine in cardiovascular disease?* Pharmacol Ther, 2010. **127**(2): p. 95-107.
40. Ranoa DRE, Kelley SL, Tapping RI, *Human lipopolysaccharide-binding protein (LBP) and CD14 independently deliver triacylated lipoproteins to Toll-like receptor 1 (TLR1) and TLR2 and enhance formation of the ternary signaling complex.* The Journal of biological chemistry, 2013. **288**(14): p. 9729-9741.
41. Feghhi S, Munday AD, Tooley WW, Rajsekar S, Fura AM, Kulman JD et al., *Glycoprotein Ib-IX-V Complex Transmits Cytoskeletal Forces That Enhance Platelet Adhesion.* Biophysical journal, 2016. **111**(3): p. 601-608.
42. Fuijkschot WW, Groothuizen WE, Appelman Y, Radonic T, van Royen N, van Leeuwen MA et al., *Inflammatory cell content of coronary thrombi is dependent*

on thrombus age in patients with ST-elevation myocardial infarction. J Cardiol, 2017. **69**(1): p. 394-400.
43. Nassiri F, Cusimano MD, Scheithauer BW, Rotondo F, Fazio A, Yousef GM et al., *Endoglin (CD105): a review of its role in angiogenesis and tumor diagnosis, progression and therapy.* Anticancer Res, 2011. **31**(6): p. 2283-90.
44. Valluru M, Staton CA, Reed MWR, Brown NJ, *Transforming Growth Factor-β and Endoglin Signaling Orchestrate Wound Healing.* Frontiers in physiology, 2011. **2**: p. 89-89.
45. Massague J, *TGFbeta signalling in context.* Nat Rev Mol Cell Biol, 2012. **13**(10): p. 616-30.
46. Assoian RK, Komoriya A, Meyers CA, Miller DM, Sporn MB et al., *Transforming growth factor-beta in human platelets. Identification of a major storage site, purification, and characterization.* J Biol Chem, 1983. **258**(11): p. 7155-60.
47. Müller II, Müller KAL, Schönleber H, Karathanos A, Schneider M, Jorbenadze R et al., *Macrophage migration inhibitory factor is enhanced in acute coronary syndromes and is associated with the inflammatory response.* PloS one, 2012. **7**(6): p. e38376-e38376.
48. *Abstracts of the 79th Annual Meeting of the German Cardiac Society. April 3-6, 2013. Mannheim, Germany.* Clin Res Cardiol, 2013. **102 Suppl 1**: p. 1.
49. Müller II, Müller KAL, Schönleber H, Karathanos A, Rath D, Vogel S et al., *Impact of counterbalance between macrophage migration inhibitory factor and its inhibitor Gremlin-1 in patients with coronary artery disease.* Atherosclerosis, 2014. **237**(2): p. 426-32.
50. Rath D, Gawaz M, *Antiplatelet Treatment for Catheter-Based Interventions in High-Risk Patients: Current Guidelines and Expert Opinion.* Hamostaseologie, 2018.
51. An D, Hao F, Zhang F, Kong W, Chun J, Xu X et al., *CD14 is a key mediator of both lysophosphatidic acid and lipopolysaccharide induction of foam cell formation.* J Biol Chem, 2017. **292**(35): p. 14391-14400.
52. Chistiakov DA, Melnichenko AA, Myasoedova VA, Grechko AV, Orekhov AN et al., *Mechanisms of foam cell formation in atherosclerosis.* J Mol Med (Berl), 2017. **95**(11): p. 1153-1165.
53. Yu XH, Fu YC, Zhang DW, Yin K, Tang CK, *Foam cells in atherosclerosis.* Clin Chim Acta, 2013. **424**: p. 245-52.
54. Kim HJ, Kim PK, Bae SM, Son HN, Thoudam DS, Kim JE et al., *Transforming growth factor-beta-induced protein (TGFBIp/beta ig-h3) activates platelets and promotes thrombogenesis.* Blood, 2009. **114**(25): p. 5206-15.
55. Li X, Kramer MC, Van Der Loos CM, Ploegmakers HJ, DE Boer OJ, Koch KT et al., *Early onset of endothelial cell proliferation in coronary thrombi of patients with an acute myocardial infarction: implications for plaque healing.* J Thromb Haemost, 2012. **10**(3): p. 466-73.
56. Hermansson C, Lundqvist A, Magnusson LU, Ullström C, Bergström G, Hultén LM, *Macrophage CD14 expression in human carotid plaques is associated with complicated lesions, correlates with thrombosis, and is reduced by angiotensin receptor blocker treatment.* International Immunopharmacology, 2014. **22**(2): p. 318-323.
57. Derksen WJ, Peeters W, van Lammeren GW, Tersteeg C, de Vries JP, de Kleijn DP et al., *Different stages of intraplaque hemorrhage are associated with different plaque phenotypes: a large histopathological study in 794 carotid and 276 femoral endarterectomy specimens.* Atherosclerosis, 2011. **218**(2): p. 369-77.

58. Reiner AP, Lange EM, Jenny NS, Chaves PHM, Ellis J, Li J et al., *Soluble CD14.* Arteriosclerosis, Thrombosis, and Vascular Biology, 2013. **33**(1): p. 158-164.
59. Saita E, Miura K, Suzuki-Sugihara N, Miyata K, Ikemura N, Ohmori R et al., *Plasma Soluble Endoglin Levels Are Inversely Associated With the Severity of Coronary Atherosclerosis-Brief Report.* Arterioscler Thromb Vasc Biol, 2017. **37**(1): p. 49-52.
60. Li CG, Bethell H, Wilson PB, Bhatnagar D, Walker MG, Kumar S, *The significance of CD105, TGFbeta and CD105/TGFbeta complexes in coronary artery disease.* Atherosclerosis, 2000. **152**(1): p. 249-56.
61. Luque A, Slevin M, Turu MM, Juan-Babot O, Badimon L, Krupinski J, *CD105 positive neovessels are prevalent in early stage carotid lesions, and correlate with the grade in more advanced carotid and coronary plaques.* Journal of Angiogenesis Research, 2009. **1**(1): p. 6.

7. Deutsche Zusammenfassung

Mit dem raschen Aufsteigen der Koronaren Herzerkrankung zur Hauptursache für Mortalität und Morbidität weltweit ist ein tiefgreifendes Verstehen und kontinuierliche Verbesserung der Therapiestrategien der zugrundliegenden Ursachen zur Voraussetzung und fortwährenden Aufgabe für Forscher und Kliniker auf der ganzen Welt geworden.

Die aktuelle Standardbehandlung für Patienten vor oder nach PCI ist eine duale antiaggregatorische Therapie mit ASS und einem Thrombozytenaggregationshemmer entweder aus der Thienopyridin-Gruppe (Clopidogrel, Prasugrel, Ticlopidin) oder mit einem nicht-Thienopyridin Derivat wie Ticagrelor oder Cangrelor. In dieser Studie wurde der Fokus auf die zwei Hauptrepräsentanten der Thienopyridin-Gruppe, Clopidogrel und Prasugrel gelegt.

Es ist bereits zuvor demonstriert worden, dass Prasugrel sowohl über eine stabilere Pharmakokinetik und –dynamik verfügt, als auch eine potentere Plättchenhemmung und einen rascheren Effektbeginn aufweist.[38]

Basierend auf der Hypothese dass Prasugrel im Vergleich zu Clopidogrel nicht nur die Größe intrakoronarer Thromben reduziert, sondern aufgrund der oben beschriebenen vorteilhaften Effekte auch ihre Komposition und somit die Thrombusarchitektur beeinflusst, war das Ziel dieser Studie diese Charakteristiken von intrakoronaren Thromben in einer Kohorte von STEMI-Patienten zu analysieren und zu vergleichen.

Für die Studie wurden insgesamt 102 Patienten aus 2 kardiologischen Zentren, die sich einer PCI und Thrombektomie unterzogen, rekrutiert. 51 Patienten dieser Kohorte erhielten ein Clopidogrel-, die andere Hälfte ein Prasugrel-Loading entweder im Rettungswagen oder bei Krankenhausaufnahme. Die Loading-Dosis war ASS 500 mg + Clopidogrel 600 mg oder ASS 500 mg + Prasugrel 60 mg entsprechend Standardklinikroutine. Die aspirierten Thromben dieser zwei Gruppen wurden im Anschluss mittels Immunhistologie weiter untersucht.

Um einen differenzierten Überblick über die Thrombenkomposition zu erhalten, wurden die histologischen Schnitte neben der Standard HE-Färbung mit einer Auswahl an molekularen Biomarkern (CD14, CD42b, CD68, CD105, MIF, Grem1, TGFß) gefärbt. Die Ergebnisse wurden dann zwischen den zwei Gruppen verglichen und in Relation zu den klinischen Befunden und Patienten-Charakteristika gesetzt.

Der größte signifikante Unterschied zwischen den zwei Gruppen zeigte sich hinsichtlich des Fibringehalts. Die intrakoronaren Thromben der Patienten, die mit Clopidogrel behandelt worden waren zeigten häufiger einen Fibrinanteil von über 50% der ausgewerteten Region im Vergleich zur Prasugrel Gruppe. Angesichts der klinischen Parameter, Risikofaktoren und weiteren Patientenmerkmale zeigten sich keine wesentlichen Unterschiede zwischen den zwei Gruppen, sodass diese Parameter als potenzielle Einflussfaktoren ausgeschlossen werden können.

Wir konnten auch einen Trend zu einer erhöhten CD14 Prävalenz in der Clopidogrel Gruppe aufweisen. Wenn auch nicht signifikant erhöht, so spricht dieser Trend doch für eine gesteigerte inflammatorische Reaktion mit erhöhter Beteiligung von Monozyten und Makrophagen in der Clopidogrel-Gruppe. Die übrigen Biomarker zeigten keine signifikanten Unterschiede zwischen den zwei Behandlungsarmen in Bezug auf ihre Expression.

Somit konnten wir zeigen, dass die Art der antithrombozytären Behandlung mit Prasugrel oder Clopidogrel einen direkten Einfluss auf die Thrombuskomposition und damit –architektur hat und dass ein Kausalzusammenhang zwischen Prasugrel-Loading und geringerem Fibringehalt in intrakoronaren Thromben von STEMI-Patienten besteht.

Weitere Untersuchungen dieser Effekte in einer größeren Patientenkohorte, auch unter Berücksichtigung der individuellen Prädisposition, wie beispielsweise Protein- und Genexpression und zusätzliche Studien in Hinsicht auf andere antithrombozytäre Therapien könnten uns helfen die zugrunde liegenden pathophysiologischen Prozesse besser zu verstehen und so neue,

möglicherweise individualisiertere Behandlungsstrategien für Patienten mit ACS zu entwickeln.

9. Contributions

Study concept and design:

Tobias Geisler, Karin Müller, Iris Müller

Acquisition of data:

Claire Chakkalakal, staff of the catheter labs at University Hospital Tübingen and Klinik am Eichert, Göppingen, Ingrid Epple, Laboratory staff University Hospital Tübingen

Analysis and interpretation of data:

Claire Chakkalakal, Karin Müller, Iris Müller, Tobias Geisler

Drafting of manuscript:

Claire Chakkalakal

Background research:

Claire Chakkalakal, Karin Müller

Critical revision:

Iris Müller, Karin Müller, Tobias Geisler

10. Acknowledgements

First, I would like to thank Prof. Dr. med. Geisler for providing me with this research topic and Prof. Dr. med. Gawaz, Head of department, for their patience and continuous confidence in me.

Many special thanks go to my supervisors PD Dr. med. Iris Müller and PD Dr. med. Karin Müller for their great commitment, ongoing support and encouragement throughout the entire project.

I further thank the laboratory staff for their help, availability and advice. My thanks go in particular to Ingrid Epple, who assisted me in all kinds of technical questions.

I would like to conclude my acknowledgements by thanking my family. My parents for everything they have done for me, for financial and moral support and in particular for instilling in me the faith that God is always with me and everything is possible with Him. I also would like to thank my brother for his spiritual and practical support, encouragement and belief in me.

Finally, thank you, Jason Rogers, love of my life and my best friend for supporting me in every possible way, for your endless patience, understanding and sacrifice, your unwavering faith in me and the blessing you have brought to my life.