Technische Universität München
TUM School of Medicine and Health

Influence of reticulated platelets on outcomes in patients with acute myocardial infarction treated with prasugrel or ticagrelor

Nina Isabella Schreiner

Vollständiger Abdruck der von der TUM School of Medicine and Health der Technischen Universität München zur Erlangung einer Doktorin der Medizin (Dr. med.) genehmigten Dissertation.

Vorsitz: apl. Prof. Dr. Ute Reuning

Prüfende der Dissertation:

1. Priv.-Doz. Dr. Maria Isabell Bernlochner
2. apl. Prof. Dr. Jürgen Pache

Die Dissertation wurde am 21.12.2023 bei der Technischen Universität München eingereicht und durch die TUM School of Medicine and Health am 05.06.2024 angenommen.

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I. Summary

It´s mature – immature platelets could become a biomarker in cardiovascular disease

Results of the ISAR REACT 5 reticulated platelets substudy

Background

Reticulated or immature platelets are young and pro-thrombotic RNA-rich thrombocytes which play an important role in numerous pathological conditions. Especially in patients with acute coronary syndrome (ACS) reticulated platelets have been accounted for adverse events. Nevertheless, their significance in ACS patients treated with the potent $P2Y_{12}$ receptor inhibitors ticagrelor and prasugrel has not been investigated yet. For this reason the ISAR REACT 5 substudy was called into action with its primary aim to prospectively evaluate reticulated platelets as a predictor of the primary ischemic endpoint consisting of death, myocardial infarction or stroke at one year in patients with ACS randomized to prasugrel or ticagrelor.

Methods and Results

The immature platelet fraction (IPF) was assessed in the first 48h after randomization using a fully automated system in a total of 577 patients. We stratified the study patients according to the median IPF value: IPF^{high} (IPF>median) and IPF^{low} (IPF≤median). IPF values in % (median [IQR]) within the first 48h did not differ between the two study groups: 3.6 [2.5,5.2] % in the prasugrel group and 3.6 [2.5,5.4] % in the

ticagrelor group (p=0.882). The incidence of the primary endpoint (MACE) was significantly higher in the IPF^{high} (IPF>3.6%) group compared to the IPF^{low} (IPF≤3.6%) group: 13.0% vs 7.1% (HR_{adj} 1.74 [1.02,3.00], p=0.044), independently from the assigned drug (p_{int} = 0.159). No significant association between IPF and BARC 3-5 bleeding was observed. ADP-induced platelet aggregation correlated weakly but highly significantly with IPF in patients treated with prasugrel (r=0.22, p=0.005) while no significant correlation was detected in patients treated with ticagrelor (r=0.09, p=0.257).

Conclusion

Independently from drug treatment, the biomarker IPF was associated with the primary endpoint and therefore offers the potential as a promising tool for the prediction of adverse cardiovascular events in ACS patients treated with prasugrel or ticagrelor.

II. List of abbreviations

ACS	Acute coronary syndrome
ADP	Adenosine diphosphate
AHA	American Heart Association
AMI	Acute myocardial infarction
BARC	Bleeding Academic Research Consortium
bIPF	baseline immature platelet fraction
BMI	Body mass index
CABG	Coronary artery bypass grafting
CAD	Coronary artery disease
COX	Cyclooxygenase
CYP2C19	Cytochrome P450 2C19, metabolic enzyme
DAPT	Dual antiplatelet therapy
DNA	Desoxyribonucleic acid
ECG	Electrocardiogram
ESC	European Society of Cardiology
GFR	Glomerular filtration rate
G_i-protein	Inhibitory G protein
GP IIb/IIIa	Glycoprotein IIb/IIIa
GRACE score	Global Registry of Acute Coronary Events
H-IPF	Highly-fluorescent Immature Platelet Fraction
HR	Hazard ratio
IPC	Immature platelet count
IPF	Immature platelet fraction
IPF%	Immature platelet fraction

IQR	Interquartile range
LD	Loading dose
LDL	Low-density lipoproteins
MACE	Major adverse cardiovascular events
MD	Maintenance dose
MEA	Multiple electrode aggregometry
MPV	Mean platelet volume
mRNA	Messenger RNA, messenger ribonucleic acid
NSTE-ACS	Non-ST elevation acute coronary syndrome
NSTEMI	Non-ST elevation myocardial infarction
PCI	Percutaneous coronary intervention
PLT-O	Optical platelet count
PTCA	Percutaneous coronary intervention
RBC	Red blood cells
RNA	Ribonucleic acid
ROC	Receiver operating characteristics
RPs	Reticulated platelets
RP%	Immature platelet fraction
SD	Standard deviation
STEMI	ST-segment elevation myocardial infarction
TO	Thiazole orange
TPO	Thrombopoietin
TxA_2	Thromboxane A_2
URL	Upper reference limit
WBC	White blood cells

1. Introduction

Cardiovascular disease cause about one third of all deaths in the world [1], with ischemic heart diseases being nominated as the “world´s biggest killer” by the World Health Organization [A]. Pathophysiologically, atherogenesis is the causal origin of cardiovascular disease.

1.1 Role of platelets in atherogenesis

Atherogenesis, the process of developing atheromatous plaques in the intima of the vessel wall, involves a complex interplay between several different cell types and circulating factors, leading over various stages from fatty streaks to vulnerable atheromas. The atherosclerotic risk factors embrace amongst others smoking, elevated ApoB/ApoA1 ratio, hypertension, diabetes, abdominal obesity, psychological factors, unhealthy nutrition, alcohol consumption and physical inactivity [2, 3]. Nevertheless, genetic factors are also implicated, including gene expression in platelets [4] and platelet phenotype [5]. These risk factors can trigger the genesis of plaques by causing chronic stress and endothelial dysfunction, leading to reactive oxygen species. Low-density lipoproteins (LDL) constitute another driving factor for the formation of vascular damage. LDL accumulates in the subendothelial layer and becomes sensitive to modifications like oxidation [6]. Oxidative stress can nurture numerous effects such as endothelial inflammation, apoptosis, immune responses, reduced expression of endothelial nitric oxygen synthase and altered adhesion of leucocytes and thrombocytes [6, 7]. While under physiologic conditions platelets do

neither adhere to the intact endothelium nor are activated by it, pathologic events leading to inflammations activate the vascular endothelium which in turn induces platelet activation. Following, adherent platelets start the coagulation cascade, recruit circulating leucocytes, attach and inflame them [6, 8]. As a consequence, leucocytes, monocytes and T-lymphocytes are recruited and transmigrate into the intima (**Figure 1**). There, monocytes transform into macrophages expressing scavenger receptors, incorporate cholesterol and become foam cells. This promotes the development of fatty streaks as well as an elevated plaque susceptibility to rupture and consecutive thrombus formation [6].

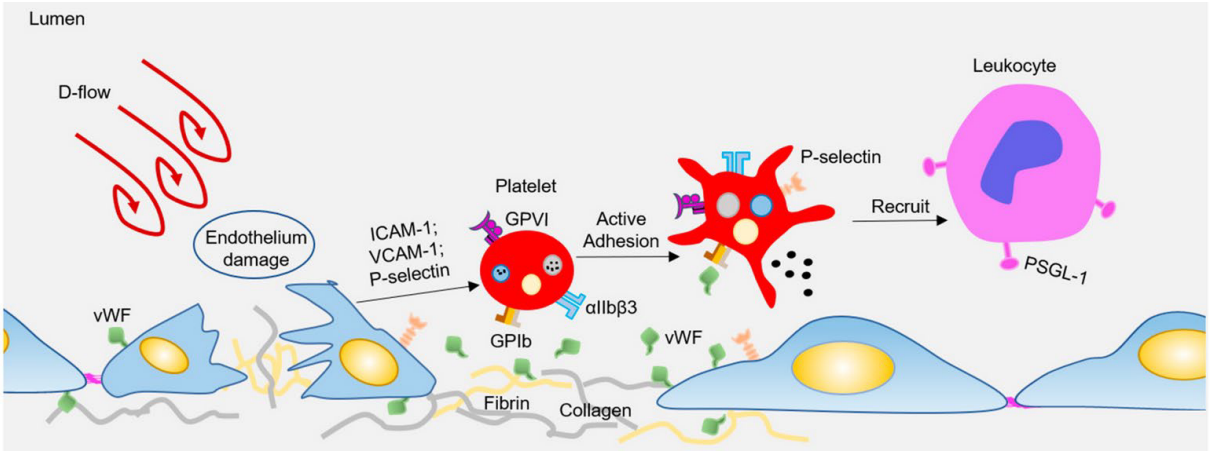

Figure 1: Disturbed flow-regulated platelets participate in the plaque formation. Disturbed flow (D-flow) activates endothelial cells (ECs), resulting in elevated expression of adhesion molecules and deposition of adhesion proteins. All these adhesion molecules and proteins interact with platelets via surface receptors, leading to platelet activation. Activated platelets recruit circulating leukocytes by P-selectin or other releasing inflammatory factors, therefore participating atherogenesis. Adapted from [9].

1.2 Role of platelets in thrombus formation and plaque rupture

Acute coronary syndromes (ACS), including unstable angina and myocardial infarction (AMI) with or without ST-segment elevation (STEMI and NSTEMI), are life-threatening diseases that remain one of the most causes of high morbidity and mortality worldwide, despite advances in treatment [10]. The central mechanism in the development of ACS comprises the rupture or erosion of the coronary plaque and subsequently platelet adhesion, activation, aggregation with thrombus formation [6, 11]. Plaque disruption leads to an exposure of central components like subendothelial collagen, inducing an activation of thrombocytes and the coagulation cascade, resulting in the formation of intracoronary thrombus (**Figure 2**) [12, 13]. Thrombus may be occluding the coronary artery partially or completely. STEMI patients often show complete occlusion while partially occlusions are without any ST-segment elevation and therefore categorized as having non-STEMI or unstable angina [12, 13]. Coronary occlusion creating a reduced blood flow and therefore an imbalance between supply and demand, provokes symptoms of ischemic chest discomfort. The endpoint of ischemia is myocardial infarction, as a result of myocardial cell death [14]. Consequently, platelets play an important role in atherosclerosis by providing the inflammatory basis for plaque formation before occluding the vessel by thrombosis upon plaque rupture [8].

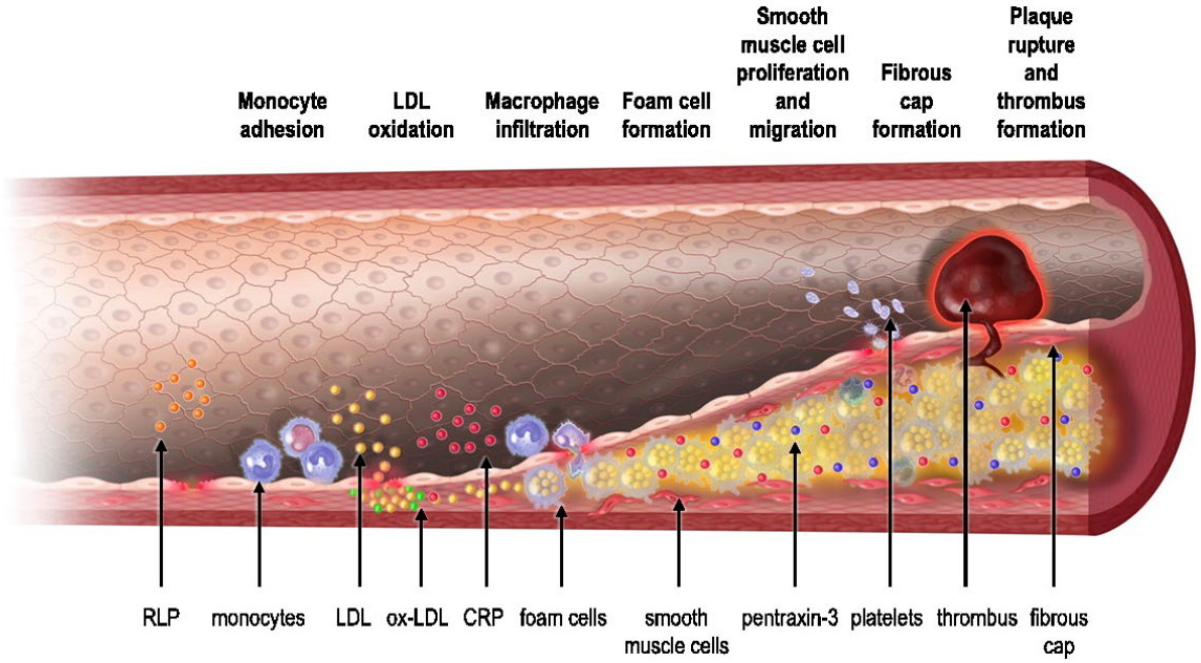

Figure 2: Atherosclerosis is a multistep process, ranging from endothelial dysfunction to plaque development, progression, and rupture, leading to thrombus formation and cardiovascular events. CRP = C-reactive protein, RLP = remnant like particle. Adapted from [15].

1.3 Clinical definition of acute myocardial infarction (AMI)

The clinical definition of acute myocardial infarction (AMI) is based on the presence of acute myocardial injury with clinical evidence of myocardial ischaemia and with disclosure of an increase and/or fall of cardiac biomarker levels (preferably cardiac troponin) with at least one value above the 99th percentile URL (upper reference level) and one further manifestation, such as symptoms of myocardial ischaemia, development of pathological Q waves on the ECG, ST-segment elevation or depression on the ECG, loss of viable myocardium or detection of intracoronary thrombus by angiography or autopsy [12, 14, 16, 17].

On the basis of an ECG it can be differentiated between those with acute chest pain and persistent (> 20 min) ST-segment elevation (STEMI) and those with acute chest pain but without persistent ST-segment elevation [18]. The latter can be divided into non-ST-segment elevation myocardial infarction (NSTEMI) and unstable angina pectoris (**Figure 3**).

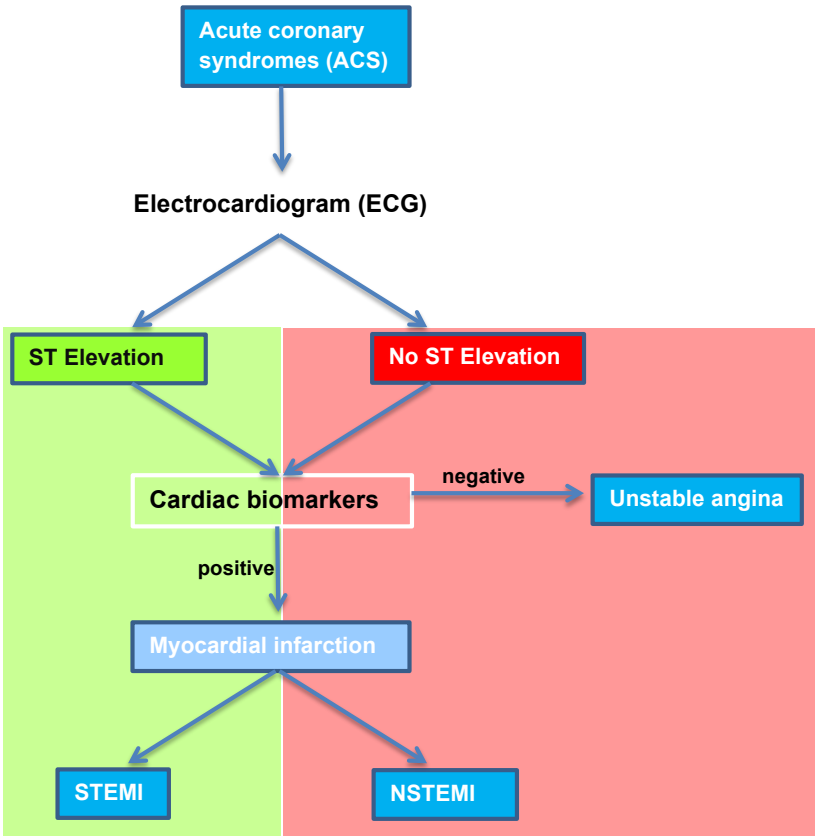

Figure 3: Clinical classification of ACS. Created and modified after https://www.heartonline.org.au/articles/pathophysiology/pathophysiology-of-acute-coronary-syndrome-and-heart-failure#pathophysiology-of-ischaemia (29.11.2021; 11:30); https://www.msdmanuals.com/de-de/heim/herz-und-gef%C3%A4%C3%9Fkrankheiten/koronare-herzkrankheit/akute-koronarsyndrome-herzinfarkt,-myokardinfarkt,-instabile-angina-pectoris (29.11.2021; 11:31) and https://www.wikiwand.com/en/Acute_coronary_syndrome (23.11.2021; 11:32)

The cardinal clinical manifestation of this imbalance between blood supply and requirement is often experienced as chest pain, encompassing epigastric, arm wrist or jaw ache or irradiating in this body regions and lasting usually for at least 20 minutes. Symptoms like unexplained nausea, weakness, dizziness or syncope or persistent dyspnea can also be included [14]. Generally, ischemic signs vary to a great deal between individuals. Elderly patients and women often show more atypical symptoms [19, 20].

1.4 Therapy of AMI

1.4.1 Interventional therapy

The gold standard in the therapy of AMI comprises immediate reperfusion by primary percutaneous coronary intervention (PCI) combined with modern antithrombotic therapy.

Respectively, current guidelines recommend for STEMI patients an emergent reperfusion therapy via primary PCI, otherwise – if the time frame of 120 minutes after diagnosis is exceeded - fibrinolysis is the method of choice [21]. In patients with non-ST elevation acute coronary syndrome (NSTEMI or unstable angina) treatment is similar to STEMI patients. In accordance with the classification into risk groups by risk factors, an ischemia guided and an early invasive strategy can be distinguished [22-24]. Immediate invasive strategy (< 2 h) is recommended by the ACC/AHA and ESC guidelines for (very) high risk patients (GRACE score), while at an intermediate risk

score, the timeline should be of < 72 h for the invasive approach. Whereas for patients with low GRACE risk scores, an ischemia guided method is indicated [24, 25].

In ACS patients with an unsuitable anatomy for PCI or complications due to myocardial infarction, emergent coronary artery bypass graft (CABG) surgery should be considered [18, 21, 24].

1.4.2 Drug therapy of ACS

Additionally, next to the pillar of invasive strategy, ACS patients receive peri- and post-procedural antithrombotic therapy, consisting of anticoagulation mostly with unfractionated heparin (UFH) and antiplatelet therapy [21, 24].

In view of the fact that platelets can influence the pathogenic course of ACS and play a key role in the mechanism of plaque development, plaque rupture and thrombus formation, it is of paramount importance to apply platelet inhibiting medication in ACS patients in order to prevent further unfavourable events. Dual antiplatelet therapy has evolved to be the cornerstone in the medicinal ACS treatment [26-30]. Contemporary guidelines recommend the long-term treatment a combination of aspirin (cyclooxygenase (COX) inhibitor acetylsalicylic acid (ASA)) and a potent $P2Y_{12}$ adenosine diphosphate (ADP) receptor antagonist in form of prasugrel or ticagrelor. Only in case of contraindications against the last named, clopidogrel should be applied [21, 24, 31, 32] (exemplary for NSTEMI in **Figure 4**). Thereafter a long-lasting mono therapy with aspirin, alternatively clopidogrel, has become the medication of choice [21, 24].

Recommendations for antithrombotic treatment in patients with non-ST-elevation acute coronary syndromes undergoing percutaneous coronary intervention

Recommendations	Class[a]	Level[b]
Pre-treatment and antiplatelet therapy		
Aspirin is recommended for all patients without contraindications at an initial oral loading dose of 150–300 mg (or 75–250 mg i.v.), and at a maintenance dose of 75–100 mg daily long-term.[681,683,721]	I	A
A $P2Y_{12}$ inhibitor is recommended in addition to aspirin, maintained over 12 months unless there are contraindications such as an excessive risk of bleeding.[701,702,722,723] Options are:	I	A
• Prasugrel in $P2Y_{12}$-inhibitor naïve patients who proceed to PCI (60 mg loading dose, 10 mg daily dose).[701]	I	B
• Ticagrelor irrespective of the preceding $P2Y_{12}$ inhibitor regimen (180 mg loading dose, 90 mg b.i.d.).[702]	I	B
• Clopidogrel (600 mg loading dose, 75 mg daily dose) only when prasugrel or ticagrelor are not available or are contraindicated.[722–724]	I	B

Figure 4: Antithrombotic therapy of ACS based on the guideline for NSTEMI. Recommendation for pre-treatment and antiplatelet therapy in NSTEMI patients undergoing PCI. Adapted from [31].

1.4.3 $P2Y_{12}$ receptor inhibitors

In the process of haemostasis and thrombus formation, especially the $P2Y_{12}$ receptor plays a fundamental role. $P2Y_{12}$ receptors are 7-membrane spanning proteins which are connected to G_i-proteins binding adenosine 5´diphosphate (ADP) [33] and are found on platelets (**Figure 5**) as well as on some other cell types [34]. With the activation of platelets and the release of the contents from their granules, ADP is set free and binds to platelet $P2Y_{12}$ receptors, which results in the amplification of reactions towards other agonists, stabilization of platelet aggregates and support of the pro-coagulant activity of thrombocytes [35-37]. Therefore, the $P2Y_{12}$ receptor is the target of several antagonists interrupting the pathological cascade of thrombosis in order to prevent cardiovascular events; among them clopidogrel, prasugrel and ticagrelor.

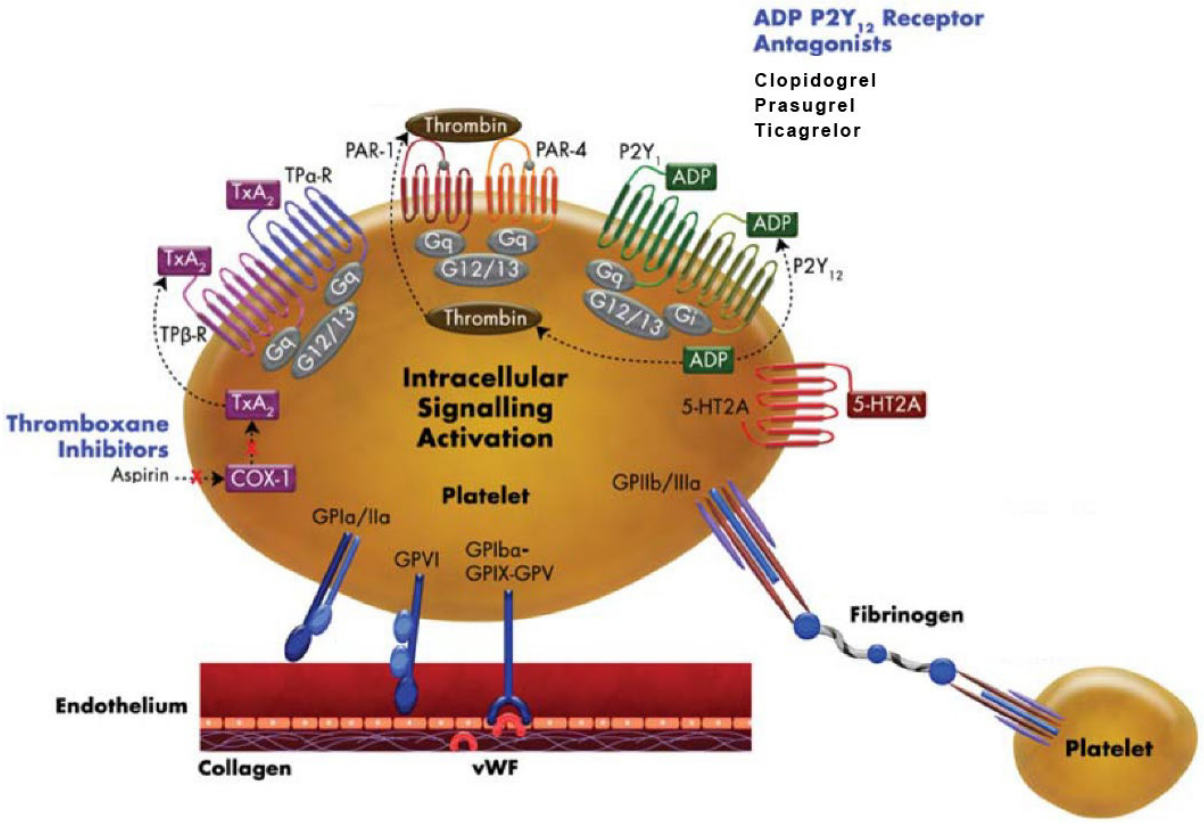

Figure 5: Pharmacological starting points for the inhibition of platelet aggregation. COX-1 (cyclooxygenase-1); vWF (von Willebrand factor); GP (glycoprotein); PAR (protease activated receptor). Modified from [38].

Patients undergoing PCI are at high risk of acute ischaemic complications and recurrent atherothrombotic events [39, 40]. Previously, it has been shown that DAPT with aspirin and the $P2Y_{12}$ inhibitor clopidogrel reduces the risk of unfavourable outcomes like death, reinfarction or stroke [41, 42]. Clopidogrel is a platelet inhibitor that irreversibly obstructs the $P2Y_{12}$ receptor of platelets [43]. This thienopyridine-type $P2Y_{12}$ receptor inhibitor has been a standard for DAPT for many years and is still widely used [44]. Yet, it is associated with numerous drawbacks on account of its pharmacological features, including its vast inter-individual response variability [45] – mostly caused by the CYP2C19 gene [46] -, its delayed onset of effect [47] and its

modest $P2Y_{12}$ receptor inhibition [48]. Therefore, the two novel $P2Y_{12}$ receptor antagonists prasugrel and ticagrelor have been developed.

Prasugrel is a third-generation oral thienopyridine ADP-receptor antagonist. Like clopidogrel it binds irreversibly to the $P2Y_{12}$ receptor and its effects last for the lifetime of the thrombocyte. However, although prasugrel is also a prodrug, its metabolism differs from clopidogrel´s metabolism as its transformation into the active component requires only a one-step metabolic activation with cytochrome P450 liver enzymes (clopidogrel requires two steps). Therefore, it has not only a more rapid onset of action, but also a less variable effect in inhibiting platelet aggregation [43, 49, 50].

A number of studies have demonstrated the advantage of prasugrel over clopidogrel in patients with ACS: TRITON TIMI 38 (Trial to Assess Improvement in Therapeutic Outcomes by Optimizing Platelet Inhibition with Prasugrel - Thrombolysis in Myocardial Infarction) was a dual-antiplatelet therapy with aspirin, comparing prasugrel with clopidogrel in patients with ACS undergoing PCI. Prasugrel was administrated after diagnostic angiography in patients undergoing PCI and reduced the rate of cardiovascular causes, nonfatal AMI and nonfatal stroke [43, 51]. Moreover, the PRINCIPLE TIMI 44 (Prasugrel in Comparison to Clopidogrel for Inhibition of Platelet Activation and Aggregation – Thrombolysis in Myocardial Infarction 44) trial included patients undergoing cardiac catheterization with planned PCI and evidenced that prasugrel had a predominant effect in platelet aggregation inhibition in comparison to clopidogrel [52]. These findings are supported by the GENERATIONS trial [53] as well as by the FEATHER trial [54] and the TROPICAL-ACS trial [55], all three showing that prasugrel induced a greater antiplatelet effect than clopidogrel.

Nonetheless, the application of prasugrel has also its limits. In this regard the TRITON TIMI 38 study revealed an increased risk of major bleeding events in patients treated with prasugrel [51]. When prasugrel was compared to clopidogrel in patients with NSTEMI or unstable angina *not* undergoing revascularization in the TRILOGY ACS trial, there was no significant advantage of prasugrel in regarding protection from ischaemia [56]. By contrast, in the ACCOAST study prasugrel was given *before* catheterization with the result of an increased bleeding risk [57, 58]. Additionally, the application of prasugrel is in general not recommended to patients ≥ 75 years or a body weight < 60 kg due to an elevated bleeding risk and a doubtful benefit [21, 59].

Unlike prasugrel, ticagrelor belongs to the chemical class of cyclopentyl triazolopyrimidine. It is a direct-acting oral and reversible non-competitive inhibitor of the $P2Y_{12}$ receptor. This reversible process allows the functional recovery of thrombocytes within approximately 48 hours. As ticagrelor does not need biotransformation for activation (the active composition itself is ingested), it provides a more rapid onset of action than clopidogrel [32, 43, 50]. Similar to prasugrel, ticagrelor enables a faster, better and more stable $P2Y_{12}$ receptor inhibition [60]. Various trials expressed the superiority of ticagrelor over clopidogrel: The PLATO (Platelet Inhibition and Patient Outcome) study compared both antiplatelet agents in patients with ACS and confirmed that therapy with ticagrelor resulted in a significantly reduction in the incidence of vascular death, non-fatal AMI and stroke, while there was no higher risk of overall major bleeding [61]. These findings are in line with the SWEDEHEART trial where patients treated with ticagrelor showed a reduced rate of death, AMI or stroke, but with an increased risk of bleeding [62]. Regarding the outcome, these trials contributed to verify the benefit of ticagrelor over clopidogrel. Besides, both in patients

treated with PCI as well as conservative treatment and CABG, ticagrelor showed superiority to clopidogrel [63, 64].

Despite the superiority of prasugrel and ticagrelor over clopidogrel, randomized studies directly comparing these two drugs were lacking. For this reason the ISAR REACT 5 trial was called into life, comparing head-to-head the efficacy and safety of the two novel $P2Y_{12}$ receptor antagonists [65].

1.5 ISAR REACT 5 study

The ISAR REACT 5 trial (Intracoronary Stenting and Antithrombotic Regimen: Rapid Early Action for Coronary Treatment 5) was an investigator-initiated, randomized, open-label, phase IV, multicentric study [65]. It was the first and the only study directly comparing the efficacy of prasugrel and ticagrelor in ACS patients. Its primary aim was the comparison of the pharmacodynamic effects of prasugrel versus ticagrelor and its impacts on ischaemic and bleeding outcomes. The primary endpoint consisted in the composition of death, myocardial infarction or stroke at one year after randomization [66]. Altogether, 23 facilities in Germany and Italy participated, including altogether 4018 patients, with a follow-up time of one year [67, 68]. Patients were eligible for enrolment if they were hospitalized for an ACS (STEMI, NSTEMI or unstable angina) and were planned to undergo diagnostic coronary angiography. They were randomly assigned to ticagrelor or prasugrel with a randomization ratio of 1:1 (**Figure 6**).

Concomitant medication consisted in a loading and a maintenance dose of acetylsalicylic acid (aspirin) [65].

The results of study demonstrated that treatment with prasugrel reduced significantly the incidence of the primary endpoint: prasugrel 6,9% vs. ticagrelor 9,3%; P=0,006 (**Figure 7**). Besides, there was no substantial difference of major bleeding events between the two groups: prasugrel 4,8% vs. ticagrelor 5,4%; P=0,46 (**Figure 8**) [68]. Subsequently, prasugrel decreased further adverse events, but not at the expense of bleeding incidents. Consequently, the discoveries of the ISAR REACT 5 study found their way already into current guidelines and clinical practice [24, 25].

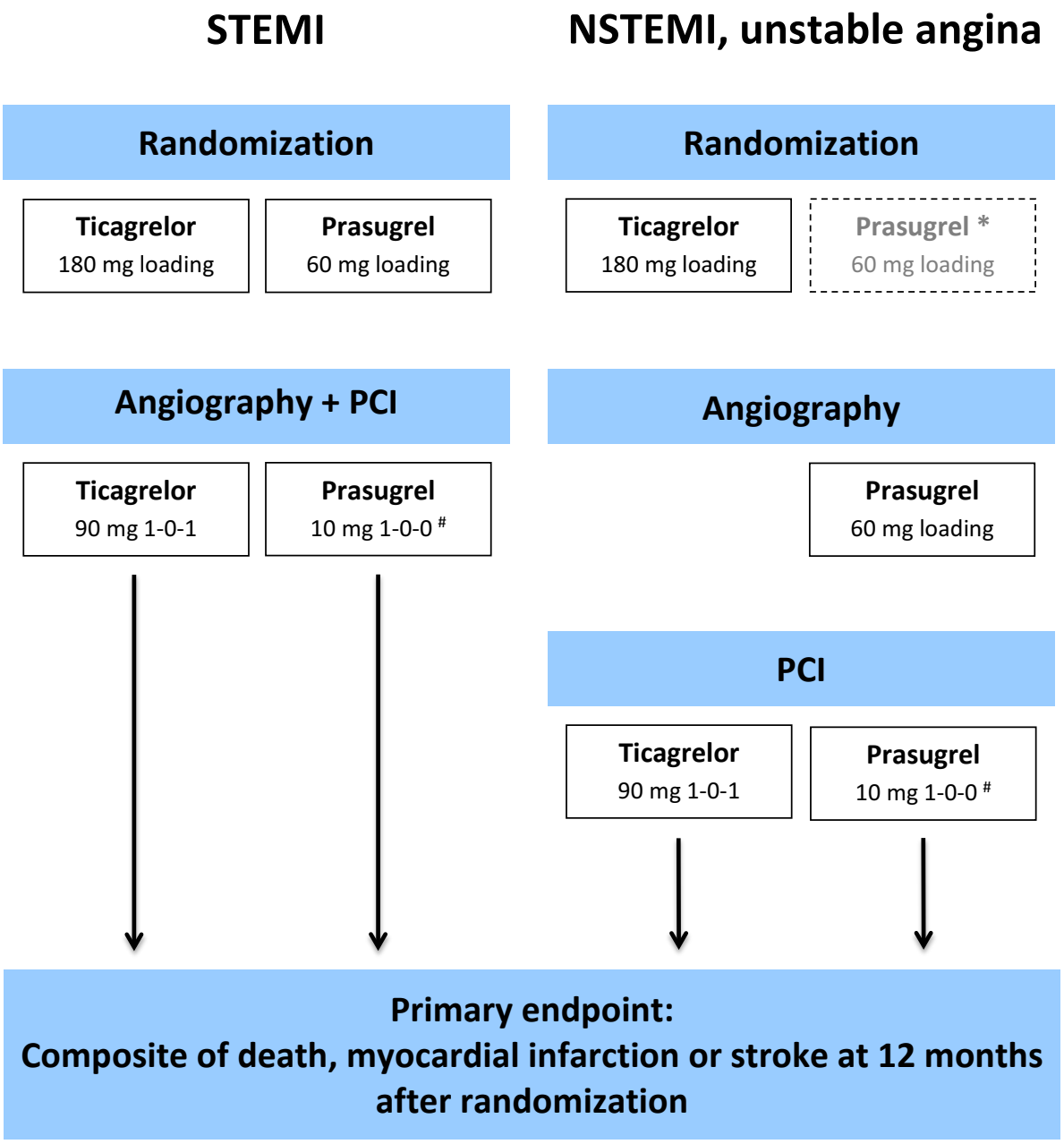

Figure 6: ISAR REACT 6 study schedule. Adaptation from [65].
* in patients with known coronary anatomy
# Prasugrel 5 mg in patients ≥ 75 years of age or with a weight < 60 kg

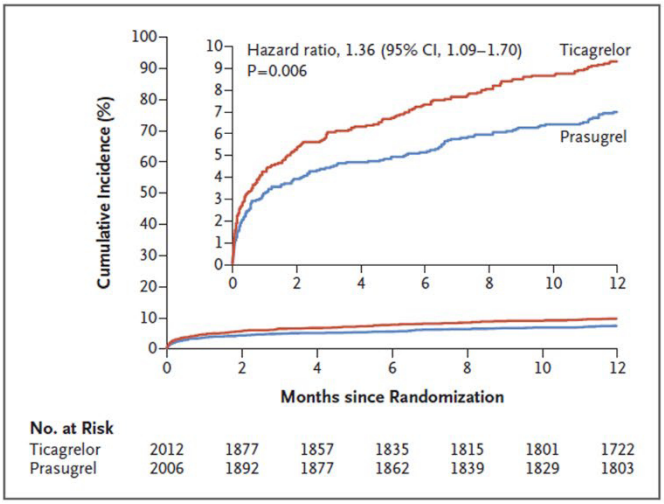

Figure 7: Cumulative incidence of the primary endpoint at one year. Adaptation from [68].

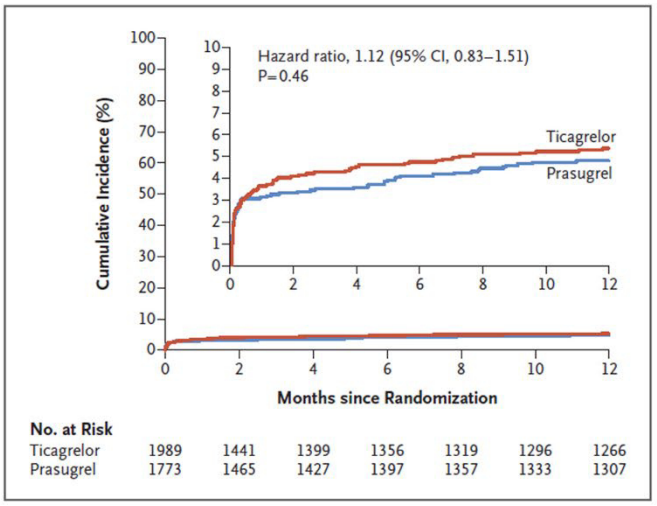

Figure 8: Cumulative incidence of the safety endpoint (BARC type 3 - 5) at one year. Adaptation from [68].

1.6 Reticulated platelets (RPs)

1.6.1 Definition of RPs

RPs are young and immature thrombocytes, recently released from the bone marrow. They represent the youngest platelets in the circulation. Even though platelets are anucleate, especially their younger forms contain mRNA in their cytoplasm mainly as residue from megakaryocytes during thrombopoiesis, thus representing a reticulated platelet marker [69, 70]. Measuring these RPs in the circulating blood reflects the release of thrombocytes from megakaryocytes and consequently gives indirectly information about thrombopoietic activity and platelet turnover [71, 72]. Several studies showed that the majority of RPs (> 60%) were in the large platelet group and constituted > 15% of all large thrombocytes [73] and that the volume of platelets is

being reduced as they undergo a process of maturation and age, including a change in chemical components [74, 75].

A current transcriptome analysis revealed a differential expression of more than 1700 genes in RPs compared with mature thrombocytes from healthy human subjects [76]. Furthermore, evidence is growing that RPs are even able to regulate translation by pathways like pre-mRNA processing and spliceosome, thus controlling synthesis of pro-thrombotic and pro-inflammatory proteins [77, 78]. It is even hypothesized that the stability of messenger mRNAs is influencing the life span of the platelet [79].

RPs are enzymatically and metabolically more active in comparison to mature platelets: Several studies evidenced the hyperactivity of RPs, showing a higher adhesiveness to collagen and a considerably greater aggregation [80, 81]. Additionally, it was noted that RPs have a greater predisposition to participate in thrombosis under shear-stress conditions corresponding to coronary artery stenosis in comparison with mature thrombocytes, implying the thrombotic potential of young platelets [82]. In this way, these cells have a higher prothrombotic potential through a higher amount of pro-thrombotic mRNA [76], they aggregate faster with collagen [80], express greater levels of procoagulant surface protein such as glycoprotein (GP) IIb/IIIa [83] and P-selectin as well as cyclooxygenase-1 and -2 (COX-1 and COX-2) [84] and produce more thromboxane A_2 (TxA_2) [85, 86]. Since TxA_2 is a strong vasoconstrictor and platelet aggregator, RPs are very reactive and could be able to result in occlusion of coronary arteries [87]. In this way thrombocytes assume essential functions in atherothrombotic events [88] and are medically as well as clinically relevant [81].

1.6.2 Measurement of RPs

A major advance in RPs research was made when flow cytometry was adopted to evaluate RPs based on RNA staining by thiazole orange (TO), providing the first structured staining method for RPs analysis [89]. This approach became the gold standard protocol to detect and study RPs with flow cytometry [71, 90-92]. Even their name derives from their similarity to erythrocyte reticulocytes when stained with TO [93]. TO is sensitive to RNase and thus RNA, making it suitable to mark immature platelets, characterized by their greater RNA proportion [71]. Nonetheless, though its clinical potential, flow cytometric measurement has also its limitations, comprising a missing standardization, diverging values of RPs depending on the methods applied as well as the need for special equipment, making it difficult for common daily use in clinics [72, 92, 94].

In the past few years new technologies have been emerged in autoanalyzers, containing impedance, optical measurement and fluorescence, making measurements of RPs in routine diagnostics cheaply and easily possible, thus overcoming the limitations of prior methods. With the introduction of haematology analyzers, quantification of RPs in clinical settings became feasible and inexpensive. There are a few point-of-care systems (Sysmex, Abbott and Mindray) that allow quantitative analysis of RPs. In this regard, Sysmex (Kobe, Japan) is the most commonly used system in the clinical practice and was the first diagnostic analyzer to introduce the immature platelet fraction (IPF) [95-97].

1.6.3 Role of RPs as biomarkers

In recent years RPs have been investigated in different patient collectives. The vast majority of studies that investigated RPs as biomarkers used IPF as variable. In this regard it was observed that IPF is a striking risk parameter in ACS patients for further adverse cardiovascular events. Cesari et al. postulated RPs as predictor for an increased risk of cardiovascular death in patients with ACS since an accelerated platelet turnover produces more active and aggressive thrombocytes [98]. Various studies proposing that platelet markers are modified in ACS [99, 100] sparked the search for thrombocyte markers in chest pain patients.

Recently, the research of immature thrombocytes moved further into the spotlight as in the last years elevated RPs have been described in patients with arterial thromboses involving cerebrovascular disease [101], stent thrombosis [102], ACS [103] as well as cardiac surgery [104]. Multiple studies have detected increased levels of IPF in different clinical conditions, especially in the cardiovascular field but also in hematologic patients [105-108]. RPs have also been characterized in indispositions including diabetes mellitus type 2 [109-111] and sepsis [112, 113]. There even seems to be a linkage between the outcome of COVID-19 patients and IPF [114-117]. Apart from this the count of RPs can be heightened by cardiovascular risk factors like smoking and diabetes [83, 98, 118] as well as cardiac [119] and non-cardiac surgeries [120, 121].

The majority of studies investigating RPs was performed in patients with cardiovascular disease. In this regard a significant incremental increase of RPs in

patients with CAD was described [103]. Furthermore it was reported that patients with ACS had higher levels of RPs, especially the ones with STEMI [104], followed by Non-STEMI and unstable angina [103, 104, 122]. Another study explored the time course of circulating RPs after AMI and showed that RPs remained elevated for the first 2 months after AMI and decreased after one year, linking RPs with long-term prognosis [123]. Altogether, these studies emphasized elevated levels of RPs in high risk cohorts as well as in acute and chronic diseases.

1.6.4 Influence of RPs on the efficacy of antiplatelet therapy

One of the main focuses of RP research in cardiovascular disease was based on the correlation of RPs in blood with an insufficient response to antiplatelet therapy. In patients with coronary artery disease (CAD) on therapy with aspirin decreased anti-platelet efficacy was observed: Several trials indicated that RPs are associated with reduced antiplatelet effects of aspirin and also greater aspirin resistance [73, 84, 102]. In patients with aspirin therapy and an accelerated platelet turnover, newly produced RPs released into the bloodstream - still unexposed to aspirin - present uninhibited COX-1 activity, possibly causing platelet aggregation [124]. Similarly, other irreversible platelet inhibitors are likely to be influenced by a high platelet turnover and a number of newly released platelets from the bone marrow. The underlying mechanism for this effect is due to the fact that the active metabolites of the irreversible platelet inhibitors have only a short timeframe in which they can bind to platelet receptors. As with RPs

it is more unlikely that they are blocked by irreversible inhibitors like aspirin and thienopyridines (clopidogrel, prasugrel).

Ergo, multiple investigations have observed an association between RPs and hyporesponsiveness to several antiplatelet medication: They could demonstrate that in healthy people as well as in ACS patients – each having an increased rate of RPs – an impaired effect of clopidogrel or DAPT with aspirin and clopidogrel was received [125, 126]. Besides, patients treated with clopidogrel having an insufficient antagonism and subsequently high platelet reactivity have a higher risk of cardiovascular complications, while low platelet reactivity is an indicator for bleeding [30, 48, 55]. Also, a significant correlation between RP levels and platelet reactivity on therapy with prasugrel was noticed [127-129]. Notably, the efficacy of prasugrel is superior to clopidogrel regarding platelet inhibition [51]. Beyond that, correlation between the rate of immature thrombocytes and response to antiplatelet therapy in patients with CAD was proven [73, 130]. Accordingly, elevated levels of RPs were associated with reduced response to antiplatelet therapies (**Figure 9**), for instance aspirin alone [84], clopidogrel alone [125, 126, 131], DAPT with aspirin and clopidogrel [73] and prasugrel [127, 128]. But – interestingly – there is no association with the reversible platelet inhibitor ticagrelor [132, 133]. Contrarily to prasugrel, ticagrelor is already an active drug, not needing metabolic activation. This enables together with a longer half-life than thienopyridines a more continuous inhibition of RPs. Consequently, prasugrel inhibits RPs to a smaller extent than ticagrelor [133].

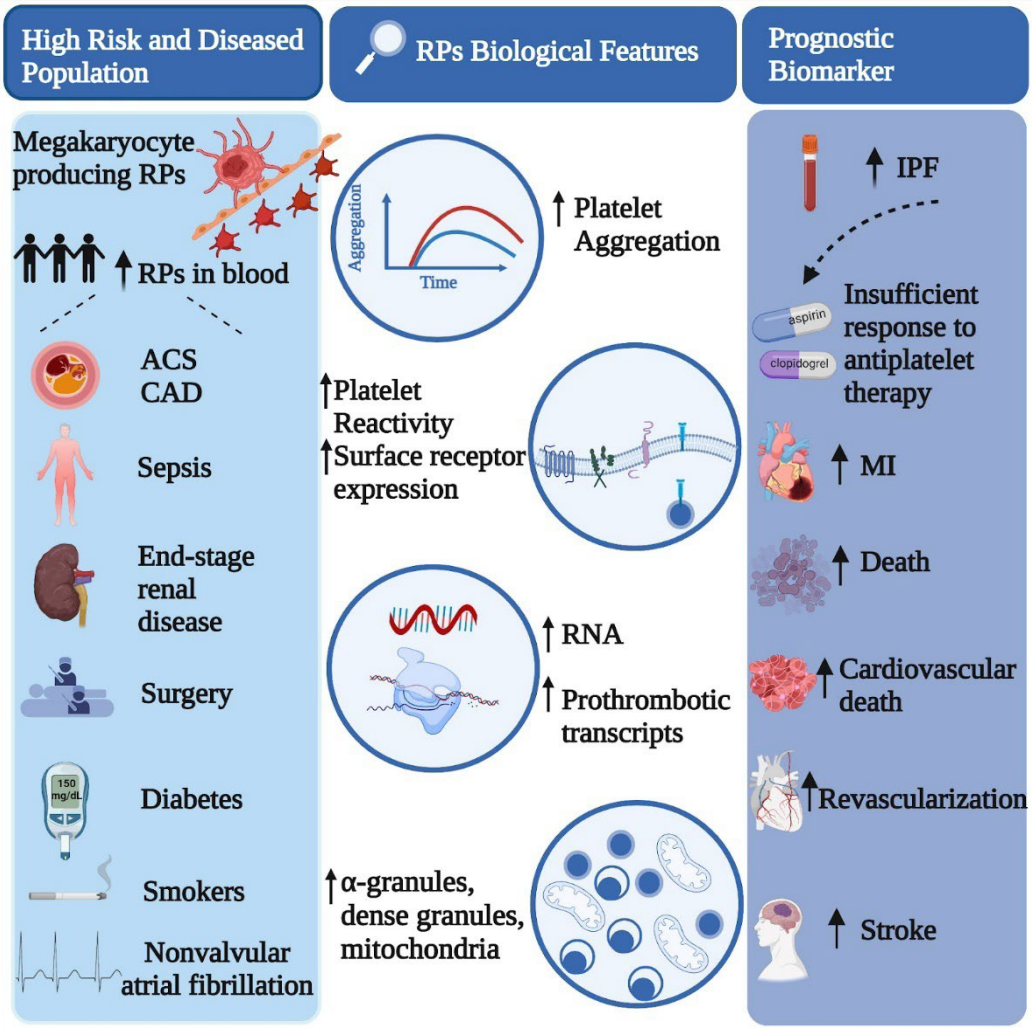

Figure 9: Reticulated platelet features. Adapted from [134].

2. Study objectives

It is known that RPs negatively influence the efficacy of thienopyridines (clopidogrel, prasugrel) but not the efficacy of ticagrelor [73, 125, 127, 129-133] . Nevertheless, to date no data regarding the prognostic relevance of RPs in patients with these drugs exists. And the prognostic relevance of RPs has been almost exclusively assessed in cardiovascular patients on DAPT with aspirin and clopidogrel [125, 126, 135, 136]. Until now, data regarding the association between IPF and adverse cardiovascular events in patients treated with potent $P2Y_{12}$ inhibitors including prasugrel and ticagrelor has been missing. On this account the ISAR REACT 5 reticulated platelet substudy has been launched. The primary aim of this pre-specified reticulated platelet substudy was to investigate the prognostic role of IPF as a predictor of the composite primary endpoint consisting of death, myocardial infarction or stroke at one year after randomization in a large cohort of ACS patients who were randomly assigned to receive either prasugrel or ticagrelor. Secondary aims consisted in the incidence of severe bleeding (BARC 3 – 5) at 12 months after randomization in association with RPs as well as in the correlation between ADP and IPF.

3. Methods

3.1 Study design of ISAR REACT 5 reticulated platelets substudy

All patients of the two participating centers in Germany (Klinikum rechts der Isar and Deutsches Herzzentrum München, both in Munich) from the ISAR REACT 5 trial were included in this reticulated platelets substudy who had at least one valid IPF value within 48 hours after randomization. If multiple measurements of IPF values were accessible from the same patient, the first value available was chosen. All patients gave written informed consent before entering the study [133].

For the correlation analysis between ADP induced platelet aggregation and IPF existed further exclusion criteria [133]:

- No reception of DAPT after angiography
- GPIIb/IIIa antagonists were administered
- Patients having on-clopidogrel maintenance treatment before inclusion into the ISAR REACT 5 study

For the correlation analysis, only values from patients who underwent PCI and who had received a study drug loading dose were included. Moreover, blood samples for this analysis had to be obtained at the same time point.

3.2 Blood sampling

Venous whole blood was obtained from patients at hospital admission and within 48 hours after randomization. For IPF measurement, blood samples were collected into 2,7 ml plastic tubes containing the anticoagulant Ethylenediaminetetraacetic acid (1,6 mg/ml EDTA, Sarstedt, Nuembrecht, Germany). For measurement of ADP-induced platelet aggregation, blood samples were collected into 2.7 ml plastic tubes containing hirudin for a final concentration of 525 antithrombin units (ATU) /ml blood (Sarstedt, Nuembrecht, Germany). Blood samples were sent to our in-house clinical chemistry department by pneumatic tube immediately after collection from each patient. After arrival in the clinical chemistry department, the blood sample was immediately proceeded to measurement of IPF and other immature platelet parameters. For measurement of ADP-induced platelet aggregation, blood samples were stored for 30 minutes at room temperature and afterward proceeded to platelet aggregation measurement.

3.3 Measurement of IPF and H-IPF

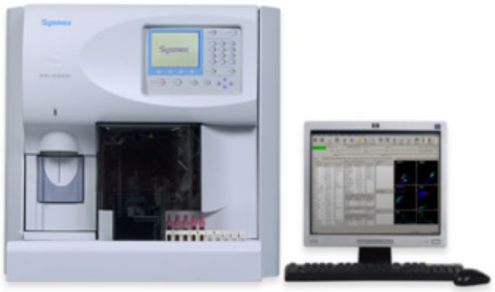

Figure 10: Sysmex XE-5000. Adapted from https://www.sysmex-europe.com/n/products/products-detail/xe-5000.html 27.09.2021, 11:07

RPs were measured as percentage of the total optical platelet count (IPF) by flow cytometry, using the fully automated system Sysmex XE-5000 or Sysmex XN (Sysmex, Kobe, Japan). For the majority of patients (n=400, 189 patients (68.7%) in the ticagrelor group and 211 patients (69.9%) in the prasugrel group, p=0.837) the Sysmex XE-5000 device (Sysmex, Kobe, Japan) was applied. For the remaining patients (n=177, 86 patients (31.3%) in the ticagrelor group and 91 patients (30.1%) in the prasugrel group) the Sysmex XN device (Sysmex, Kobe, Japan) was used.

Both systems determine the red blood cell count and platelets by impedance and can implement an additional optical platelet measurement in the reticulocyte channel to differentiate between mature and immature platelets. Two fluorescent dyes containing polymethine and oxazine penetrate the cell membrane, staining the RNA in reticulocytes (young erythrocytes) and immature thrombocytes. The stained cells then transverse a stream, onto which a laser light from a semiconductor laser diode is focused. By passing through the sensing zone of the flow cell, each cell scatters the

focused light. A photodetector recognises the forward scattered light (cell volume) and fluorescence intensity (RNA content) and transforms it into an electrical impulse.
In this way, the following characteristics of cells can be determined:

- Forward scattered light: gives information about cell volume
- Side scattered light: internal cell structures
- Side fluorescence light: information about DNA/RNA

Immature thrombocytes are identified by their greater size and their higher RNA substance than mature platelets. The quantity of generated pulses is directly proportional to the number of cells travelling through the sensing zone in a specified time. A computerized algorithm (Sysmex IPF Master) then separates the mature thrombocytes (represented as blue dots in platelet scattergrams; **Figure 11**) from RPs (displayed as green dots) by the intensity of forward scattered light and fluorescence. RPs distinguished by this method are designated as immature platelet fraction (percentage of the total optical platelet count, IPF%) [96, 97, 137, 138], [B]. The highly-fluorescent immature platelet fraction (H-IPF) parameter, representing the percentage of RPs with a superior amount of RNA and therefore with a very high fluorescence, is another factor that can be received [126]. Further possibly analyzed parameters are the red blood cells (RBC) along with the white blood cells (WBC) [97]. By this means Sysmex offers a practical method for routine complete blood count measurements with the ability to a reliable quantification of RPs with a high throughput [137].

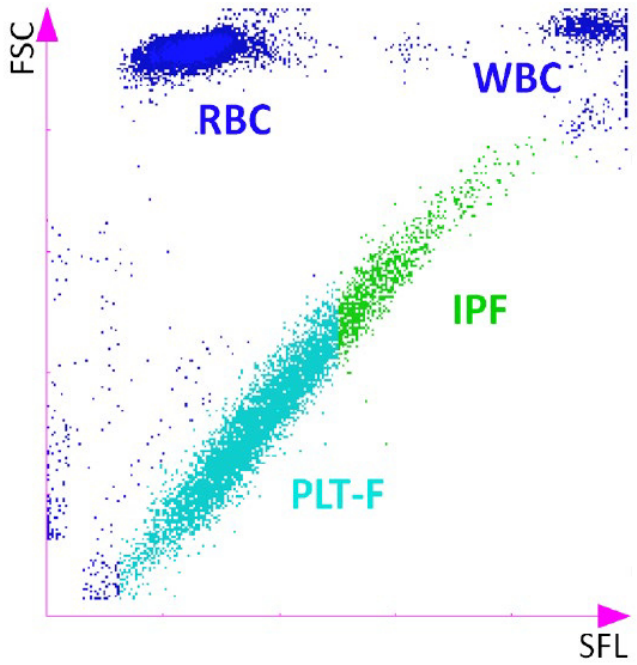

Figure 11 Scattergram (Sysmex-XN-series) measuring the immature platelet fraction (IPF) in the platelet fluorescence (PLT-F) canal, using forward scattered light (FSC; cell volume) against side fluorescence (SFL; nucleic acid amount). RBC = red blood cell, WBC = white blood cell. Adapted from [139].

The Sysmex XE-5000 device uses polymethine and oxazine to stain nucleic acid and IPF is assessed in the RETchannel, in which reticulocytes are also measured. This can cause unspecific interferences especially by red blood cell fragments and hemolysis [140]. The Sysmex XN system applies only oxazine for staining of nucleic acid, features an own PLT-F channel and has a five times higher counting volume leading to a higher reproducibility and a higher specificity for the detection of IPF [140, 141]. Despite reported differences regarding reference intervals for IPF [142, 143], both named Sysmex devices overall show very good correlation for IPF [141].

In our study the primary outcome parameter was IPF. H-IPF and IPC were also assessed. The immature platelet fraction (IPF%) was the primary directly measured

and reported parameter according to size and fluorescence intensity of the cells and defined as the larger and higher fluorescent percentage of all platelets. Additional assessed parameters involved the highly immature platelet fraction (H-IPF%), and the immature platelet count (IPC 10^3/µl), which is a calculated product received by the multiplication of the IPF with the total platelet count (IPC 103/µl = IPF x total platelet count) [98, 144]. The mean platelet volume (MPV), reflecting the average size and volume of circulating platelets, was also measured.

According to results obtained from IPF measurements within 48 hours after randomization, patients were divided into two groups based on the median of the IPF values: the IPF^{high} group included all patients with IPF > median of the entire study cohort and the IPF^{low} group including patients with IPF ≤ median of the study cohort. The same stratification was performed for the other assessed immature platelet parameters: $H\text{-}IPF^{high}$ (H-IPF>median) and $H\text{-}IPF^{low}$ (H-IPF≤median) as well as IPC^{high} (IPC>median) and IPC^{low} (IPC≤median).

3.4 Measurement of ADP-induced platelet aggregation

ADP-induced platelet aggregation values were measured using the Multiplate®Analyzer (Roche Diagnostics, Switzerland). Platelet aggregation values were quantified as the area under the curve (AUC = AU × min) of aggregation units (AU).

Figure 12: Multiplate® Analyzer. Adapted from https://www.roche.de/diagnostik-produkte/produktkatalog/systeme/multiplate-analyzer/ 27.09.2021; 10:43

ADP is one of the crucial platelet agonists binding to the $P2Y_1$ and the $P2Y_{12}$ receptor. The latter has a key function in exponentiation platelet stimulation mediated by ADP. In view of the fact that all clinically available ADP-receptor inhibitors interact only with the $P2Y_{12}$ receptor, several studies suggest that $P2Y_{12}$ specific specimen were the ideal test for these medicines [145-148].

The Multiplate® Analyzer by Roche works based on impedance aggregometry and is recommended for point of care [40] tests of platelet aggregation in whole blood. Platelet aggregometry with multiple electrodes (MEA = multiple electrode aggregometry) works by measuring a time-related increase in resistance between two electrodes due to platelet aggregation on the electrodes after pharmacological activation. The graphical representation is displayed by comparing the aggregation and time units in the coordinate system as amplitude-time curves (**Figure 13**). Among the measured variables are velocity of aggregation, maximum of aggregation and – most importantly

– the area under the curve [149], which is regarded to be an optimal parameter to mirror platelet aggregation (**Figure 14**) [150, 151].

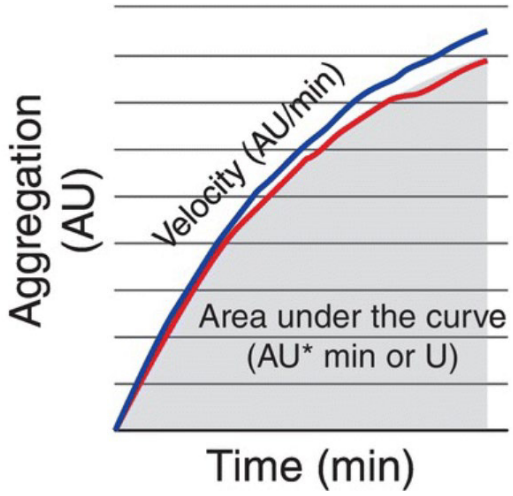

Figure 13: Graphical display of platelet aggregation measurements against time as area under the curve. Adapted from https://thoracickey.com/multiplate-analyzer/ 27.09.2021, 10:45

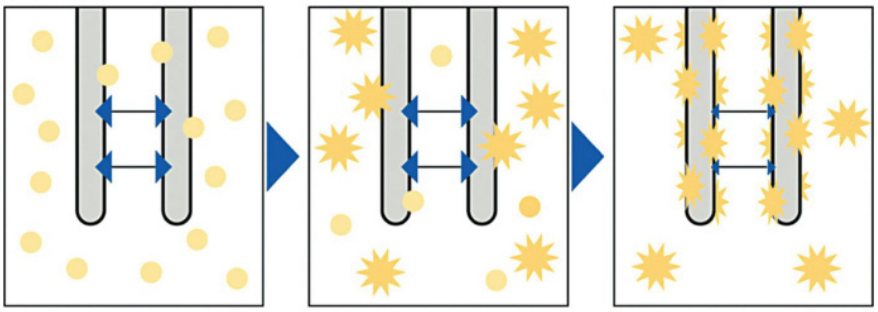

Figure 14: Functional mechanism of Multiplate® Analyzer: When platelets aggregate on the metal surface of the two electrodes, electrical impedance between the electrodes is increased and the current flow (represented as arrorws) is reduced. Adapted from https://thoracickey.com/multiplate-analyzer/ 27.09.2021; 10:45

3.5 Study endpoints

The primary endpoint was the composite of death from any cause, myocardial infarction or stroke at one year after randomization. The definition of myocardial infarction applied in this study was adapted from the Third Universal Definition of Myocardial Infarction [152]. Cardiac troponin was used as the favoured biomarker, while creatine kinase-myocardial band (CK-MB) and CK values were measured simultaneously and utilized if troponin values were not available [65]. The secondary endpoint comprised the incidence of severe bleeding as defined by the Bleeding Academic Research Consortium [98] type 3 – 5 (**Table 1**) at 12 months after randomization [153].

Table 1: Bleeding Academic Research Consortion [98]	
BARC type 3	a) Overt bleeding and a haemoglobin drop of 3 – 5 g/dL b) Overt bleeding and a haemoglobin drop of 5 g/dL c) Intracranial haemorrhage
BARC type 4	coronary artery bypass grafting-related bleeding; perioperative intracranial bleeding within 48 hours; reoperation after closure of sternotomy for the purpose of controlling bleeding; transfusion of 5 U of whole blood or packed red blood cells within a 48-hour period; chest tube output 2 L within a 24-hour period
BARC type 5	a) probable fatal bleeding; no autopsy or imaging confirmation but clinically suspicious b) definite fatal bleeding; overt bleeding or autopsy, or imaging confirmation

Legend to Table 1: Bleeding Academic Research Consortium [98]. Adaptation from [153] and Wells GA, Elliott J, Kelly S, et al., Dual Antiplatelet Therapy Following Percutaneous Coronary Intervention: Clinical and Economic Impact of Standard Versus Extended Duration. CADTH Optimal Use Report, No. 9.2b
https://www.ncbi.nlm.nih.gov/books/NBK542934/ (01.01.2022; 13:04)

3.6 Follow-up and monitoring

Follow-up was performed at 30±10 days, 6±1 and 12±1 months. Patients were contacted by telephone, hospital or outpatient visit, or structured follow-up letter. In case of adverse events related with the prior determined endpoints, source data were requested. All serious unfavourable incidents as well as primary and secondary endpoints were adjudicated by an independent committee.

3.7 Statistical analysis

Continuous data are shown as mean ± standard deviation (SD) or median with interquartile range (median [IQR]) and were analysed using the Student's t-test or non-parametric Wilcoxon rank-sum test depending on the distribution of the data. Categorical variables are shown as numbers with percentages and were compared using the chi-square or Fisher's exact tests. All statistical tests were two-sided and a p-value <0.05 was considered statistically significant. The association between IPF and clinical outcomes was tested with the Cox proportional hazard model. The same model was used for testing the interaction between the IPF and randomly assigned study drug regarding clinical outcomes. The proportional hazards assumption of the Cox model was checked and confirmed by statistical tests and graphical diagnostics (Schoenfeld residuals) for both primary end point and major bleeding. Continuous variables were entered into the model as original values without any transformation.

Multivariable cox proportional hazard models were applied to test for an independent association between IPF and the primary study endpoint and included the following variables: study group, age, gender, BMI, diabetes, GFR, admission diagnosis, treatment of ACS (conservative versus PCI). The Kaplan-Meier method was used for building even curves. Hazard Ratios are presented as HR [lower, upper 95 percentile] and score logrank test for p value. Spearman rank correlation coefficient (r) was used to describe the relationship between ADP-induced platelet aggregation and IPF. Correlations are shown as spearman coefficient r, and p value. The statistical analysis was performed using the R 3.6.0 Statistical Package (The R foundation for Statistical Computing, Vienna, Austria). A two-sided $P<0.05$ was considered to indicate statistical significance.

4. Results

4.1 Baseline characteristics

Altogether 577 patients with at least one available IPF measurement within 48 hours after randomization were included in this analysis. Baseline characteristics of patients, shown in **Table 2**, were similar in both treatment groups: patients of the prasugrel group tended to be marginally older (65.6 ± 12.1 years versus 63.7 ± 11.9 years, p=0.052) in comparison to the ticagrelor group and thrombocytes (median [IQR]) at admission were slightly higher in the prasugrel group as compared to the ticagrelor group (226 [189-267] $x10^3$/µl versus 215 [184-248] $x10^3$/µl, p=0.039).

Diagnostic angiography was performed in all except from one patient in the prasugrel group (**Table 3**). Treatment of ACS did not differ significantly between the two treatment groups and the majority of patients underwent PCI (92.4% in the prasugrel group and 94,5% in the ticagrelor group). One patient in the ticagrelor group received CABG and the remaining patients (7.6 % in the prasugrel group and 5.1 % in the ticagrelor group) were treated conservatively. There was also no difference between the two treatment groups regarding procedural characteristics and periprocedural antithrombotic therapy of patients undergoing PCI (**Table 4**).

After adjustment for all significant differences in baseline characteristics between the two Sysmex devices we did not find a significant correlation between IPF categories and Sysmex device (p adj=0.187).

Table 2: Baseline characteristics

Characteristic	Prasugrel (n=302)	Ticagrelor (n=275)	P value
Age – years	65.6 ± 12.1	63.7 ± 11.9	0.052
Women – no. (%)	71 (23.5)	54 (19.6)	0.304
Diabetes – no. (%)*	65 (21.6)	64 (23.3)	0.702
Insulin-treated – no. (%)	18 (6.0)	18 (6.6)	0.914
Current smoker – no. (%)	104 (34.7)	86 (31.4)	0.456
Arterial hypertension – no. (%)	212 (70.7)	200 (73.3)	0.551
Hypercholesterolemia – no. (%)	195 (64.8)	170 (62.3)	0.591
Prior myocardial infarction – no. (%)	57 (18.9)	49 (17.8)	0.812
Prior PCI – no. (%)	78 (25.8)	70 (25.5)	0.994
Prior CABG – no. (%)	18 (6.0)	11 (4.0)	0.376
Cardiogenic shock – no. (%)	17 (5.6)	13 (4.7)	0.764
Systolic blood pressure – mmHg	146 ± 26	145 ± 27	0.619
Diastolic blood pressure – mmHg	83 ± 15	83 ± 16	0.843
Heart rate – beats/min	77 ± 16	78 ± 19	0.634
Weight < 60 kg	16 (5.3)	11 (4.0)	0.589
Body mass index – kg/m²	27.5 ± 4.6	27.5 ± 4.3	0.898
Creatinine – µmol/L	93.9 ± 32.1	93.4 ± 24.1	0.840

GFR – ml/min/1,73 m²	73.7 ± 20.0	74.3 (19.2)	0.728
Sysmex XE5000, no. (%)	211 (69.9)	189 (68.7)	0.837
Diagnosis at admission			0.364
Unstable angina – no. (%)	33 (10.9)	21 (7.6)	
NSTEMI – no. (%)	130 (43.0)	118 (42.9)	
STEMI – no. (%)	139 (46.0)	136 (49.5)	
Coronary angiography – no. (%)	301 (99.7)	275 (100)	1.000
Treatment strategy – no. (%)			0.204
PCI	279 (92.4)	260 (94.5)	
CABG	0	1 (0.4)	
Conservative therapy	23 (7.6)	14 (5.1)	
Aspirin at admission – no. (%)*	109 (37.5)	94 (35.5)	0.691
Clopidogrel at admission – no. (%)†	15 (5.2)	10 (3.8)	0.562
Betablocker at admission – no. (%)‡	91 (31.3)	81 (30.6)	0.930
Statins at admission – no. (%)§	96 (33.0)	83 (31.3)	0.742

Legend to Table 2: Data are mean ± standard deviation or counts (%); CABG indicates coronary artery bypass grafting; GFR, glomerular filtration rate; NSTEMI, non-ST-elevation myocardial infarction; PCI, percutaneous coronary intervention; STEMI, ST-elevation myocardial infarction. *Aspirin at admission was not available in 2 patients (1 in the prasugrel group and 1 in the ticagrelor group). †Clopidogrel at admission was not available in 2 patients (1 in the prasugrel group and 1 in the ticagrelor group). ‡Betablocker at admission was not available in 2 patients (1 in the prasugrel group and 1 in the ticagrelor group). §Statins at admission was not available in 2 patients (1 in the prasugrel group and 1 in the ticagrelor group).

Table 3: Angiographic characteristics

Characteristic	Prasugrel (n=301)	Ticagrelor (n=275)	P value
Access site			0.859
Femoral – no. (%)	294 (97.7)	267 (97.1)	
Radial – no. (%)	7 (2.3)	8 (2.9)	
No of diseased coronary vessels			0.361
No obstructive CAD – no. (%)	13 (4.3)	11 (4.0)	
One vessel – no. (%)	65 (21.6)	77 (28.0)	
Two vessels – no. (%)	84 (27.9)	72 (26.2)	
Three vessels – no. (%)	139 (46.2)	115 (41.8)	
Left ventricular ejection fraction* – %	49.1 ± 11.0	48.2 (10.0)	0.289
ACS treatment			0.204
CABG	0	1 (0.4)	
conservative	23 (7.62)	14 (5.1)	
PCI	279 (92.4)	260 (94.5)	

Legend to Table 3: Data are mean ± standard deviation or counts (%), CAD indicates coronary artery disease; One patient in the prasugrel group did not undergo coronary angiography. * Left ventricular ejection fraction was not available in 4 patients (2 in the prasugrel group and 2 in the ticagrelor group).

Table 4: Periprocedural characteristics

Characteristic	Prasugrel (n=279)	Ticagrelor (n=260)	P value
More than 1 lesion treated	150 (53.8)	126 (48.5)	0.253
Target vessel			0.599
Left main coronary artery – no. (%)	8 (2.9)	10 (3.9)	
Left anterior descending artery – no. (%)	125 (44.8)	128 (49.2)	
Left circumflex coronary artery – no. (%)	49 (17.6)	44 (16.9)	
Right coronary artery – no. (%)	90 (32.3)	75 (28.8)	
Bypass graft – no. (%)	7 (2.5)	3 (1.2)	
Complex lesion (type B2/C) – no. (%)	231 (82.8)	211 (81.2)	0.701
TIMI flow grade before the intervention			0.866
0 – no. (%)	102 (36.6)	100 (38.5)	
1 – no. (%)	13(4.7)	15 (5.8)	
2 – no. (%)	42 (15.1)	35 (13.5)	
3 – no. (%)	122 (43.7)	110 (42.3)	
TIMI flow grade after the intervention			0.820
0 – no. (%)	3 (1.1)	4 (1.5)	
1 – no. (%)	0	0	

2 – no. (%)	11 (3.9)	12 (4.6)	
3 – no. (%)	265 (95.0)	244 (93.8)	
Type of intervention			
Drug-eluting stent – no. (%)	241 (86.4)	212 (81.5)	0.157
Bare-metal stent – no. (%)	0	0	-
Bioresorbable vascular scaffold – no. (%)	29 (10.4)	35 (13.5)	0.334
Drug-eluting balloon – no. (%)	2 (0.7)	5 (1.9)	0.271
Plain balloon angioplasty – no. (%)	13 (4.7)	14 (5.4)	0.851
Maximal stent diameter† – mm	3.2 ± 0.5	3.2 ± 0.5	0.762
Total stented length‡ – mm	30.0 ± 13.4	31.5 ± 14.1	0.196
Successful PCI – no. (%)	270 (96.8)	253 (97.3)	0.912
Periprocedural antithrombotic medication			
Aspirin – no. (%)	261 (93.5)	244 (93.8)	1.000
Unfractionated heparin – no. (%)	275 (98.6)	259 (99.6)	0.374
Low molecular weight heparin – no. (%)	1 (0.4)	4 (1.5)	0.202
Bivalirudin – no. (%)	1 (0.4)	1 (0.4)	1.000
GPIIb/IIIs inhibitor – no. (%)	9 (3.2)	7 (2.7)	0.912

Legend to Table 4: Data are mean ± standard deviation or counts (%), CAD indicates coronary artery disease; PCI, percutaneous coronary intervention; TIMI, Thrombolysis in Myocardial Infarction. † Maximal stent diameter was not available in 8 patients (2 in the prasugrel group and 6 in the ticagrelor group). ‡ total stented length was not available in 8 patients (2 in the prasugrel group and 6 in the ticagrelor group).

4.2 No association between baseline IPF and clinical outcomes

Baseline IPF (bIPF) values (median [IQR]) at hospital admission were available in 230 patients (76.2%) in the prasugrel group and in 214 patients (77.8%) in the ticagrelor group (p=0.709) and did not differ between the two groups (3.4 [2.5,5.2] % in the prasugrel group and 3.6 [2.5,5.2] % in the ticagrelor group, p=0.425). No association between bIPF and the primary endpoint (P= 0.739) or bleeding (P= 0.247) was observed.

4.3 Significant association between IPF within 48 hours and outcomes

IPF values (median [IQR]) within 48 hours after randomization were assessed in all patients at a median time interval of 16.3 [11.5,20.8] hours and did not differ between the two study groups: IPF 3.6 [2.5,5.2] % in the prasugrel group and 3.6 [2.5,5.4] % in the ticagrelor group (p=0.882). IPF values were significantly higher in the IPF^{high} group compared to the IPF^{low} group (5.3 [4.3,6.7] % versus 2.5 [2.0,3.0] %, p<0.001).

The primary endpoint – death from any cause, myocardial infarction or stroke at 1 year after randomization – occurred in a total of 58 of 577 patients (10.1%): 26 patients (8.8%) in the prasugrel group and 32 patients (11.8%) in the ticagrelor group (p=0.23). The incidence of the primary endpoint was significantly higher in patients with IPF^{high}

values (IPF>3.6%) compared to patients with IPF^{low} values (IPF≤3.6%): 13.0% (37/284) vs 7.1% (21/293) with a HR [95%CI] of 1.897 [1.11,3.24], p=0.019 (**Figure 15**).

In the multivariate model after adjustment for potential cofactors including study group, age, gender, BMI, diabetes, GFR, admission diagnosis, treatment of ACS, IPF^{high} (HR [95%CI] = 1.744 [1.02-3.00], p= 0.044) and GFR (HR [95%CI] 9.807 [0.97-0.99], p= 0.014) remained independent predictors of the primary endpoint. Of importance, the association between IPF^{high} and the primary outcome was not dependent on the randomly assigned study drug (p for interaction 0.159). Additionally, no interaction between Sysmex device and outcomes was observed (p for interaction=0.463).

Major bleeding (BARC type 3-5) occurred in a total of 43 patients (7.5%): 23 patients (7.7%) in the prasugrel group and 20 patients (7.4%) in the ticagrelor group (p=0.875). No significant association between IPF and major bleeding (BARC 3-5) was observed (HR 1.633 [0.89,3.01], p=0.116).

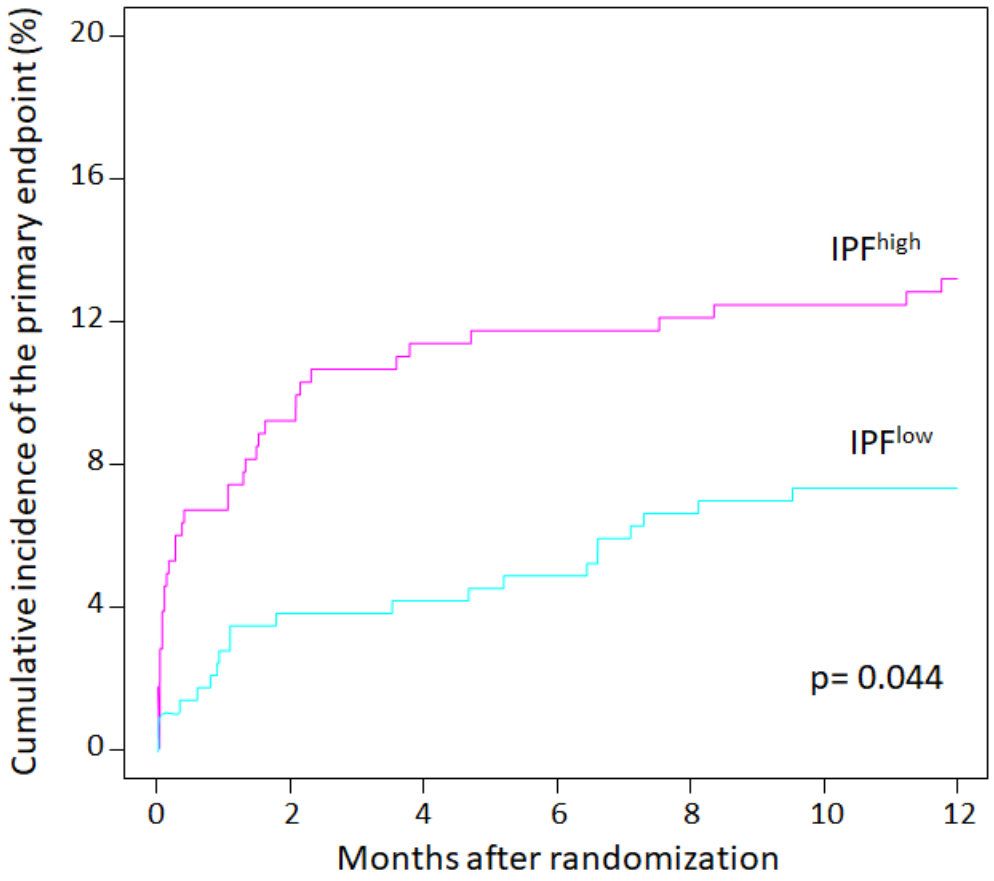

Figure 15: Cumulative incidence of the primary endpoint at 1 year. The Kaplan-Meier curves show the cumulative incidence of the primary endpoint – a composite of death, myocardial infarction, or stroke at 1 year – according to IPF^{high} (IPF>median) and IPF^{low} (IPF≤median) values. Adapted from [154].

4.4 Association between other immature platelet parameters and clinical outcomes

H-IPF values (median [IQR]) within 48 hours after randomization were available in 222 patients (73.5%) in the prasugrel group and in 210 patients (76.4%) in the ticagrelor group (p=0.488) and did not differ between the two study groups: 1.0 [0.7;1.6] % in the prasugrel group and 1.1 [0.7,1.7] % in the ticagrelor group (p=0.588). In the univariate model, there was a significant association between H-IPF^{hig} and the primary endpoint (HR 2.04 IQR [1.064-9.902] p= 0.032) independent from the randomly assigned study drug (p for interaction 0.15). However, this association was no longer significant after multivariate testing (HR 1.81 IQR [0.938,3.499] p=0.077). There was also a trend towards a higher incidence of major bleeding in the H-IPF^{high} group in the univariate model (HR 1.99 [0.98-4.03], p=0.050) which was no longer significant after multivariate testing (HR 1.82 [0.89-3.73], p=0.099).

The IPC within 48 hours after randomization was available in 212 patients (70.2%) in the prasugrel group and in 199 patients (72.4 %) in the ticagrelor group (p=0.57) and did not differ between the two study groups: 7.50 [5.40,9.95] x10^{3}/µl in the prasugrel group and 7.40 [5.4.10.0] x 10^{3}/µl in the ticagrelor group (p=0.632). No significant association between the IPC and the primary endpoint (HR 1.799 [0.93-3.48], p=0.08) or major bleeding (HR 1.57 [0.78-3.16], p=0.200) was observed.

MPV, which is known to correlate with RPs level, was available within 48 hours in 202 patients (66,9%) in the prasugrel group and in 182 patients (66,2%) in the ticagrelor group (p=0.858) and did not differ between the two treatment groups: 10.4 [9.8-10.8] fl

in the prasugrel group and 10.5 [9.9-11.2] in the ticagrelor group (p=0.139). No significant association between MPV and the primary endpoint (HR=1.13 [0.77-1.64], p=0.544) was observed.

4.5 Correlation between IPF and ADP-induced platelet aggregation

Correlation analyses between IPF values within 48 hours after randomization and simultaneously measured values of ADP-induced platelet aggregation were performed in a subgroup of 324 patients (169 patients (56.0%) in the prasugrel group and 155 patients (56.4%) in the ticagrelor group, p= 0.923) who underwent PCI, received the study drug and who had not received periprocedural GP IIb/IIIa receptor inhibitors or prior clopidogrel treatment (**Figure 16**).

In prasugrel treated patients a weak but highly significant correlation between IPF and ADP-induced platelet aggregation was observed (Spearman coefficient r=0.22, p=0.005). In patients who received ticagrelor for $P2Y_{12}$ receptor inhibition no correlation between IPF and ADP-induced platelet aggregation was observed (Spearman coefficient r=0.09, p=0.257). Even if there was a tendency, no significant association between the ADP-induced platelet aggregation and the primary end point (HR, 1.34 [0.98–1.85]; p=0.071) was observed.

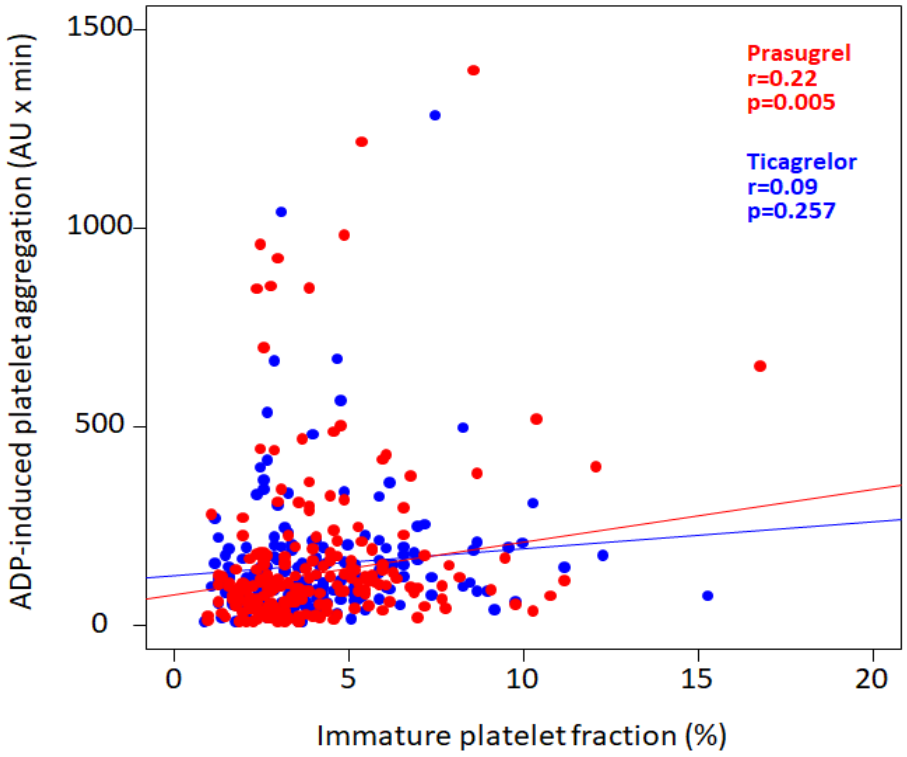

Figure 16: Scatterplot of immature platelet fraction and adenosine diphosphate-induced platelet aggregation (A) in prasugrel-treated patients (red dots) and in ticagrelor-treated patients (blue dots). Spearman's correlation coefficients (r) and corresponding p-values are provided. ADP, adenosine diphosphate; AU, aggregation units. Adapted from [154].

5. Discussion

The main finding of my thesis was that in patients with ACS elevated IPF (>3.6%) was independently associated with the combined primary study endpoint, consisting of death, myocardial infarction or stroke at 12 months, irrespectively of the application of prasugrel or ticagrelor (p for interaction 0.159).

With the discovery of their prothrombotic phenotype, RPs drew attention to themselves in the cardiovascular field. Several studies within the last decade indicated that RPs are of prognostic relevance regarding major adverse cardiovascular events (MACE) [11, 120, 128, 135]. However, the vast majority of studies in this field were conducted in patients on therapy with clopidogrel and aspirin [125, 126, 135, 136] and data with potent $P2Y_{12}$ antagonists were missing. Additionally, the larger part of these studies was performed in chronic coronary syndrome patients, not randomized or with limited patient numbers. The ISAR REACT 5 trial reticulated platelet substudy was the first study that assessed IPF as biomarkers in ACS patients who were randomly assigned to receive either prasugrel or ticagrelor for $P2Y_{12}$ receptor inhibition.

The median IPF value of 3.6% that allowed us to sufficiently discriminate between patients with or without ischemic events corresponds to the IPF value that has also been found by ROC curve analysis in another earlier study in CAD patients on therapy with aspirin and clopidogrel [98]. Interestingly, we did not observe a correlation between baseline IPF (bIPF) values measured at hospital admission and the primary ischemic endpoint. Yet this finding should be handled carefully since these values were only available from 230 patients (76.2%) in the prasugrel group and from 214 patients

(77.8%) in the ticagrelor group. However, another study by Berny-Lang et al. measured IPF immediately after hospital admission in the emergency department in patients with suspected ACS, and also did not find any prognostic relevance of IPF measured at such an early time point [100]. All other trials that assessed RPs later, a few days after the acute event, confirmed our primary findings and clearly showed an association between these cells and adverse ischemic events in CAD patients [98, 136, 155, 156]. These results suggest that the optimal time point for IPF measurement to predict ischemic events in CAD patients seems to emerge in the course of an acute event rather than being present already at baseline (e.g. at hospital admission). Still, the optimal time point of IPF measurement needs further investigation.

Although there are slight differences in regard of operating principles and reference intervals for IPF between the two applied Sysmex systems (Sysmex XE-5000 and Sysmex XN), we found no association between Sysmex device and IPF category as well as no interaction between Sysmex device and outcomes. Nonetheless, taken the differences between the two devices into account, attention should be paid to standardized measurement of IPF especially regarding the design of future studies in this area of research.

Our study indicates that the predictive value of IPF is independent from the assigned $P2Y_{12}$ inhibitor. Consistent with this observation, RPs have an increased reactivity on account of their significantly elevated activation marker expression [156], emerging to be a risk factor for atherothrombotic events also in non-cardiac patients without antiplatelet drug therapy including patients with sepsis, stroke or after non-cardiac surgery [101, 120, 121, 157]. All these results support the hypothesis that a high

number of RPs in patients is not only a parameter of high platelet reactivity on $P2Y_{12}$ receptor inhibition, but much more a consequence of atherothrombosis predicting and probably even evoking future adverse ischemic events.

It is currently not clear to what extent the association of increased IPF and ACS is a cause of clinical instability or consequence of the condition [73]. Assuming a confounded association, therapeutical interventions focusing on RPs should not influence the risk of MACE while, in case of a causal association, one could hypothesize that specific inhibition of RPs could decrease the risk of MACE [158]. This theory of a causal association is supported by the findings that RPs are enzymatically and metabolically more active, leading to an increased pro-thrombotic phenotype in comparison to more mature platelets. In addition, an increased level of RPs in blood along with an augmented platelet turnover is accompanied with a significant proportion of insufficiently inhibited platelets and therefore is likely to negatively influence the response to antiplatelet drugs [73, 125, 127, 133, 144, 159]. Consequently, these cells could not only be helpful in identifying patients at risk of increased adverse events [128], but also offer the potential for guidance of a more individualized platelet-directed treatment.

Our trial did not present a connection between other immature platelet parameters like the highly immature platelet fraction (H-IPF) or immature platelet count (IPC) and the combined ischemic endpoint. However, the evaluation of these parameters was beyond the aim of this prespecified substudy and therefore they were available only in about three quarters of patients in both study groups. H-IPF represents the largest and the highest fluorescent platelets with the greatest amount of RNA and maybe even the

most thrombogenic phenotype. Preliminary findings by Cesari et al. observed a superior association between H-IPF and cardiovascular death in ACS patients than IPF [98]. While in our study univariate testing suggested a positive correlation between H-IPF and the primary endpoint, this association was no longer significant in the multivariate model. Regarding IPC, another smaller study has found – in contrast to our findings – a positive correlation between IPC and MACE including all-cause mortality, myocardial infarction, unplanned revascularization or hospitalization for angina [156]. Reasons for these discrepant findings may include differences regarding the study design and the composition of the primary endpoint. Importantly, IPC is not a directly measured parameter but a calculated product of IPF and the total platelet count. Therefore, it does not solely correspond to the immature platelet pool in peripheral blood but also represents the influence of the total platelet count on adverse cardiovascular events. Since higher platelet count per se is positively correlated with ischemic complications [160, 161], the interpretation of this parameter regarding the predictive value of solely RPs in peripheral blood should be handled with caution and remains to be elucidated in further investigations.

We also analysed whether there was a linkage between immature platelet parameters and major bleeding (BARC type 3 – 5). Although there was a positive correlation between H-IPF and major bleeding in the univariate model, we did not observe a significant association between IPF, H-IPF or IPC and bleeding. While the latter observation is in line with prior reports by Perl et al. [128] and McDonnell et al. [162] who found even an inverse correlation, it is in contrast with the results of Freynhofer et al. [135] and Frelinger et al. [163], who encountered an association between RPs and the risk of bleeding. Pathophysiologically it is thinkable that that RPs are able to

indicate acute bleeding events since acute blood loss leads to an increased platelet production, thus increasing the amount of RPs in peripheral blood. So far there are only few studies concerning the function of RPs and bleeding events, hence being a matter of ongoing controversy. Reasons for these discrepant findings might include different clinical settings, the presence or absence of antiplatelet drugs, the time point of assessment of RPs in view of bleeding complications as well as the variety of different bleeding definitions. Anyhow, further investigation is warranted to clarify the role of these cells in bleeding events.

The mean platelet volume (MPV) is another prognostic biomarker for MACE in ACS patients [11, 164-166], which seems to be connected to platelet reactivity and atherothrombotic incidents [98]. It reports average platelet size and was especially used before the introduction of IPF, but assesses only indirectly the amount of RPs [167, 168]. In our study, MPV was available in 60-70% of patients and we did not observe a significant association between MPV measured within 48 hours after randomization and the primary ischemic study endpoint. This finding is in line with other studies constituting IPF to be a more specific and sensitive biomarker in comparison to MPV.

6. Study limitations

The limitations of this study can be summarized as follows: First, the analysis carries the known limitations of sub-studies in general, especially that the assessments were restricted to only two participating centers in Germany. Second, simultaneous measurements of ADP-induced platelet aggregation and IPF were only available in a bit more than half of the patients, not allowing to compare the prognostic relevance of both parameters. Third, measurement of other immature platelet parameters including H-IPF, MPV and IPC was not part of this prespecified substudy and therefore not available in all patients and further investigations are needed to evaluate their prognostic role in CAD patients. More limitations could be seen in the fact that the trial was conducted in an open-label manner as well as in the circumstance that the follow-up was mainly carried out via telephone instead of face-to-face appointments [68].

7. Conclusion

The ISAR REACT 5 reticulated platelet substudy was the first and largest clinical study measuring the role of RPs to predict adverse ischemic events after acute myocardial infarction in patients on therapy with potent $P2Y_{12}$ receptor inhibitors. With our trial we demonstrated that elevated levels of RPs, which were assessed by an automated haematology analyser and expressed as IPF, were independently associated with the primary ischemic study endpoint consisting of death, myocardial infarction or stroke regardless of the administration of prasugrel or ticagrelor. Our findings strengthen IPF to be a useful and promising biomarker for the prediction of adverse cardiovascular events in ACS patients treated according to current guidelines. Therefore, measurement of IPF in the acute phase of myocardial infarction may improve risk stratification in these patients. Assuming a causal association between RPs and adverse cardiovascular events based on their pro-thrombotic phenotype, knowledge about the amount and the proteome of circulatory pro-thrombotic RPs may also offer the opportunity of a personalized and an individually tailored antithrombotic therapy.

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9. Internet registry

[A] https://www.who.int/news-room/fact-sheets/detail/the-top-10-causes-of-death, 02.08.2021

[B] Sysmex XE-5000 work instructions: https://www.sysmex.de/fileadmin/media/f101/Muster-Standardarbeitsanweisung/SOP_XE-5000_11_2010.pdf, 02.08.2021

10. Acknowledgements

This project would never have been possible without the support, collaboration and guidance of various people at the Klinikum rechts der Isar and the Deutsches Herzzentrum München.

Firstly, I would like to pay my special gratitude to Dr. Isabell Bernlochner, my thesis supervisor, for granting me this interesting topic and providing continuous guidance and feedback throughout this study. Thank you for all the advice, support and patience in leading me through this project.

My sincere thanks go also to Vincent Karschin, my partner PhD student, Rosanna Gosetti and Dr. Dario Bongiovanni for our good collaboration. Without your help and us working together this project would not have been the same.

Besides I would like to thank the rest of my thesis committee, including my mentor, Prof. Dr. Wolfgang Siess.

I would also like to extend my deepest gratitude to the whole team of the ISAR REACT study, who provided me an opportunity to join their team, as well as all patients participating in this trial for enabling this project. Without you this study would never have been possible.

Last but not least I would like to thank my family – to whom I dedicate this work – for accepting nothing less than excellence from me: my parents, Roman and Rita Schreiner as well as my grandparents, Marianne and Karl Schreiner. I would like to recognize the invaluable assistance, patience and unconditional support that you all provided during my study.