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Transcriptomics and Proteomics of Platelet Heterogeneity: From RNA-Sequencing to Mass Cytometry

Melissa Klug

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Abstract

Platelets play a central role in maintaining the physiological hemostasis of human blood. Furthermore, they are significantly involved in the development of arterial thromboembolic pathophysiological events. Among the heterogeneous human platelet population, immature "reticulated" platelets (RPs) are of particular importance. In contrast to mature platelets (MPs), they have an increased thrombogenicity, negatively influence the effectiveness of anti-platelet drugs and are associated with an increased mortality. Despite progressing knowledge with regard to the biology of this platelet subpopulation, no specific biomarkers exist so far and an established characterization of the functionality of RPs is pending.

In the first study, I developed a reproducible staining and sorting protocol that enables RNA extraction of RPs for the first time. Staining of platelets with the fluorescent RNA dye Thiazole Orange allowed the sorting after RNA content (high RNA content=RPs, low RNA content = MPs). Stimulation of sorted platelets with adenosine diphosphate (ADP) demonstrated a hyper-reactive phenotype of RPs, characterized by an increased expression of the platelet activation markers P-selectin and LAMP-3. In the second study, I focused on the receptor expression diversity of platelets and designed a mass cytometry by time of flight (CyTOF) pipeline for platelet investigation from platelet-rich-plasma (PRP). The method allows the freezing of stained samples and the acquisition of a high cell number, which is essential for bioinformatic analysis. CyTOF analysis of stimulated PRP with thrombin receptor activating peptide (TRAP) and collagen related peptide (CRP-XL) showed differences in platelet reactivity. During the third study, I investigated platelet reactivity in COVID-19 patients compared to healthy donors. By using the previously developed CyTOF protocol I detected a disease specific hyperreactive platelet phenotype at rest and a dysregulated activation pattern of stimulated COVID-19 platelets.

Kurzfassung

Blutplättchen spielen eine zentrale Rolle bei der Erhaltung der physiologischen Hämostase des Blutes. Ferner sind sie maßgeblich an der Entstehung arterieller thromboembolischer pathophysiologischer Ereignisse beteiligt. Unter der heterogenen menschlichen Blutplättchenpopulation sind unreife „retikulierte“ Plättchen (RPs) besonders wichtig. Im Gegensatz zu reifen Thrombozyten (MPs) haben sie eine erhöhte Thrombogenität, beeinflussen die Wirksamkeit von Thrombozytenaggregationshemmern negativ und sind mit einer erhöhten Sterblichkeit verbunden. Trotz steigender Erkenntnisse zur Biologie von RPs existieren bisher keine spezifischen Biomarker und eine funktionelle Charakterisierung ist ausstehend.
In der ersten Studie habe ich ein reproduzierbares Zellsortierungsprotokoll entwickelt, das erstmals die RNA-Extraktion von RPs ermöglichte. Die Färbung der Blutplättchen mit dem fluoreszierenden RNA-Farbstoff Thiazol Orange ermöglichte die Sortierung nach RNA (hoher RNA-Gehalt = RPs, niedriger RNA-Gehalt = MPs). Die Stimulation sortierter Plättchen mit Adenosindiphosphat (ADP) zeigte hyperreaktive RPs, gekennzeichnet durch eine erhöhte Expression der Aktivierungsmarker P-Selektin und LAMP-3. In der zweiten Studie untersuchte ich die Diversität der Rezeptorexpression von Blutplättchen mittels einer Massenzytometrie (CyTOF)-Pipeline für die Analyse von Plättchen aus plättchenreichem Plasma (PRP). Die Methode ermöglichte das Einfrieren gefärbter Proben und die Messung einer hohen Zellzahl, was für die bioinformatische Analyse wesentlich ist. Die CyTOF-Analyse von stimuliertem PRP mit Thrombinrezeptor-aktivierendem und kollagenverwandtem Peptid zeigte signifikante Reaktivitätsunterschiede. Während der dritten Studie untersuchte ich die Plättchenreaktivität bei COVID-19-Patienten im Vergleich zu gesunden Spendern. Mittels der zuvor entwickelten CyTOF-Pipeline entdeckte ich einen krankheitsspezifischen hyperreaktiven Phänotyp im Ruhezustand und eine fehlregulierte Aktivierung von stimulierten COVID-19-Thrombozyten.

Contents

1 Introduction

1.1 Human Platelets - Generation and Biological Function

Even before platelets were known as such, several scientists over the past 100 years took interest in the small blood cells and investigated their part in the composition of blood [1] or their role during thrombosis [2]. In 1910, James Wright was the first to refer to them as they are known today while investigating at first attempts the platelet origin and production [3]. It took until the 21st century to fully understand platelet production, the so-called thrombopoiesis [4], and the underlying principle of megakaryopoiesis, the maturation and differentiation of megakaryocytes [5].

The process starts in the bone marrow with pluripotent hematopoietic stem cells (HSC) and their ability to differentiate into all lineages of circulating blood cells [6, 7]. Then, the long journey begins with the immature megakaryocytes production that underlies several differentiation steps: From HSC into multipotent progenitor cells, followed by differentiation into committed megakaryocyte-progenitor cells [8, 9]. The latter ones become immature megakaryocytes when triggered by key regulator thrombopoietin [10], transcription and growth factors as well as hematopoietic cytokines [11, 12]. Thrombopoietin also plays a key role in the process afterwards, called endomitosis [13]. It is characterized by persistent DNA replication inside the immature megakaryocytes without resulting in cell proliferation, leading to cells containing up to 64 times the normal amount of DNA [14]. Alongside the ploidy, the size of megakaryocytes also increases during endomitosis [15]. Importantly, these characteristics allow the cells to gather organelles, proteins, granules and several kinds of ribonucleic acid (RNA) [16]. Later on, these compounds are passed on to platelets after the maturated megakaryocytes have docked to the blood vessel membrane and formed

pseudopodia [9, 17]. After release into the circulating blood flow, the resulting pro-platelets further release individual platelets, a process that is accelerated by shear stress [18].

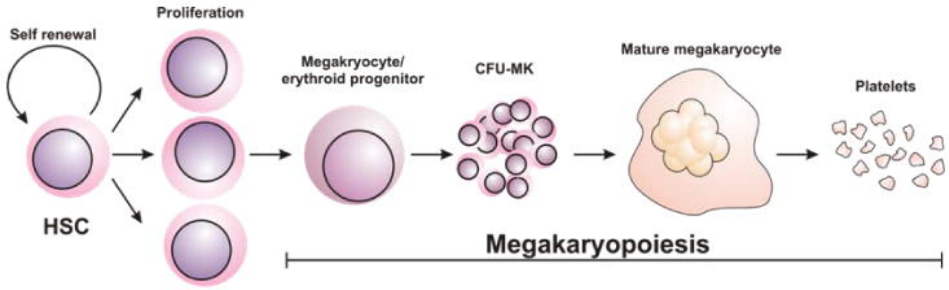

Figure 1.1: Overview of megakaryopoiesis. Megakaryocytes are derived from the hematopoietic stem cell and proliferate and differentiate under the influence of thrombopoietin [19].

All these newly produced platelets have only a short lifespan of up to 10 days [20], partly predetermined by the limited resource of anti-apoptotic protein Bcl-xL [21]. This theory is supported by the use of Bcl-xL knock-out mice which show decreased numbers of circulating platelets [22]. Despite this inability of de novo production due to lacking a nucleus, platelets are equipped with several mRNAs and components which enable them to perform RNA processing and translation on their own [23, 24, 25]. Some of these processes are signal-dependent and regulated by platelet activation [25, 26, 27, 28]. While the number of platelets in healthy human blood should be at 150-400 billion platelets per liter, 100 billion platelets need to be produced daily considering their short lifespan [29]. Chronic and acute diseases [30, 31, 32] as well as myeloproliferate neoplasms, inflammation and stress [33, 34, 35] can lead to an upregulation of platelet production and a subsequently increased platelet count in blood. This condition with more than 450 billion platelets per liter of blood is called thrombocythemia. In contrast to that, a disturbance in the balance of platelet production and clearance can lead to the opposite, a condition with less than 50 billion platelets per liter of blood [36]. This disease condition describing an abnormally low platelet count is referred to as thrombocytopenia and underlies either an increased platelet elimination or a decrease in platelet production [37].

Most prominently, platelets are known to be key regulators in hemostasis. In particular,

this refers to maintaining the functional integrity of blood vessels [9]. Generally, platelet activation is suppressed by endothelial cells with the activation of antithrombin III or the use of adenosine diphosphatase (ADPase) to break down Adenosine 5'-diphosphate (ADP) signal [38]. Yet, the situation changes upon blood vessel damage, when endothelial cells and tissue factor, known as coagulation factor III or tissue thromboplastin, actively initiate platelet activation, eventually leading to the formation of a hemostatic plug [39]. Exposed collagen initiates further platelet activation as they bind to the fibers via von Willebrand factor (VWF) [40]. The resulting release of α-granule contents like surface receptors and soluble platelet activators triggers the recruitment of more platelets to the site of injury [41]. Additionally, platelets bind to fibrin and fibronectin, which stabilizes the increasing thrombus [42, 43]. In this way, platelets ensure the sealing of the injured vessel after internal or external damage as well as the continuation of a normal blood flow [44].

Apart from their hemostatic role, platelets are also involved in immune response. It has been shown that they express several chemokine receptors, which allow platelets to detect allergens or viruses [45]. Moreover, platelets actively interact with leukocytes in peripheral blood and platelet-neutrophil aggregates have been described and correlated with pro-thrombotic phenotypes [46]. On top of that, platelets directly participate in neutralizing not only viruses but also bacteria and other pathogens by digestion and subsequent granule release [47, 44, 48]. In releasing metabolically active mitochondria that initiate inflammatory responses, platelets underline their additional role as immune cells [49]. In contrast to that, platelets can also worsen the outcome of several diseases. During tumor progression, they have been shown to interact with tumor cells directly. For example, platelets are shading tumor cells from immune cells, allowing them to bypass an immune response [50, 51].

1.2 Platelet Structure

Quiescent platelets in healthy humans are elliptically shaped [52]. With a diameter of only 1.5 - 3 µm they present the smallest particulate component of blood [53, 54]. Upon activation, platelets do not only undergo a shape change but also show an increase in surface. This

results in an overall higher mean platelet volume [55, 56]. In contrast to their roundly shaped non-activated form, activated platelets exhibit protrusions of plasma membrane, so called pseudopodia [57]. Despite their differences, there are several structural characteristics that all platelets have in common.

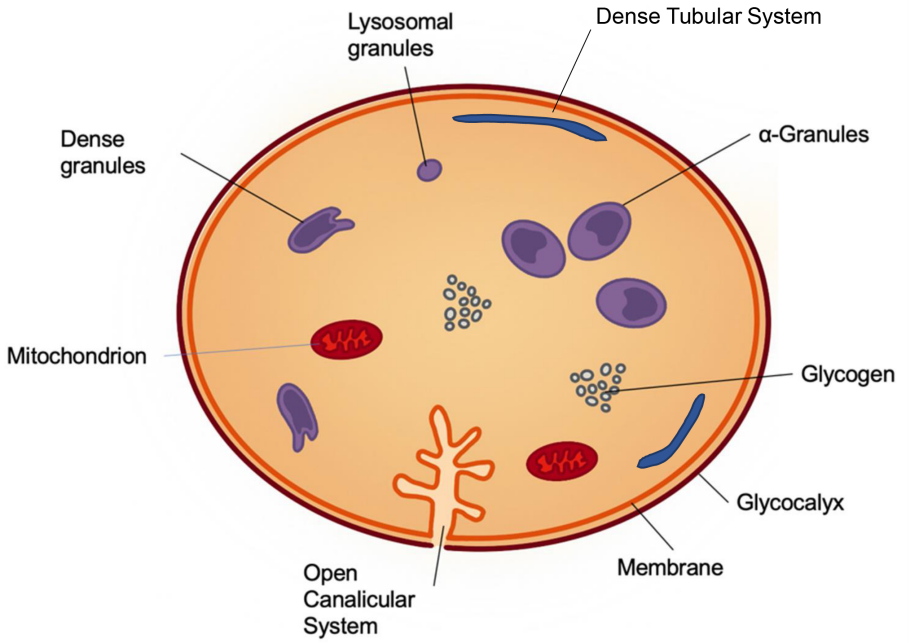

Figure 1.2: Schematic drawing of a quiescent platelet. This scheme shows a simplified unactivated platelet and its common compounds (Figure altered with permission from Marina Biasi).

A layer made of several glycoproteins (GPs), the glycocalyx, covers the platelet plasma membrane [58]. As familiar from other cell types, the platelet plasma membrane consists of the typical polarized phospholipid bilayer sheet [59]. In platelets, the asymmetric distribution of phospholipids is essential for platelet function [60]. This structure is changed upon platelet activation: it triggers the release of several second messengers, arachidonic acid and the assembly of platelet factor Va by the plasma membrane [61].

Microtubuli as well as the microfilament network account for the maintenance of platelet structure and stability [62]. As a side effect, these structures and their resulting acting forces are the main determinants of platelet size at the stage of fresh platelet production [63].

Besides the role of platelet mitochondria during inflammation [49], they are mainly responsible for providing energy at rest and maintaining the energy level despite increased need during platelet activation [64, 65]. Next to mitochondria, endoplasmic reticulum, golgi complexes [66], glycogen and glycosomes [67], the platelet cytoplasm contains three different forms of granula: α-granules, dense granules (δ-granules) and lysosomes [68]. These platelet characteristic storage loci incorporate proteins and other substances important for platelet function. Dense granules mainly store ADP, Adenosine-5'-Triphosphate (ATP), Ca^{2+}, serotonin and pyrophosphate [69, 70], substances known to promote aggregation [71, 72]. Also the most abundant storage units, α-granules, contain several adhesive proteins as well as growth and coagulation factors which influence platelet aggregation and adhesion [73]. Lysosomal granules on the other hand, are similar to lysosomes of nucleated cells and mainly degrade proteins and extracellular components [74]. For this purpose, they contain hydrolases and other components necessary for protein degradation [75].

Together with the dense tubular system, the open canalicular system builds up the most important part of platelet membrane systems. The latter one is connected to the plasma membrane via its twisted canals and represents an enlargement of the plasma membrane system [76, 77]. It is accessible from the extracellular space through pores and is able to transport several substances and either absorb or release them [78, 79]. In contrast to that, the dense tubular system is derived from the endoplasmic reticulum that the platelets have received from megakaryocytes during thrombopoiesis [80]. It mainly functions as Ca^{2+} storage [81]. Despite their different roles within the platelet, both systems join forces and interact in terms of Ca^{2+} signaling [82]. During activation, several changes in the described systems and pathways occur. These alterations and shifts in platelet structure as well as activation pathways and markers are addressed in the next chapter.

1.3 Platelet Activation

Platelet activation induces important biomolecular and functional changes in platelets within a few seconds. Easiest to observe is the activation-triggered change of the elliptically or

disk-like platelet to a spherical structure [83, 84]. Mostly driven by the polymerization of actin filaments [85], the process of spreading is followed by the formation of filopodia, through which platelets can connect. The more the platelet stretches out, the more its contents like granules and other organelles are moved to the center [86]. Upon fusion with the open canalicular system, they are eventually excreted [87]. Next to an increased amount of free Ca^{2+} in the cytoplasm, the cytoskeleton is also modified by other signaling pathways [88]. With the phosphoinositide pathway activation leading to changes in the plasma membrane [89], direct control of the actin cytoskeleton is possible [90]. In addition, Rho GTPases also influence and control the cytoskeleton as well as several other physiological processes within the platelet [91]. Their activation is mainly triggered through downstream signaling of platelet receptors [92]. Changes in the microtubule skeleton resulting in the formation of a central microtubule coil, are additional factors of activation-related change of platelet shape [93, 94].

1.3.1 Activation Pathways and Cellular Interaction

Behind the activation and shape change of platelets lay a series of reactions, building up on one another [95]. Several receptors on resting platelets and their downstream activation cascades are triggered by contact with surface or soluble platelet stimuli [96, 97, 98]. ADP, thrombin, collagen or arachidonic acid metabolite thromboxane A2 (TXA2) are such stimuli, which upon release by activated platelets trigger activation of further platelets. The initiated downstream G-protein coupled receptor (GPCR) signaling processes are described below.

ADP, one of the most prominent stimuli, binds to GPCRs $P2Y_1$ and $P2Y_{12}$ after its release by platelet dense granules or discharge from injured endothelial tissue [99, 100, 101]. Binding to the $P2Y_1$ receptors leads to subsequent phospholipase C (PLC) signaling activation [102], while stimulation of the $P2Y_{12}$ receptor results in the initiation of the protein kinase C pathway [103]. Both pathways eventually lead to an increase of intracellular Ca^{2+} [104, 105]. Additionally, $P2Y_{12}$ receptor stimulation induces the dephosphorylation of vasodilator-stimulated phosphoprotein (VASP), a regulator protein of the actin cytoskeleton. As inhibition of the $P2Y_{12}$ receptor leads to phosphorylation of VASP in return, it is possible to measure $P2Y_{12}$ receptor- mediated platelet activation with the (de)phosphorylation levels of VASP

[106]. Moreover, the binding of ATP to the P2X1 receptor induces additional Ca^{2+} influx [107, 108]. Further ADP release is enabled by the following conformational change and degranulation processes [109, 110]. This results in signal amplification which initiates further platelet activation [101].

Besides ADP-mediated platelet activation, also thrombin-mediated activation is well described in literature [111]. Known to be a strong platelet agonist, thrombin binds to protease-activated receptors (PARs) PAR1 and PAR4 on the membrane of human platelets [112]. The subsequent activation of downstream signaling events leads to the recruitment and activation of more platelets [113] and the activation of GPIIbIIIa receptors [114]. Via binding of fibrinogen, GPIIbIIIa receptors enable the formation of stable platelet aggegrates [115]. Furthermore, thrombin transforms fibrinogen into fibrin, which stabilizes these aggregates [116].

Collagen has also been subject to several studies investigating its role in platelet interaction and activation [117, 118, 119]. Binding to collagen receptor GPVI leads to degranulation of α- and dense granules accompanied by strong platelet activation [120, 121]. While collagen also binds to the receptor complex GPIb-IX-V via VWF, it can additionally bind to the GPIa/IIa (CD49/CD29) receptor directly [122]. Therefore, endogenous collagen is considered a strong platelet activator, equal to thrombin [95, 123].

Another soluble agonist is TXA2, which acts as an enhancing factor for other stimuli [124]. Through binding to the GPCR thromboxane receptor [125] it induces a positive feedback reaction [126]. As downstream signaling cascades, PLC and Rho signaling pathways are initiated via coupled G-proteins [127, 114], leading to platelet shape change and aggregation [98]. Activated platelets then synthesize more TXA2, the primary product of cyclooxygenase-1 (COX-1) [128, 129].

In addition to the most prominent platelet agonists, also other substances like serotonin [130], adrenaline [131], prostaglandin E2 [132] $CaCl_2$ [133] or epinephrine [134] have been described to be soluble platelet activators. How platelet activation can be observed on the platelet surface, is subject of the next subsection.

1.3.2 Platelet Activation Biomarkers

During laboratory experiments, several of the above-mentioned receptors are used to measure platelet activation. Most familiar in flow cytometry experiments is the use of the adhesion protein P-Selectin (CD62P) as a marker for platelet activation [135, 136]. CD62P interacts not only with other platelets but also with leukocytes and neutrophils through binding to P-Selectin glycoprotein ligand-1 (PSGL-1), expressed on the cell surfaces [137, 138]. Before activation and degranulation lead to the translocation of CD62P to the platelet surface, it is stored inside the α-granules [139, 140]. Also, the glycoprotein CD63 (LAMP-3) is only expressed on the surface upon activation and previously located on the membrane of lysosomal granules [141, 142].

Other activation markers used in experimental settings during my doctoral project include CD107 (LAMP-1), CD154 (CD40-ligand) and IgM PAC1, the latter one recognizing the activated GPIIbIIIa receptor. After conformational change of agonist-stimulated platelets, PAC1 binds to the now exposed fibrinogen receptor of integrin αIIb3 [143]. Like P-Selectin, CD154, also called CD40-ligand (CD40L), is located in the platelet α-granules, exposed to the surface upon degranulation [144]. Besides its role during hemostasis and thrombus stabilization [145], CD40L also triggers a direct inflammatory response [146]. In a similar pattern, lysosomal-associated-membrane-protein 1 (LAMP-1) is translocated from the lysosomal membrane to the platelet surface upon activation [147].

Though constitutively expressed on the platelet surface, the following receptors show altered functionality patterns during platelet activation. Some of the platelet glycoproteins have the ability to sense injured blood vessels via binding free VWF or collagen [148]. The activation of collagen receptor GPVI and its direct binding to collagen triggers the platelet integrins to switch to a high affinity state [122]. Additionally, GPVI-mediated downstream signaling initiates the release of granule content [149]. The GPIb-IX-V receptor complex, consisting of VWF receptor units CD42a-d (GPIX, GPIbα, GPIbβ, GPV) connects to stationary VWF on collagen [150]. Also, the GPIIbIIIa receptor reaches an activated form, recognized by PAC1. Its activation is mainly driven by the platelet agonist amplification processes described before [151, 126, 114] and activation via Ca^{2+} binding [152]. In its activated and high affinity state,

GPIIbIIIa is able to bind fibrinogen and therefore contributes to platelet-platelet-aggregation [153].

1.4 Platelet Heterogeneity - Reticulated Platelets

Transmembrane receptors as well as other platelet components are not equally distributed between platelets. While platelets are equipped with megakaryocyte-derived mRNA and possess the ability to process and translate this RNA [25], the composition of their RNA pool differs [154]. Reasons behind this heterogeneous content within the platelet population might be a discriminative transfer of mRNA during thrombopoiesis [27] as well as the platelets' capability of performing RNA exchange with other cells [155, 156]. In addition, particular RNA decay over time might influence or emphasize transcriptome- and subsequently also proteome heterogeneity [23]. The latter one is characterized by variations in surface receptor expression among the platelet population at health [157] and during several disease conditions [158, 159, 160]. Furthermore, platelet diversity also appears in terms of size [161] and granule density [162, 163]. While a laboratory-based platelet subgroup distinction relying on the basis of size differences has been prominent in the past [161], recent technologies allow for platelet discrimination using other parameters.

Currently, one platelet subgroup differing from the other platelets in terms of containing more RNA content has been subject to recent studies. These immature or reticulated platelets (RPs) have first been described in 1969 as younger platelets, more involved in platelet aggregation compared to other platelets [164]. These RPs are defined differently when discussed in the clinical or laboratory context.

In present clinical studies, RPs have shown prothrombotic capabilities [165]. In these studies, the reticulated platelet fraction is defined by a point of care system like Sysmex, Abbot or Mindray hematology analyzers. Most commonly used, the Sysmex system (Kobe, Japan) quantifies RPs in clinical routine by using a nucleic acid specific dye in an optical fluorescent channel [166, 167]. It measures the percentage of RPs in the total number of platelets in the bloodstream as immature platelet fraction (IPF%) with an in-house designed gating system.

Additionally, the system provides the immature platelet count (IPC 10^9/L), product of total platelet count and IPF% [168]. Although RP measurements by point-of-care methods are fast, cheap and highly feasible for routine diagnosis in the clinics, they are limited to analyze only quantitative parameters of RPs. These methods do not allow downstream biological characterization of the detected RP population. In order to investigate the biology of RPs and their correlation with the negative outcome of several diseases, it requires laboratory-based sorting methods that permit further usage of the sorted cell populations.

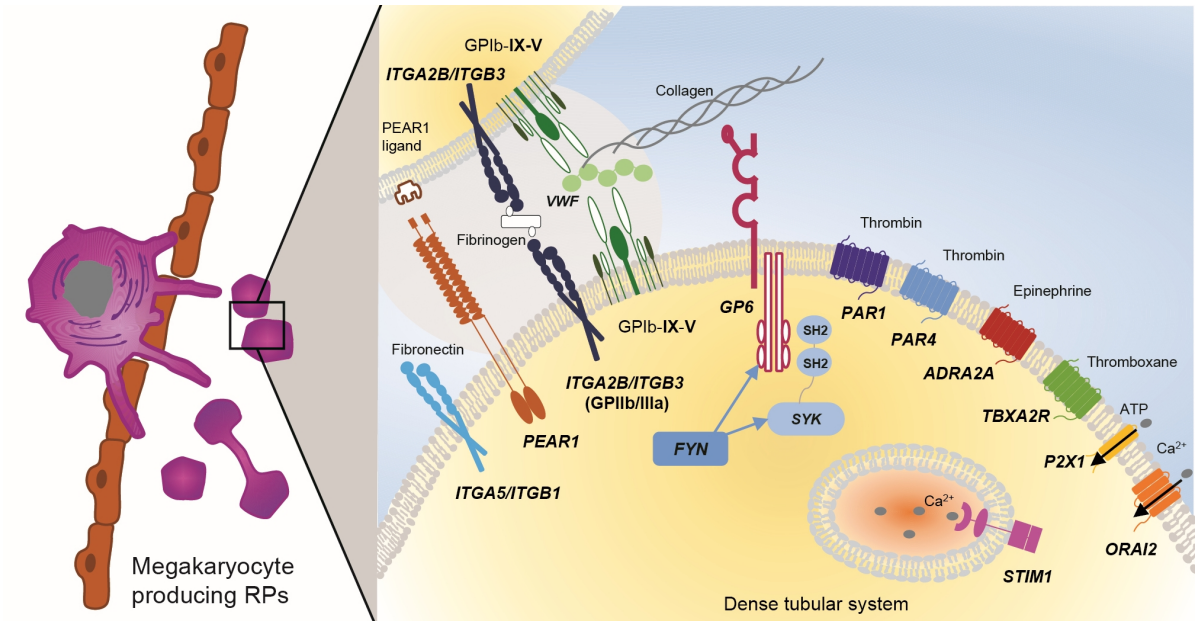

Figure 1.3: **Schematic illustration of prothrombotic transcripts enriched in reticulated platelets (RPs).** Summary of essential adhesion and activation receptor transcripts enriched in RPs of healthy donors. Significantly upregulated transcripts are drawn in bold font. Nonbold characters and unfilled forms are not significantly upregulated but are included to complete the illustration (own Figure [169]).

In the laboratory context, RPs can be detected with a flow cytometer. The staining with fluorescent dye thiazole orange (TO) makes it possible to separate platelets by their RNA content [170, 171], a technique that was standardized in the earliest publication part of this dissertation [136]. RNA-seq after sorting allows the identification of upregulated transcripts in RPs (Fig.1.3). These flow cytometry-detected RPs have been shown to moderately correlate with the RPs determined by hematology analyzers [172, 173]. Biological investigations of these RPs by several groups showed a hyper-reactivity of RPs in healthy donors [174] and

in patients after dual antiplatelet therapy [175, 176]. Additionally, RPs, defined as platelets with higher TO-staining, predominantly participated in thrombus generation [177]. In our study, we investigated the hyper-reactive behavior of sorted RPs by capturing their higher response to activation stimulus ADP in contrast to sorted mature platelets (MPs) in healthy donors [169]. Other studies have conducted similar experiments, detecting higher P-Selectin levels on the membrane surface [178]. Both the associated hyperactivity and especially the pro-thrombotic characteristics of RPs make them an interesting study subject in the clinical context.

1.5 Pathophysiological Roles of Reticulated Platelets

RPs are of particular interest in biomedical research, because high RP-levels in blood are associated with the incidence of adverse events and a worse prognosis in several diseases. In combination with their pro-thrombotic and hyper-reactive behavior, these characteristics made them the target of several researchers. Apart from the cardiovascular context, RPs are also elevated in diabetic patients, in smokers [32, 179] and after surgery [180, 181]. These findings contribute to making RPs an appealing drug target both in and outside the cardiology field.

In cardiologic settings, RPs are of particular interest: In the last 15 years, several studies investigated the connection of high levels of RPs and a deficient reaction to dual antiplatelet treatment, consisting of aspirin and different $P2Y_{12}$ receptor inhibitors [182, 165, 183]. The most prominent and currently used antiplatelet drugs targeting the $P2Y_{12}$ receptor are prasugrel [184], clopidogrel [185] and ticagrelor [186]. Although aspirin irreversibly inhibits the TXA2 secretion via blockage of COX-1, [187] it does not prevent platelet activation in all patients to the desired extent [188]. While some patients could be non-responders due to genetic reasons or an acquired resistance [189], most aspirin-resistant patients are generally at higher risk for adverse cardiovascular events [190]. Additionally, a higher abundance of RPs resulting from an elevated platelet-turnover is another reason behind a lacking aspirin response [32]. Overall, a higher abundance of RPs in patients generally worsens the outcome

of disease progression. By assessing the absolute number or fraction of immature platelets, several studies have stated that a high IPF predicts an insufficient response to dual antiplatelet treatment [191, 192, 193]. Additionally, as shown in Fig.1.4, a high immature platelet count was found to be associated with cardiovascular death and thrombotic events in chronic and acute coronary syndrome patients [194, 195].

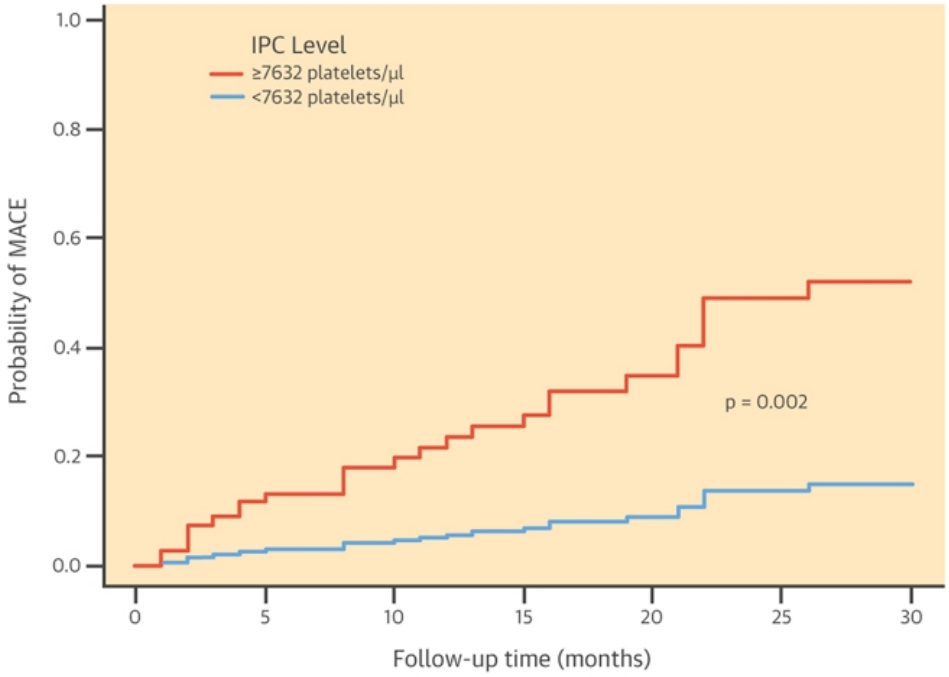

Figure 1.4: **Cox Proportional Hazards Model for MACE at Follow-Up** After adjustment for diagnosis at time of enrollment, initial treatment, age, history of heart failure, smoking, hematocrit, and platelet count, the level of immature platelet count (IPC) was found to be significantly associated with major adverse cardiovascular events (MACE) [195].

1.5.1 Chronic Coronary Syndrome

Chronic coronary syndrome describes the pathological progression of coronary artery disease. While the state of chronic disease progression can be kept at bay or can even be improved by using medication, lifestyle changes or via surgery, it can also worsen abruptly [196]. The stable phase of coronary artery disease can be naturally asymptomatic or influenced by medication [197]. If symptoms already arise at this stage, they are similar to acute coronary syndrome and

mostly include angina pectoris. It is typically described as a substernal pain or pressing heavy feeling which can occasionally move into the shoulder and arm [198]. Furthermore, angina pectoris can be classified into different categories according to the Canadian Cardiovascular Society [199, 200]. Depending on the genetic and coronary risk factors, the velocity in which disease progression deteriorates, differs [201]. Most commonly, the acute phase is initiated by the rupture and increase of atherosclerotic plaques and the subsequent blocking of normal blood flow. During this phase, symptoms like chest pain and shortness of breath are likely to occur or worsen [197, 202]. At the same time, further platelets circulating in the blood are triggered by the present plaque material, increasing the size of thrombi [203]. Eventually, the oxygen transport to the heart cells is impaired, leading to cell death, ischemia and myocardial infarction [204, 197]. Myocardial infarction as one subtype of acute coronary syndrome [205] is, after an initial phase of drug loading and after percutaneous coronary intervention (PCI), treated similarly to chronic coronary syndrome.

Standard treatment of chronic coronary syndrome involves the use of aspirin as well as a potent $P2Y_{12}$ inhibitor [206] as described before (chapter 1.4.1). Briefly, studies in the last decade have shown that this standard therapy significantly lowers the chance of adverse cardiovascular events if continued after PCI [207, 208]. Nonetheless, a high platelet turnover and the presence of an abnormal level of RPs in the blood can impair the effect of this treatment [193]. This characteristica as well as the pathophysiological role of RPs in general were the initial reason for the research performed during the course of my PhD.

1.5.2 Platelets during SARS-CoV-2 infection

At the beginning of the pandemic, an increase in cardiovascular events in COVID-19 patients was reported by clinicians worldwide [209, 210, 211]. While some studies reported an aberrant coagulopathy [212, 213, 214], others discovered resulting higher venous and arterial thrombotic risks [215, 216, 217]. Additionally, severe COVID-19 cases correlate with an increase of ischaemic stroke [218]. Generally, stroke was also reported in young SARS-CoV-2 individuals [219] and discovered more frequently in COVID-19 patients compared to influenza patients [220]. As previously described in chapter 1.3, platelets play an important role in

maintaining a normal blood flow [221] while high platelet reactivity influences coagulopathy. Therefore, it was not clear if the hypercoagulopathy reported during SARS-CoV-2 infection was directly linked to a higher reactivity in platelets [222, 223, 224]. The mechanisms behind this thrombo immune dysregulation are still not fully understood.

1.6 Aim of the thesis

Overall, I planned to characterize platelet expression heterogeneity in health and disease. In the clinical context, it is important to understand platelet biology as platelets are involved in several diseases. As reticulated platelets (RPs) show treatment resitance during dual antiplatelet therapy, a deep characterization of this subgroup is needed to identify better therapies for patients with high RP levels.

1. In the first project on which this thesis is based on, I aimed to lay the foundations to investigate the biology of RPs. In order to get there, my first step was to develop a sorting technique for RPs and MPs, which later on makes RNA-sequencing of both subgroups possible.

2. In addition to that, I planned to investigate platelet heterogeneity beyond the RPs and MPs subgroups by using CyTOF. Thus, I aimed to develop a CyTOF protocol to analyze platelets from platelet-rich plasma (PRP) allowing a broader investigation of heterogeneity. Within the CyTOF project, I planned to work on data analysis optimization to set a standard for differential expression analysis in platelets.

3. As an upregulation of cardiovascular and thrombotic events was observed in COVID-19 patients, the question arose if these observations could be directly linked to platelets and a higher reactivity during SARS-CoV-2 infection. Therefore, I planned to examine the role of platelets during SARS-CoV-2 infection. With the previously developed CyTOF strategy, I aimed to investigate platelet reactivity at rest and after stimulation in COVID-19 patients compared to healthy donors.

2 Material and Methods

This section describes the study design, reagents, experimental procedures and bioinformatic analysis techniques used in this work. Catalog numbers are indicated at first mentioning.

2.1 Study Design and Participants

All subprojects included in this thesis required the recruitment of healthy blood donors and patient donors. From each healthy donor, I collected 12-20 mL venous blood into 3 mL vials containing citrate dextrose A (Sarstedt AG & Co.KG, 05.1165.001). From the patients, 12-20 mL were collected directly at the ward by medical doctors. Patients were recruited at the Klinikum rechts der Isar, Department for Internal Medicine I, Cardiology, at the Technical University of Munich. Healthy donor blood collection was performed by myself. All study participants gave written informed consent according to the declaration of Helsinki. Additionally, the three studies have been approved by the local ethics committee of the Technical University Munich (TUM, ethikkomission@mri.tum.de) Ismaninger Straße 22, 81675 Munich (Ethic no. 352/18 S-AS, for COVID-19 study: additional approval number 147/20).

2.2 Blood Sampling and PRP generation

During the first project, I optimized the preparation of platelet-rich-plasma (PRP) and set a standard for our laboratory. In brief, I prepared PRP immediately after each blood collection within 30 minutes after withdrawal. Per 3 mL of whole blood, the PRP yield was between

500 µL and 1.5 mL. The whole-blood vials were centrifuged at room temperature (RT) for 10 minutes at 200 x g in a benchtop centrifuge (rotina 420 R, Hettich GmbH & Co. KG, 4701 4706) without brake. At this step, the blood plasma containing the platelets was separated from the erythrocytes and the leucocyte-rich buffy coat. Afterwards, the plasma phase was transferred to a 15 mL RNase free tube (Greiner Bio-One, 188261) using a 1 mL pipette (Eppendorf AG, 3124000121). All steps were carried out with caution to prevent platelet activation. Additionally, 0.6 U ADPase/ml (Apyrase from potatoes; Sigma-Aldrich, A6535-100UN) and 1 µM prostaglandin E1 (PGE1; Sigma-Aldrich, 538903) were added to the plasma. Mixing was induced by rolling the tube carefully on the bench. Then, the plasma was incubated in a 37°C water bath for 30 minutes. Further platelet aggregation was prevented by the addition of 5 mM ethylenediamine tetraacetic acid (EDTA; Invitrogen, Thermo Fisher Scientific, 5575020) and the pH was balanced by adding 1 mM citric acid (Sigma-Aldrich, 251275). Again, the tubes were rolled without inversion to gently mix the substances and immediately centrifuged at 800 x g for 15 minutes without brake using the same benchtop centrifuge. After discarding two-thirds of the supernatant, another inhibition cocktail was added to the sample containing 0.6 U ADPase/mL, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; Sigma-Aldrich, 15630080), 1 µM PGE1 and 1 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA; Sigma-Aldrich, E3889). Following this, the pellet was resuspended carefully with a 1 mL pipet and the resulting PRP was ready for downstream experiments.

2.3 Transcriptomic Methods

2.3.1 PRP Staining for Sorting

Fluorescence activated cell sorting (FACS) required previous staining with fluorescent antibodies. In total, 300 µL PRP was stained with platelet marker CD41 (1:200 v/v; Biolegend, 303729) for positive selection, as well as deoxyribonucleic acid (DNA) binding dye (1:500 v/v; Vybrant DyeCycle Ruby Stain, Thermo Fisher Scientific, V10309) for negative selection.

Sorting after RNA content additionally required staining with 600 µL Thiazole Orange (TO; BD ReticCount, Becton Dickinson, 349204) for a final concentration of 6.7 µg/mL. After 30 min incubation at RT in the dark on a rotator at slowest speed (Heidolph instruments, 544–41200-00), I magnetically labeled the samples with CD61-MicroBeads (Miltenyi Biotec, 130-051-101) for 10 min. Then, I centrifuged the samples at 1000 x g for 12 min. Pellet resuspension followed in 2-3 mL Ca^{2+} and Mg^{2+} free phosphate buffered saline (PBS; Gibco, Thermo Fisher Scientific, 10010023) containing 0.6 U/mL ADPase, 1 mM EGTA and 1 µM PGE1. All samples were filtered through a 35 µM cell strainer (Thermo Fisher Scientific, 10585801) and transferred to a 15 mL lo-bind tube (Eppendorf, 0030122216).

2.3.2 Sorting of RPs and MPs

Platelets were sorted at the Microbiology sorting facility (CyTUM MIH) by trained operators using a FACS Aria III (Becton Dickinson). I applied the gates as shown in Fig. 2.1. Briefly, the first gate in the forward scatter area/side scatter area (FSC-A/SSC-A) plot excluded other cells based on size, while doublets were excluded in gate two in the FSC-width (FSC-W)/FSC-A plot. In the DNA stain/CD41 plot, I selected for platelets (CD41+/DNA-). The final plot Thiazole Orange/SSC-A included all previous gates. Here, I defined platelets with the highest RNA content (15% highest TO signal) as RPs and platelets with the lowest RNA content (30% lowest TO signal) as MPs. Of these two populations, the operators sorted up to 2 x 10^7 cells into 15 mL lo-bind tubes containing 500 µL Ca^{2+} and Mg^{2+} free PBS. After reaching the capacity of 1 x 10^6 cells per tube, tubes were replaced and stored on ice until the end of the sort.

2.3.3 Magnetic-Based Separation

For the RNA isolation procedure, a PBS-based RNA isolation buffer with pH 7.2 was prepared containing 0.5% bovine serum albumin (BSA; Gibco, Thermo Fisher Scientific, 15260037) and 2 mM EDTA.

Immediately after sorting, I performed magnetic-based concentration of the sorted populations

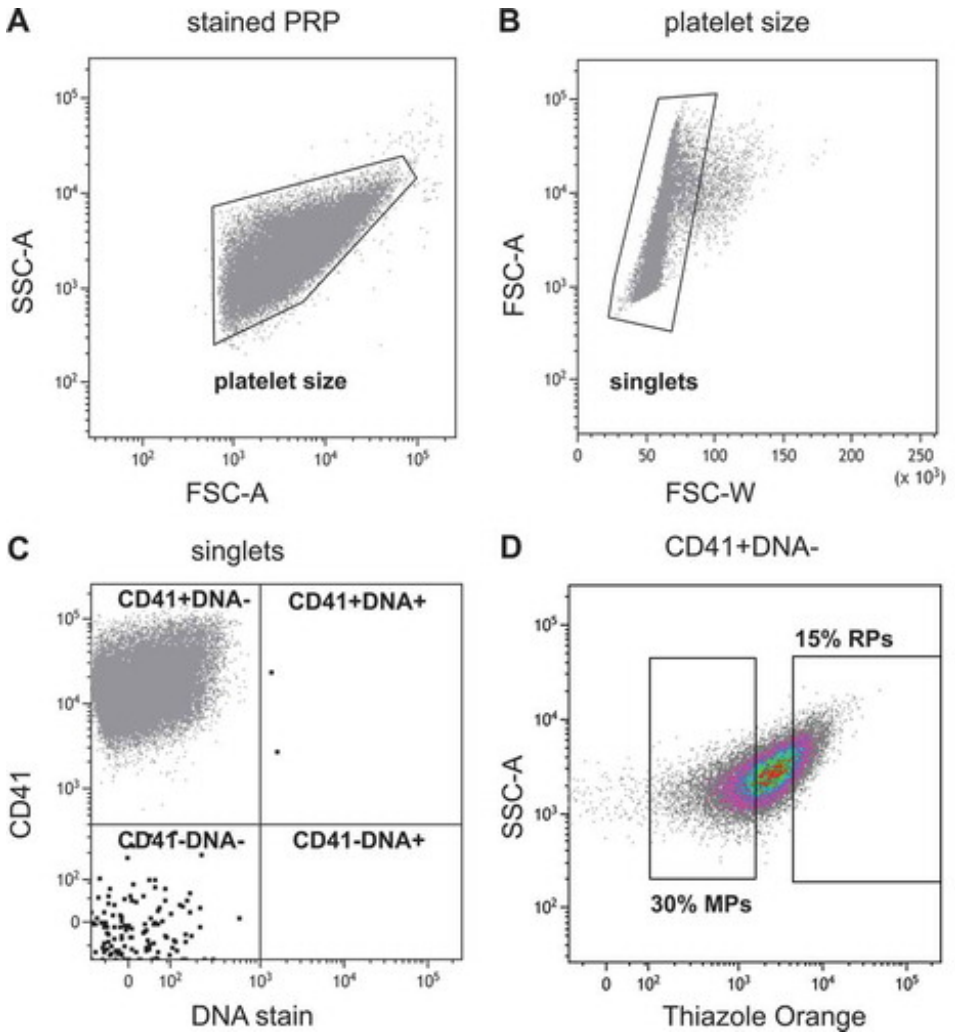

Figure 2.1: Flow Cytometry sorting strategy. (A) Applied gate to stained platelet-rich plasma (PRP) separating the platelets by size using FSC/SSC. (B) Doublets are excluded by a high FSC-width. (C) Sorting strategy after CD41 and DNA staining for platelet isolation. Platelets are selected by gating CD41 positive and DNA negative events. CD41-BV = 421 nm, DNA stain Ruby = 638 nm. (D) Gating strategy for RNAlow platelets (mature platelets, MPs) and RNAhigh platelets (reticulated platelets, RPs). Platelets with the 15% highest TO signal are defined as RPs, platelets with the lowest 30% TO signal as MPs (TO = 488 until 550 nm). TO: Thiazole Orange, BD FACS Aria III, SSC: Side Scatter, FSC: Forward Scatter. RPs: reticulated platelets, MPs: mature platelets [136].

using MS columns (Miltenyi biotec, 130-042-201) and a magnetic activated cell sorting (MACS) separator magnetic field attached to a MACS multistand (both Miltenyi biotec, 130-042-109 and 130-042-303). During the process, the sorted samples were kept on ice to keep the RNA intact. Following the manufacturer's instructions, I cleaned the columns three times with 500 µL RNA isolation buffer after loading the column with the sample. Then, I eluted the platelets bound to the magnets into a 1.7 mL RNAse free tube (Axygen, Corning, MCT-175-X) using 1 mL RNA buffer. The eluted samples were pelleted at 12,000 x g for 10 min at 4°C, resuspended and homogenized in 700 µL QIAzol (Qiagen, 79306) using a vortex mixer (Heidolph instruments). Until RNA extraction, homogenized cells were stored in QIAzol at -80°C.

2.3.4 RNA Extraction

For RNA extraction, I used the miRNeasy micro kit (Qiagen, 217084) and followed the manufacturer's instructions. Briefly, 140 µL chloroform (Merck, 102445) was added to the homogenized cells in 700 µL Qiazol and thoroughly mixed by shaking. After a 3 min incubation on the bench, samples were centrifuged for 15 min at 12,000 x g. RNA was then extracted from the upper aqueous phase following the miRNeasy Micro Handbook. At the final step, the RNA was eluted in 20 µL RNase-free water from the kit and stored at -80°C until further processing or shipment to our collaborators at the Department of Cardiovascular Medicine at the Humanitas Clinical and Research Center Irccs of the Humanitas University in Rozzano, Italy (Humanitas).

2.4 Mass Cytometry

CyTOF allows the exploration of receptors expressed on the platelet membrane and can detect differences in platelet subgroups. In addition, this method allows the analysis of platelet behavior at rest and after activation. Functional assays investigating the role of different platelet activators ADP, thrombin receptor activating peptide (TRAP) or collagen related

peptide (CRP-XL) can be used to examine the platelet activation cascade.

My publication "Mass cytometry of platelet-rich plasma: a new approach to analyze platelet surface expression and reactivity" shows the development of an optimal protocol to analyze platelets from PRP. Therefore, I described the CyTOF methods in most detail within the paper and I will only briefly present them in this section.

2.4.1 Panel Preparation

Before the actual experiments, I designed a panel which I used in all further mass cytometry experiments. In summary, all receptors known to be expressed on the platelet surface to a high- medium extent were chosen to be coupled to metals known to be detected to a low extent. Surface markers that are known to be expressed to only a low extent on the platelet membrane were chosen in a form in which they were coupled to metals emitting a high detectable CyTOF signal. Table 2.1 shows the finalized panel including all receptor functions and the antibodies with their attached metals.

Table 2.1: Mass Cytometry Panel

Antigen	**Common Name**	**Biological Function**	**Clone**	**Type** [1]	**Metal**	**Tag** [2]	**Fabricator**
CD3	T-cell Receptor T3, TCR-CD3 complex	Adaptive immune response	UCHT1	IgG	Er170	c	Fluidigm
CD9	Tetraspanin	Cell Adhesion	SN4 C33A2	IgG	Yb171	c	Fluidigm
CD29	Integrin β1	Collagen Receptor Unit	TS2/16	IgG	Gd156	c	Fluidigm
CD31	PECAM-1	Cell Adhesion	WM59	IgG	Nd145	c	Fluidigm
CD36	GPVI	Collagen Receptor	5-271	IgG	Sm152	c	Fluidigm
CD40	TNFRSF5	Induction of Immuno-globulin Secretion	5-C3	IgG	Nd142	c	Fluidigm
CD41	Integrin αII	Alpha Unit of Fibrinogen Receptor	HIP8	IgG	Y89	c	Fluidigm
CD42a	GPIX	Von Willebrand Factor Receptor Unit	GR-P	IgG	Pr141	i	Thermofisher

[1] all antibodies are monoclonal, final concentration 0.5 mg/ml
[2] c=commercial, i=in-house

CD42b	GPIbα	Von Willebrand Factor Receptor Unit	HIP1	IgG	Nd144	c	Fluidigm
CD45	PTPRC	Positive Regulator of T-cell Coactivation	HI30	IgG	Sm154	c	Fluidigm
CD47	MER6	Adhesion Receptor for THBS1 on Platelets	CC2C6	IgG	Bi209	c	Fluidigm
CD61	Integrin β3	Beta Unit of Fibrinogen Receptor	VI-PL2	IgG	Nd146	c	Fluidigm
CD62P	P-Selectin	Cell Adhesion, Activation Marker	KO-2-7	IgG	Dy161	i	Thermofisher
CD63	LAMP-3	Cell Adhesion, Activation Marker	H5C6	IgG	Nd150	c	Fluidigm
CD69	CLEC2C	Lymphocyte Signal Transmission	FN50	IgG	Dy162	c	Fluidigm
GPVI	GPVI	Collagen Receptor	HY101	IgG	Lu175	i	Thermofisher
PAC1	Activated αIIbβ3, Activated GPIIb-IIIa	Fibrinogen/ Von Willebrand Receptor	PAC1	IgM	Gd155	i	BD Biosciences
PAR1	F2R	Thrombin Receptor	ATAP2	IgG	Sm147	i	Thermofisher
PEAR1	JEDI	Platelet Endothelial Aggregation Receptor	492621	IgG	Yb174	i	Novusbio
CD107a	LAMP-1	Cell Adhesion, Activation	H4A3	IgG	Eu151	c	Fluidigm
CD154	CD40L	Interaction with Endothelial Cells	24-31	IgG	Er168	c	Fluidigm

2.4.2 Antibody Conjugation

Commercially available and ready-to use antibodies for CyTOF analysis were preferably used for the experiments. Nonetheless, the following antibodies were not available in a metal-conjugated state and had to be conjugated in-house: CD42a, CD62P, GPVI, PAR1 (all four Thermo Fisher Scientific, MA1-91023, MA1-81809, 14-9813-81 and 35–2200) as well as PAC1 (BD Biosciences, 340535) and PEAR1 (Novus Biologicals, MAB4527).

Briefly, I coupled 100 mg of these carrier-free antibodies to a metal-labeled X8 polymer from the Maxpar X8 Multimetal Labeling Kit (Fluidigm Sciences, 201300). By following

the Maxpar Antibody Labeling Protocol (Fluidigm Sciences), I coupled the antibodies to isotopically enriched lanthanide metals (provided in the Maxpar X8 Multilabel kit, see Table 2.1 for the corresponding metals). The manufacturer recommends the use of IgG antibodies only. In case of PAC1 it has been previously shown that although it is an IgM antibody, the coupling reaction with the recommended kit works and that the antibody is suitable for CyTOF analyses [159]. All labeled antibodies were stored in an antibody stabilization buffer (Boca Scientific, 131050) at a concentration of 0.5 mg/mL at 4°C until use.

2.4.3 Antibody Validation

Before the first use, the custom-conjugated antibodies were validated. For this process, I added 0.5 µL of the respective conjugated antibody to one drop of calibration beads (Fluidigm Sciences, 201078). After a 15 min incubation step, I washed the sample twice by adding 1.5 mL PBS and centrifuging at 300 x g for 10 min. Additionally, I performed two more wash steps with 1.5 mL de-ionized water (MiliQ water, Merck) at the same centrifugation speed. Acquisition followed resuspending the sample in 200 µL de-ionized water.

2.4.4 Staining

Platelet-rich-plasma was obtained from each donor as described in section "Blood sampling and PRP generation". Per sample, 600 mL PRP were mixed with 400 µL PBS and stained with 1 µL Cell-ID Cisplatin (Fluidigm Sciences, 201064) for a final concentration of 5 µM Cisplatin per mL. After a 5 min incubation period, I added 5 mL cell staining buffer (CSB; Fluidigm Sciences, 201068), centrifuged the samples at 300 x g for 5 min, and resuspended the pellets in 50 µL CSB. For each experiment, one baseline sample consisting of non-stimulated platelets and 1-2 activated samples (stimulated platelets) were produced. Depending on the experiment, platelets were either activated by adding 10 µM TRAP (Bachem, 4031274) and/or 2 µg/mL CRP-XL (Cambcol Laboratories, CRP-XL). After incubating for 2 min, I added 50 µL of a previously mixed antibody-cocktail to all samples and incubated them at room temperature for 30 min (see Table 2.1 for antibody-metal information). Two washing steps,

each consisting of adding 2 mL CSB and centrifuging at 300 x g for 5 min, were followed by a cell fixation step. The pellet was resuspended in 1 mL of 1.6% formaldehyde (Thermo Fisher Scientific, 28906) in PBS and left at 4°C overnight. Cells were centrifuged at 800 x g for 10 min the next day before the pellet was resuspended in up to 50 µL residual solution. A 1 h incubation with 125 nM Iridium in 1 mL MaxPar Fix and Perm Buffer (Fluidigm Sciences, 201067) at room temperature preceded a centrifugation at 800 x g for 5 min. Afterwards, as a final washing step, 2 mL CSB were added to the samples which were centrifuged at 800 x g for 5 min. The pellet was resuspended in 10% dimethyl sulfoxide (DMSO, Sigma-Aldrich, D2650) in fetal bovine serum (Thermo Fisher Scientific, F7524) and frozen at -80 °C until acquisition.

2.4.5 Final Preparation

After thawing on ice, the samples were shortly spun down and transferred to a 5 mL polystyrene tube (Thermo Fisher Scientific, 10100151). In total, I performed four wash steps. I added 2 mL CSB for the first two washing steps, subsequently 2 mL de-ionized water for the last two. All centrifugation steps were carried out at 800 x g for 5 min with subsequent removal of supernatant. The pellet from the final run was stored at 4°C until measurement within 24 hours.

2.4.6 CyTOF Measurement

Cells were resuspended to a concentration of 1 x 10^6 platelets/mL in 10% EQ beads in cell acquisition solution (CAS; Fluidigm Sciences, 201240) before acquisition. Then, I acquired the samples at the Helios instrument (Fluidigm Sciences) using the recommended acquisition parameters (lower convolution threshold 400, event length 10–150, sigma 3, signal subtraction 0). Based on the experiment, I measured between 300,000 and 500,000 events per sample at an acquisition rate of 200-350 events/second.

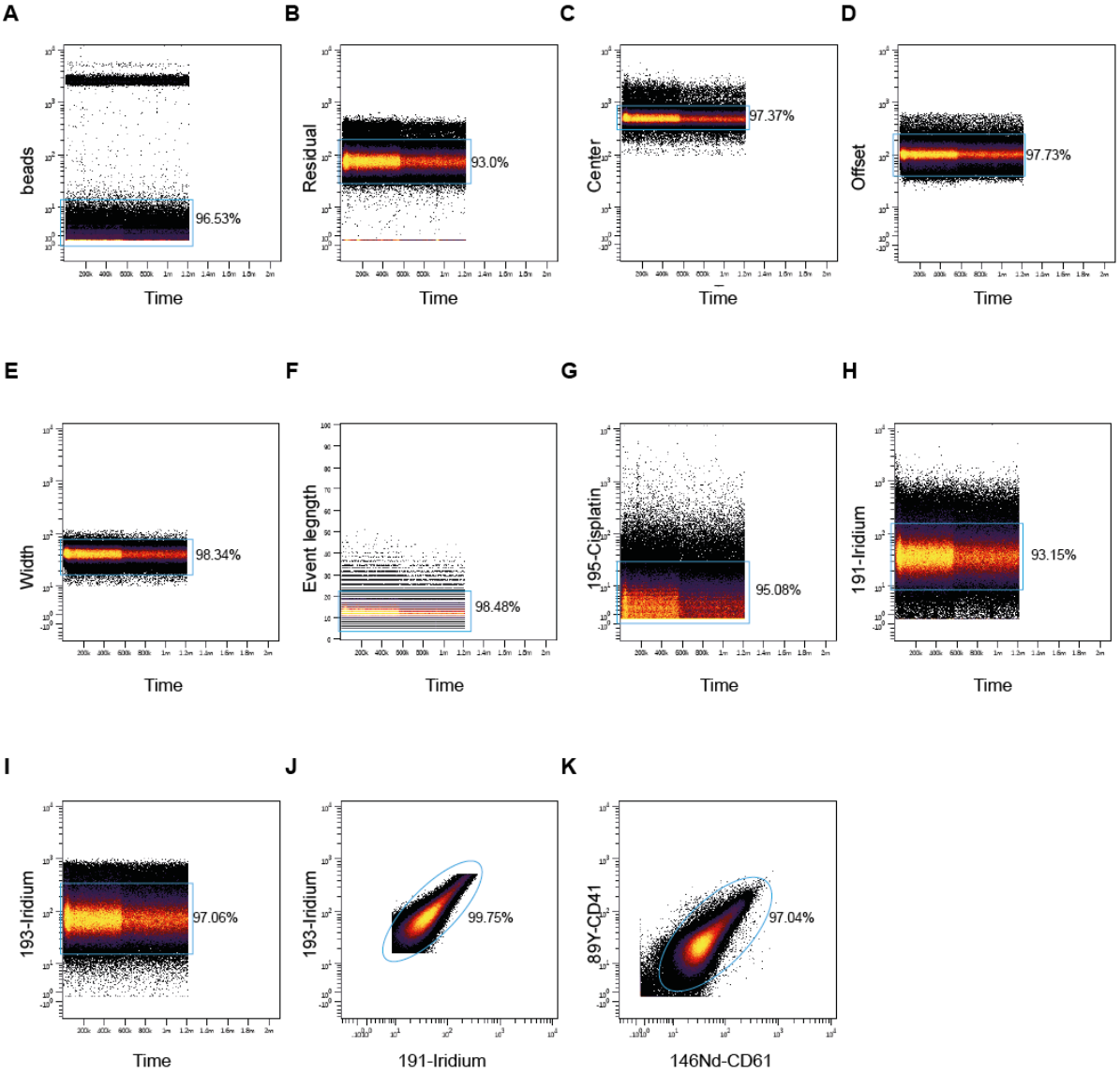

Figure 2.2: Gating protocol on PRP sample. A) Exclusion of beads, B-E) clean-up according to the Gaussian discrimination, F) selecting event length for low range intensity, G) live-dead cell discrimination by only excluding cisplatin stained cells, H-J) RNA containing cells stained with 191/193-Iridium, K) gating only CD41 and CD61 positive cells to exclude debris and contamination. Percentages show the % of parent population. In the last gate, 62.22% of the total acquired events are left [225].

2.4.7 Normalization and Gating

Normalization was performed immediately after measurement using the Fluidigm software v7.0. Gating was performed on the normalized .fcs files according to the Maxpar clean-up protocol (Approach to Bivariate Analysis of Data Acquired Using the Maxpar Direct Immune Profiling Assay, Fig.2.2 A-I). For platelet specific analysis, gates J and K were added (Fig.2.2 J-K). I performed all gating processes using the Cytobank software v8.1 (premium.cytobank.org). For further processing, I exported the gated .fcs files and transformed them by factor 5 using the in-house developed cytometry analysis using shiny (CYANUS) application (https://exbio.wzw.tum.de/cyanus/ [226]). All following bioinformatic analyses were performed on these transformed files.

2.4.8 Computational Data Analysis - Preprocessing and Visualization

I performed differential expression analysis using the CYANUS shiny app. Design of the project and app development was performed by three bioinformatic master students under the co-supervision of me and Olga Lazareva. Therefore, the students designed the app for my analysis needs and it was used throughout the project from the point of data transformation, having first looks at the expression distribution, dimensionality reduction etc. [226]. In short, I will describe the normal workflow that I used whenever working with the app. First, I inserted the previously gated data which were exported from premium.cytobank.org and uploaded them to the main page. Alongside, also a panel file including the markers, their metals, and corresponding overall function of being either a negative marker, a type marker or a state marker was uploaded as a .csv file (Table 2.2. The latter markers are supposed to change their expression through activation. The third file needs to be a meta file, describing the .fcs files with regard to sampleID, patientID, condition, activated_baseline, timepoint and other valuable information. After upload, the data was transformed with cofactor 5 using an inverse hyperbolic sine transformation before continuation [227, 228]. In many cases, it was necessary to downsample to a defined cell number, so that all files contain the same amount of cells. First steps include several quality checkpoints, for example the generation of an pseudobulk-level multi-dimensional-scaling plot (MDS) plot based on the median marker

expression values, in which similar samples within the same condition should cluster together. On the second page, dimensionality reduction techniques are available as data visualization to get a broad overview of the samples. Furthermore, clustering analysis can be performed using the FlowSOM algorithm, which uses a Self-Organizing Map (SOM) [229].

Table 2.2: Panel file used for upload to the CYANUS web application

fcs_colname	**antigen**	**marker_class**
Dy161Di	CD62P	state
Dy162Di	CD69	type
Er168Di	CD154	state
Er170Di	CD3	none
Eu151Di	CD107a	state
Gd155Di	PAC1	state
Gd156Di	CD29	type
Lu175Di	GPVI	type
Nd142Di	CD40	none
Nd144Di	CD42b	type
Nd145Di	CD31	type
Nd146Di	CD61	type
Nd150Di	CD63	state
Pr141Di	CD42a	type
Sm147Di	PAR1	type
Sm152Di	CD36	type
Sm154Di	CD45	none
Y89Di	CD41	type
Yb171Di	CD9	type
Bi209Di	CD47	type
Yb174Di	PEAR	type

2.4.9 Computational Data Analysis - Differential Expression

After completion of these steps, differential marker expression analysis is possible. In contrast to differential cluster abundance analysis, which is used to compare the proportions of cell types across experimental conditions per cluster, differential marker expression analysis

focuses on the different marker expression per condition, either overall or cluster-wise. In the following, I only used differential marker expression analysis. For data visualization, boxplots are generated displaying the desired populations next to one another. Then, an algorithm determining the statistical difference between two groups can be chosen. In my case, I first ran limma, which compares median expression data and is completed within several seconds. For some applications it was necessary to use CyEMD, an algorithm designed and adapted in-house [226] using the calculation of the earth mover's distance (EMD). The EMD compares two histograms of marker expression and calculates the amount of work that is needed to transform one histogram into the other. Using this non-parametric approach, differences that are not visible on the median level can be found. It takes all data points into account and is the algorithm of choice when investigating differences in markers that have a high zero inflation (mainly activation markers CD107a and CD154). A high zero inflation is characterized by many cells showing only very weak expression of a certain marker which results in the median being 0. Limma and other algorithms comparing only the median expression will compare 0 against 0 while CyEMD will take all values into account and may find differences that are present. The CyEMD reports a p value and Cohen's effect size. Differences between several groups with a p value < 0.05 were considered significant.

2.4.10 Clustering and Dimensionality Reduction Analysis

I performed clustering analysis mostly using Cytobank v8.1 (premium.cytobank.org), as most plots were generated before the CYANUS shiny app was finished. Of note, there are currently no techniques available on the Cytobank platform that allow for differential marker expression or differential cluster abundance analysis. For dimensionality reduction on the other hand, Cytobank offers several algorithms which reflect the needs of different experiment settings. In most cases, visual stochastic network embedding (viSNE) analysis is the dimensionality reduction method of choice to gain a broad overview of the different samples and marker expression among subpopulations. Platelet subpopulations are far less heterogeneous than most cells that researchers using CyTOF analysis work with. Hence, the algorithms are designed for showing the differences in expression of several sub cell

types which have experimentally proven lineage markers. In the case of platelet analysis, subpopulation analysis is a field which requires new ideas and de novo definition of which kind of subtypes might be present. Using only viSNE or uniform manifold approximation and projection (UMAP) analysis, a real estimation is not possible. Therefore, I mainly used the FlowSOM algorithm for clustering analysis and visualized the clusters on viSNE plots [229].

2.5 Methods Covid Study

2.5.1 Patient Characteristics

For this study, the blood of 8 patients with symptomatic SARS-CoV-2 infection, hospitalized at the Klinikum rechts der Isar, was collected between March and May 2020 as described above. Infection was confirmed by IgM antibodies against COVID-19 in the blood or a positive reverse transcriptase-polymerase chain reaction (PCR) test result. Additionally, 11 healthy donors who tested negative for SARS-CoV-2 infection were recruited. In the weeks after blood collection, none of them developed any symptoms. All donors were between >18 and <70 years and did not have any pre-existing conditions except for arterial hypertension and were taking no medication except for antihypertensive drugs. Further exclusion criteria were platelet disorders like thrombocytopenia (<100 G/L) or thrombocytosis (>500 G/L) as well as a decreased leukocyte count (<1 G/l), decreased hemoglobin levels (<10g/dL) or a renal dysfunction (glomerular filtration rate <60ml/min).

2.5.2 Mass Cytometry

Throughout the study, healthy and COVID-19 patient samples were prepared, stained and measured alongside at the CyTOF, using the antibody panel shown in Table 2.1.

2.5.3 Software

Gated and pre-preprocessed samples (as described previously) were used for the computational analysis. Python package v0.11.1 [230] was used to build and evaluate all models. Further processing after using the Cytobank platform was done with R 4.0 (R Development Core Team, 2005) and Python 3.6 (Python 3 Reference Manual, 2009).

2.5.4 Computational Analysis

We used UMAP (using CATALYST v1.12.1) based on 16 markers (all markers excluding activation markers and negative marker CD3) for dimensionality reduction and to visualize activation marker expression across the platelet population. For an equal analysis, we randomly downsampled to the lowest number of acquired events (41,525 events) and built mixed-effect linear models for both groups, TRAP-stimulated and non-stimulated samples. Sample-wise median signal intensity was treated as a dependent variable, whereas disease status was treated as fixed and the patientID as a random effect [231]. In order to evaluate if activation is significantly influenced by the disease status, we built a linear model with an interaction term for TRAP-stimulated and non-stimulated samples. Finally, we used the Benjamini-Hochberg method for multiple hypothesis correction of the p values (false discovery rate <0.05) [232].

2.5.5 Differential Analysis - COVID-19 Patients and Healthy Donors

We used the following linear mixed-effect model to investigate differential expression between COVID-19 patients and healthy donors, in more detail to test the difference of the sample-wise median marker expressions. Regression analysis was used to assess statistical significance.

$$Y_{ij} = \beta_{0j} + \beta_{cj} x_{ci} + \gamma_i + \epsilon_{ij} \tag{2.1}$$

In this model, Y_{ij} is the median expression of the j-th marker for i-th patient, x_{ci} is a binary

variable that specifies whether a patient i belongs to case or control group, and $\gamma_i \tilde{} N(0, \sigma_{2i})$ is a random intercept for each patient, allowing us to unravel the variance within-sample and within-group.

We used slope coefficients β_{cj} to test the linear relationship between the independent variable x_c and the dependent variable Y for significance:

$$\begin{aligned} H_0 &: \beta_{cj} = 0 \\ H_1 &: \beta_{cj} \neq 0 \end{aligned} \tag{2.2}$$

2.5.6 Differential Analysis - Baseline and TRAP-Stimulated Samples

Moreover, we investigated the reaction capacity of quiescent COVID-19 and controls upon stimulation. Therefore, we compared the slope coefficients for the covariate that matches activation in patients and healthy donors separately. The following model was used for control samples:

$$Y_{hj} = \beta_{0j} + \beta_{aj} x_{ah} + \gamma_h + \epsilon_{hj} \tag{2.3}$$

Here, h belongs to a set of indices for all controls, whereas x_{ah} is a binary variable that indicates if a sample h was TRAP-stimulated. For every marker j, several slopes βaj were computed which show an average linear increase in expression after stimulation.

For the slope analysis in COVID-19 patients we used a similar model:

$$Y_{pj} = \beta_{0j} + \beta_{aj} x_{ap} + \gamma_p + \epsilon_{pj} \tag{2.4}$$

Here, p belongs to a set of indices for all COVID-19 patients. Direct comparison of β_a coefficients from the model (3) and model (4) do not allow us to reason that the difference

between slope coefficients is statistically significant. Instead, a single model with an interaction effect term was used to test for a statistically significant difference in the reaction to TRAP stimulation between patients and controls.

A resulting significance of the Interaction effect means that the combination of activation status and patient condition (disease or control) has a significantly larger effect on median signal intensity than compared to the sum of the individual factors alone. Thus, the following model was used:

$$Y_{ij} = \beta_0 + \beta_a x_{aij} + \beta_c x_{cij} + \beta_{intij} x_{intij} + \gamma_i + \epsilon_{ij} \tag{2.5}$$

In this model, x_{int} is an interaction term defined as $x_a \times x_c$. The slope coefficient for the interaction β_{int} was then tested for statistical significance as shown in equation 2.

2.5.7 Clustering Analysis Using FlowSOM

Unlike the FlowSOM analysis that I mainly performed during my projects, the clustering analysis for the COVID data was not run with the algorithm embedded in Cytobank. Instead, we manually ran the clustering analysis using FlowSOM [229], compensated, transformed and scaled the data. Then, cells were distributed on a 10 x 10 self-organizing map. Based on the relative change in area under the cumulative distribution function curve, we selected to perform meta clustering in 12 clusters as more clusters indicated no improvement. Using a linear mixed-effect model, a p value was calculated for each cluster-marker pair:

$$Y_{ij} = \beta_0 + \beta_{cj} x_{ci} + \gamma_i + \epsilon_{ij} \tag{2.6}$$

In this model, Y_{ij} belongs to the median expression of the j-th marker for i-th cluster, whereas x_{ci} is a binary variable indicating if cell population Y_{ij} belongs to the case or the control group, and $\gamma_i \tilde{} N(0, \sigma_{2i})$ is a random intercept for each cell subpopulation. Finally, we tested

slope coefficients β_{cj} between the independent variable x_c and the dependent variable Y for significance of their linear relationship.

3 Publications

3.1 Publication 1: Sorting and Magnetic-Based Isolation of Platelets

Citation

The article entitled “Sorting and magnetic-based isolation of reticulated platelets from peripheral blood” was published online at Taylor & Francis on 11 February 2020 and in print on 2 January 2021 (www.tandfonline.com).

Full Citation:
“Isabell Bernlochner, Melissa Klug, Ditya Larasati, Moritz Von Scheidt, Donato Santovito, Michael Hristov, Christian Weber, Karl-Ludwig Laugwitz & Dario Bongiovanni (2021) Sorting and magnetic-based isolation of reticulated platelets from peripheral blood, Platelets, 32:1, 113-119, DOI: 10.1080/09537104.2020.1724923.”

3.1.1 Summary and Author Contributions

Summary

In this study, we developed a reproducible approach for detection, isolation and collection of reticulated platelets (RPs) from peripheral blood. RPs are known to be larger platelets that contain more RNA compared to others and have been shown to have a prothrombotic and hyperactive behavior. In several clinical settings like in diabetic patients or after myocardial infarction, RP levels are increased and correlate with mortality and adverse cardiovascular events. Moreover, high RP levels predict an insufficient response to dual antiplatelet treatment with aspirin and P2Y12 inhibitors, which makes them a potential drug target. The lack of an

RP specific marker which would allow selection and their instability make their processing challenging. Studies in the past have investigated RPs using flow cytometry. Based on their size (FSC/SSC) and RNA-content after staining with Thiazole Orange (TO), analysis was possible but not standardized. Furthermore, until now a method to sort and extract RNA from RPs has been lacking. Therefore, we developed a standardized protocol that allows detection, isolation and collection of RPs from whole blood by using RNA-specific staining with TO. The additional magnetic platelet-specific labeling made it possible to reach sufficient RNA output in high quality. This protocol allows the continuation with RNA extraction and deep biological downstream analysis of the sorted platelets, for example with RNA-sequencing.

Author Contribution

M.K. refers to Melissa Klug, author of this dissertation. D.B., D.S. and I.B. had the initial idea behind the project, while M.K. carried out all experiments in this paper, some assisted by former master student D.L.. The first draft of the paper was written by M.K., while I.B. and D.B. wrote the main discussion part. M.H. operated the FACS Aria III and gave input on sorting parameters. M.K. prepared all figures and tables upon consultation with D.B. and I.B.. Interpretation of results was done by M.K., D.L., D.B., I.B., D.S. and M.H., while these next to M.S., C.W. and K.L. revised the paper.

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Year of publication: 2021
Name: Melissa Klug
Street address: Ismaninger Strasse 22
Town: Munich
Postcode/ZIP code: 81675
Country: Germany
Email: melissa.klug@tum.de
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METHOD ARTICLE

Check for updates

Sorting and magnetic-based isolation of reticulated platelets from peripheral blood

Isabell Bernlochner[1,2*], Melissa Klug [1,2*], Ditya Larasati[1], Moritz Von Scheidt[2,3], Donato Santovito [4], Michael Hristov[4], Christian Weber[2,4], Karl-Ludwig Laugwitz[1,2], & Dario Bongiovanni [1,2,5]

[1]*Medical Department 1, Cardiology, Klinikum Rechts Der Isar, Technische Universität, Munich, Germany,* [2]*DZHK (German Center for Cardiovascular Research), Partner Site Munich Heart Alliance, Munich, Germany,* [3]*Department of Cardiology, Deutsches Herzzentrum München, Technische Universität, Munich, Germany,* [4]*Institute for Cardiovascular Prevention (IPEK), Ludwig-Maximilians-University Munich, Munich, Germany, and* [5]*Department of Cardiovascular Medicine, Humanitas Clinical and Research Center, Rozzano, Milan, Italy*

Abstract

Reticulated Platelets (RPs) are large, RNA-rich, prothrombotic and hyperactive platelets known to be elevated in high-risk populations such as diabetics and patients with acute coronary syndrome. High levels of RPs correlate with mortality and adverse cardiovascular events in patients with coronary artery disease as well as with an insufficient antiplatelet response to thienopyridines and aspirin after percutaneous coronary interventions, making them an appealing drug target. However, processing of platelets is challenging and no specific marker for RPs exists. Until now, the gold standard laboratory-based method to study them is based on the flow cytometric measurement of their cell size and their RNA-content with the fluorescent dye Thiazole Orange (TO). Nevertheless, standardized protocols for staining and processing of RPs are missing and the existing techniques were not applied for cell sorting. We provide here a structured and reproducible method to detect, isolate and collect RPs from peripheral blood by RNA-specific staining with TO implementing several platelet inhibitors as well as magnetic labeling allowing sufficient cell recovery and deep biological investigation of these platelets.

Keywords

Immature platelets, platelet isolation, reticulated platelets

History

Received 14 September 2019
Revised 21 December 2019
Accepted 28 January 2020
Published online 13 February 2020

Method

Materials

Biological Materials

Fifteen to eighteen milliliters of human peripheral blood. Caution: all experiments involving human subjects must comply with the declaration of Helsinki and be carried out following national and institutional guidelines. Written informed consent must be obtained.

Reagents and Consumables

Safety-Multifly needle 21G with tube 80 mm, DEHP-free - Sarstedt Catalog #: 85.1638.203

3 mL S-Monovette® tubes, Citrate 3.2% (1:10), 66 × 11 mm, green EU code, paper label, 50/inner box sterile – Sarstedt Catalog #: 05.1165.001

15 mL conical tubes – Thermo Fisher Scientific Catalog #: 10136120

5 mL round-bottom polystyrene tubes – Thermo Fisher Scientific Catalog #: 10100151

5 mL tubes with cell strainer – Corning Catalog #: 10585801

15 mL conical tubes – Eppendorf Catalog #: 0030122151

1.7 mL RNase-free tubes – Corning Catalog #: MCT-175-X

Adenosine 5′-diphosphatase, Apyrase from potatoes (10 U/100 µL in PBS stock) – Sigma-Aldrich Catalog #: A6535-100UN

Prostaglandin E_1 ($C_{20}H_{34}O_5$ 1 mg/mL DMSO stock, 0.1 mg/mL in PBS) – EMD Millipore Corp. Catalog #: 538903

EDTA ($C_{10}H_{14}N_2Na_2O_8$ * $2H_2O$, 0.5 M, pH 8.0) – Invitrogen Catalog #: 5575020

Citric Acid ($HOC(COOH)(CH_2COOH)_2$, 1 M stock) – Sigma-Aldrich Catalog #: 251275

HEPES ($C_8H_{18}N_2O_4S$, 1 M buffer solution, pH 7.2–7.5) – Thermo Fisher Scientific Catalog #: 15630080

Ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid ($[-CH_2OCH_2CH_2N(CH_2CO_2H)_2]_2$; EGTA, 0.5 M stock, pH 8) – Sigma-Aldrich Catalog #: E3889

PBS (Ca2+ and Mg2+ free; pH 7.4) – Gibco Catalog #: 10010023

BSA (Bovine Albumin Fraction V 7.5%) – Gibco Catalog #: 15260037

BD ReticCount Thiazol Orange (TO, 0.01mg/mL, 200 mL, store at 4°C) – BD Catalog #: 349204

Brilliant Violet 421 anti-human CD41 antibody – Biolegend Catalog #: 303729

Vybrant DyeCycle Ruby Stain – Thermo Fisher Scientific Catalog #: V10309

CD 61 magnetic beads, MicroBeads – Miltenyi Biotec Catalog #: 130-051-101

MS columns – Miltenyi Biotec #: 130-042-201

*These authors contributed equally

Correspondence: Isabell Bernlochner, Department of Cardiology, Klinikum Rechts Der Isar, Ismaningerstr. 22, Munich 81675, Germany. E-mail: isabell.bernlochner@tum.de

Table I. Troubleshooting.

Step	Problem	Possible Reason	Solution
3	Leucocyte contamination	Buffy coat was aspirated	Only take the upper part of plasma. Use DNA-dye to gate only for DNA negative cell (s. steps 14 and 26).
6	Aggregation	Platelets activated	Do not pipet up and down, control concentration of all inhibitors (ADPase, PGE_1, EDTA, citric acid).
7	No pellet visible	Platelets disrupted	Use centrifuge with acc/dec settings at the lowest level.
17	Activation/ aggregation	Fast shaking activated the platelets	Only shake at the lowest speed, if that is too fast: do not put the sample on shaker but tip against the tube every 5–10 min.
27	No visible TO stain	TO staining not sufficient	Make sure to mix the sample after TO addition at steps 11–17.
34	Bubbles	Plunger was pushed too slowly	Increase the speed of gently pushing the plunger. Pull the column out of the solution while pushing.
35	No pellet	Platelets float in supernatant	Increase the centrifugation speed or time.
37	Low outcome of platelets	Not enough beads bound to platelets	Mix the beads before use.

Equipment

BD FACS Aria III Cell Sorter – BD Biosciences Catalog #: 648282

Neubauer counting chamber – Brand Catalog #: 717805

Rotating shaker – Heidolph Instruments Catalog #: 544-41200-00

MACS separator Octomacs – Miltenyi Biotec #: 130-042-109

MACS Multistand – Miltenyi Biotec #: 130-042-303

Procedure

Blood withdrawal, timing 5–10 min

(1) Take 4 × 3 mL venous blood from a donor into tubes with 3.2% citrate dextrose. Use a Safety-Multifly needle no smaller than 21 gauge. Alternatively, a Safety-Multifly needle connected to a 20 mL syringe containing citrate can be used. Throw away the first 1 mL of blood to prevent hemolysis and turn the tubes up and down 1–2 times to mix blood and citrate. If using a tourniquet, apply it loosely to not activate platelets during the procedure.

CRITICAL: Proceed with no delay to the next step. In order to avoid platelet activation and RNA degradation, we suggest to process all samples immediately after phlebotomy. Consult Table 1 for the whole troubleshooting guideline.

Production of platelet-rich-plasma, timing 1.5 h

(2) Centrifuge the vials at 200 × *g* for 10 min at room temperature without brake.

CRITICAL: Set acceleration and deceleration to the lowest level.

(3) The sample is now separated into red blood cells (RBC), buffy coat and plasma. Take only the plasma phase. Depending on the donor, take ~1–2 mL of plasma per vial and collect it in a fresh 15 mL tube. Be careful to not aspirate the buffy coat.

(4) Add apyrase to prevent ADP-mediated platelet activation (0.6 U ADPase/mL) to the plasma. For example, add 6 µL of the aliquot solution to 1 mL of plasma. Additionally, add 1 µM prostaglandin E_1 (e.g. 10 µL from the PGE_1 aliquot to 1 mL of plasma). Carefully tip against the tube to mix.

(5) Incubate the sample in a 37°C water bath for 30 min.

(6) Further platelet aggregation can be prevented by adding 5 mM EDTA (e.g. 10 µL stock/mL plasma) and reducing the pH adding 1 mM citric acid (e.g. 10 µL of stock/mL plasma). Gently roll the tube without inversion to mix, do not pipet up and down. CRITICAL: EDTA irreversibly denaturates many proteins including the integrin complex GPIIb/IIIa. Consider avoiding the usage of EDTA if downstream proteomic investigation is planned.

(7) Immediately centrifuge the sample at 800 × *g* for 15 min at room temperature without brake.

CRITICAL: Set acceleration and deceleration to the lowest level.

(8) Discard the upper two-thirds of the solution and add the following reagents to the lower one-third of the sample: 0.6 U Apyrase/mL (e.g. 6 µL of stock/ml), 20 mM of HEPES (e.g. 20 µL of stock/ml) and 1 mM EGTA (e.g. 10 µL of stock/ml). Carefully resuspend the pellet in the lower one-third of the solution.

CRITICAL: Use large pipette tips (e.g. 1 mL tips) for resuspension to prevent platelet activation.

(9) Add 1 µM PGE_1 (e.g. 10 µL from stock/ml), mix by tipping against the tube and incubate the sample for 5 min at room temperature.

Platelet Counting, timing 10 min

(10) To our knowledge, no automatic counter can detect cells under 4 µm diameter. Thus, we suggest counting platelets using the traditional method with a counting chamber: Take 10 µL of the PRP and add 90 µL of PBS. Analyze 10 L of this diluted PRP using a Neubauer counting chamber. CRITICAL: A minimum of 100 platelets must be counted.

Staining, timing 45 min

(11) Prepare an unstained control sample by adding 50 µL of the PRP solution to 200 µL PBS.

(12) Addition of Thiazole Orange and CD 41 antibody:

Dilute the PRP to 6×10^5 platelets/µL PRP. Take ~1.8×10^8 platelets (e.g. 300 µL of PRP containing 6×10^5 platelets/µL PRP). Add Thiazole Orange reaching the concentration of 6.7 µg/mL (e.g. 600 µL of 0.01 mg/mL stock) and 1:200 v/v CD41-BV (e.g. 4.5 µL).

CRITICAL: The whole staining procedure should be carried out in the dark.

NOTE: Thiazol Orange is light sensitive and must be stored at 2–8°C in the dark. It can be used until the expiration date. Further information can be found in the manufacturer's protocol.

(13) Cover the sample in aluminum foil and put it on a shaker for 10 min, rotating at the slowest speed.

(14) OPTIONAL Add 1:500 v/v DNA dye Vybrant Red (e.g. 1.8 µL for 300 µL PRP and 600 µL TO) and gently roll the tube without inversion to mix.

(15) Incubate the sample for additional 20 min on the shaker at the slowest speed.
(16) Addition of magnetic beads:

Add 18.5 µL CD61-magnetic beads/10^7 cells. Keep a ratio of 5/1, solution/beads (e.g. 333.3 µL CD61-magnetic beads to 300 µL of PRP containing ~1.8×10^8 platelets). Add PBS to sustain the volume ratio of 1 part beads to 5 parts of total volume (1:5 v/v ratio), (e.g. 433.3 µL PBS; v/v 1666 µL solution/333.3 µL beads) and mix well by pipetting up and down with a 1 mL pipette tip.

CRITICAL: Mix the beads well before use to ensure the distribution of the magnetic tags.

(17) Cover the sample again and incubate 10 min on the shaker rotating at the slowest speed.

CRITICAL: Comply with suggested incubation time with TO for a total of 30 min.
Prepare samples for Flow Cytometry, timing 15–20 min

(18) Centrifuge the sample and the unstained control at 1000 × *g* for 12 min with brake.
(19) Aspirate the supernatant by applying vacuum, being careful not to mobilize the pellet.
(20) Add 400 µL PBS containing 1 mM EGTA and 0.6 U/mL Apyrase (e.g. 4 µL and 2.4 µL of stock, respectively) to the unstained sample and resuspend.

Add 2000 µL PBS containing 1 mM EGTA and 0.6 U/mL Apyrase (e.g. 20 µL and 6 µL of stock, respectively) to the sample and resuspend.

(21) After resuspension, add 1 µM PGE1 to both samples and incubate for 5 min at room temperature.
(22) Filter each solution through a 35 µm cell strainer of a 5 mL round-bottom polystyrene tube and put on ice.
(23) Prepare 6 to 8 RNase-free low-bind collection tubes for the sorting by adding 1 mL PBS to each tube. Store the tubes on ice until ready for collection.

Sorting at the Flow Cytometer, timing 1.5–2.5 h

(24) Gate for platelet size at appropriate FSC threshold in an FSC/SSC log plot, excluding the cells with the largest FSC and SSC pattern (Figure 1A).

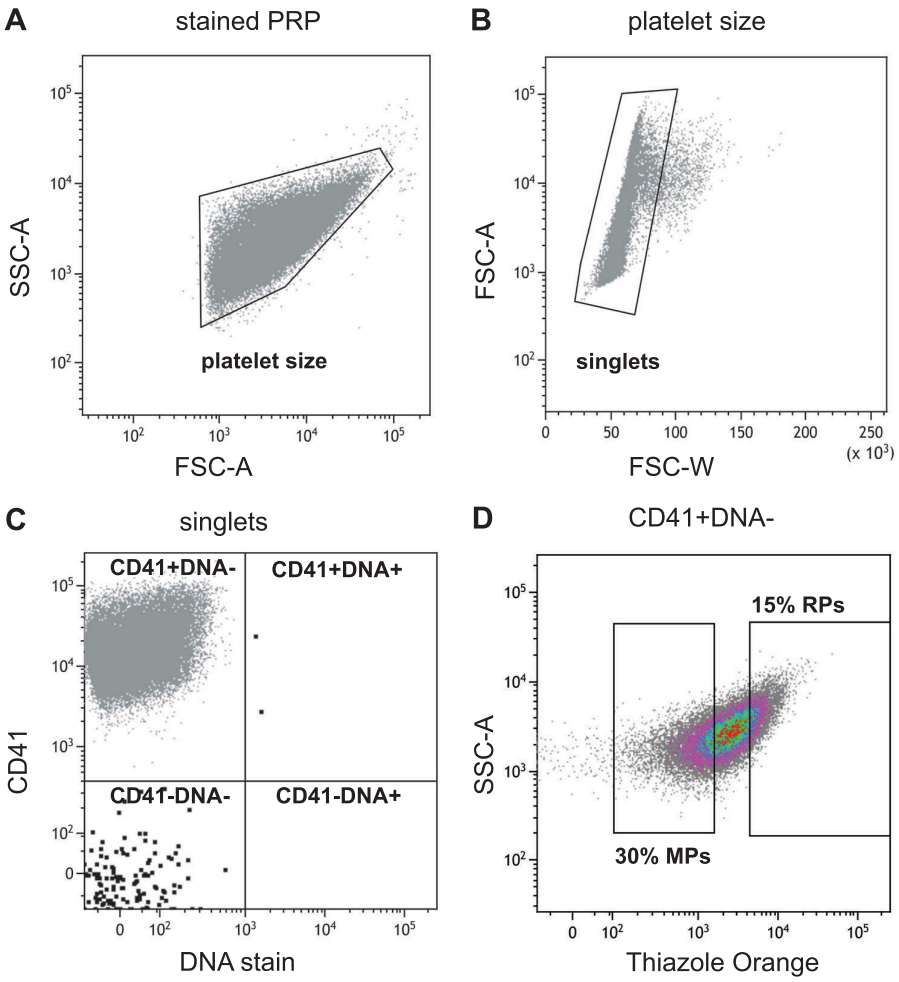

Figure 1. Flow Cytometry sorting strategy. (A) Applied gate to stained platelet-rich-plasma (PRP) separating the platelets by size using FSC/SSC. (B) Doublets are excluded by a high FSC-width. (C) Sorting strategy after CD41 and DNA staining for platelet isolation. Platelets are selected by gating CD41 positive and DNA negative events. CD41-BV = 421 nm, DNA stain Ruby = 638 nm. (D) Gating strategy for RNA^{low} platelets (mature platelets, MPs) and RNA^{high} platelets (reticulated platelets, RPs). Platelets with the 15% highest TO signal are defined as RPs, platelets with the lowest 30% TO signal as MPs (TO = 488−550 nm). TO: Thiazole Orange, BD FACS Aria III, SSC: Side Scatter, FSC: Forward Scatter. RPs: reticulated platelets, MPs: mature platelets.

(25) Exclude doublets in the FSS-W (linear)/FSC-A (log) plot by gating out cells with a higher FSC-width (Figure 1B).
(26) Gate for CD41 positive and DNA negative (CD41+/DNA-) events to avoid leucocyte contamination (Figure 1C).
(27) For the TO gating strategy plot TO fluorescence against SSC. The 15% TO-brightest cells consist of RPs (RNA^{high}). OPTIONAL: the 30% TO-lowest (RNA^{low}) can be used as control for mature platelets (MPs). (Figure 1D).

Sort the RPs by using the 70 µL nozzle at purity precision and (optional) the MPs populations.

CRITICAL: Verify purity after sorting (Figure 1E,F).

(28) Using a BD FACS Aria III, up to 6×10^6 platelets can be sorted into 15 mL low binding tube in around 45 min.

Magnetic Separation, timing 30–60 min

(29) Prepare a buffer containing 0.5% BSA and 2 mM EDTA in PBS (e.g. add 2.67 mL of BSA stock and 200 µL of EDTA stock to 36 mL PBS, store at 4°C).
(30) Place 2–4 columns in a magnetic field of a MACS separator and put a collection basin underneath.
(31) Prepare the columns by rinsing 500 µL of buffer per column.
(32) Pour the cell suspensions onto the columns.

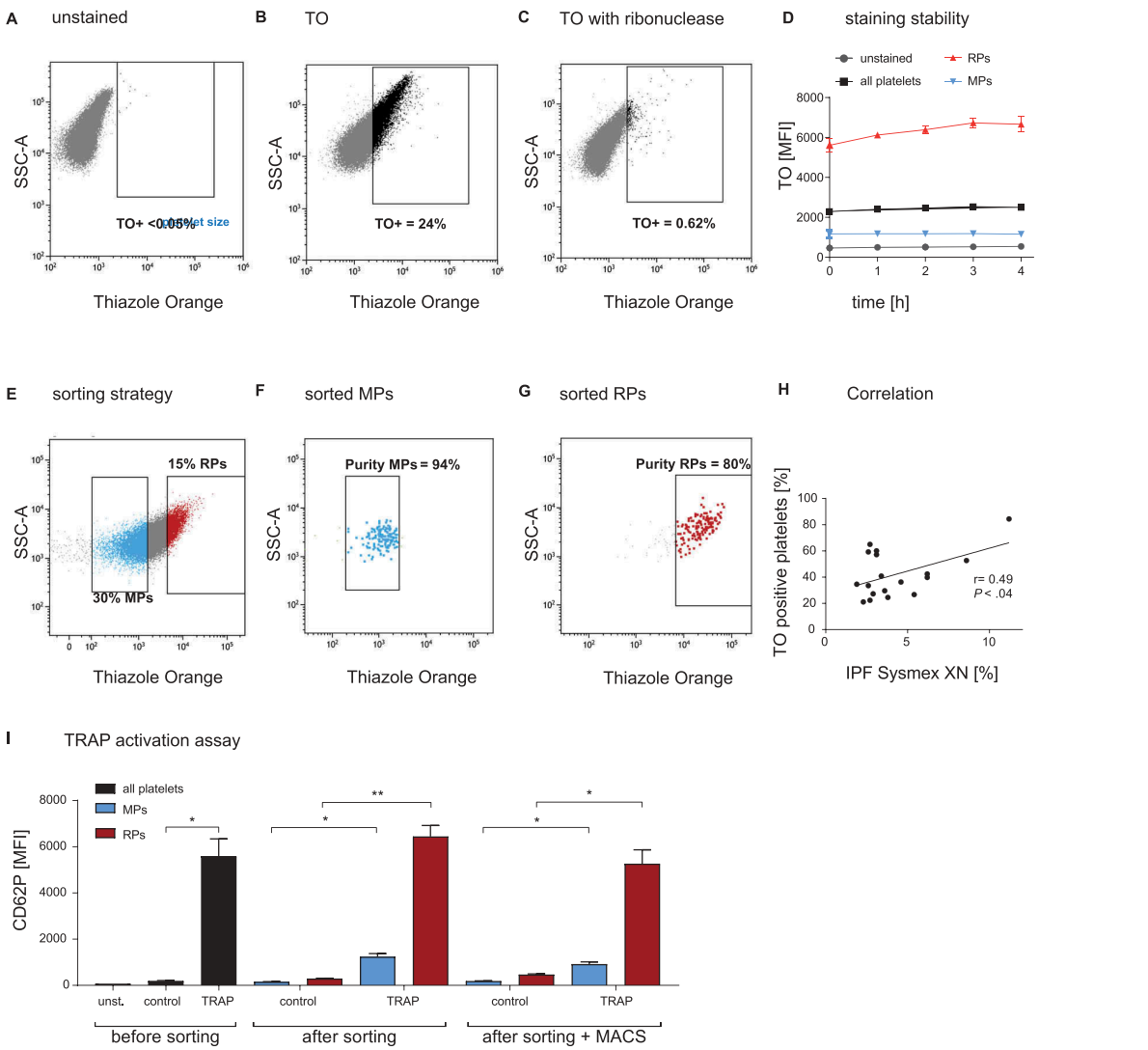

Figure 2. Validation. (A–C) RNase-treatment of platelets after permeabilization with saponin: dot plots showing (A) the unstained sample, (B) >24% TO positive events for the TO stained sample and (C) 0.62% TO positive events with the use of RNase and TO. (D) Staining signal stability of TO staining over time from four healthy subjects (median and range; h = hours). One-way ANOVA did not detect any statistical differences from one to 4 h (MPs $P = .83$, RPs: $P = .28$, all platelets: $P = .13$). (E) Gating strategy for RNA^{low} platelets (mature platelets, MPs) and RNA^{high} platelets (reticulated platelets, RPs). Platelets with the 15% highest TO signal are defined as RPs, platelets with the lowest 30% TO signal as MPs (TO = 488 − 550 nm). (F) Exemplary purity analysis after sorting of MPs and the RPs (G) gated populations. (H) Scatter plot showing the correlation of TO positive cells (based on unstained controls) with the immature platelet fraction (IPF) measured with the point of care system Sysmex XN in 18 patients with CAD. (I) TRAP activation assay showing the CD62P expression of PRP before sorting and sorted MPs and RPs after sorting and after MACS column separation (for methods see supplemental file). A concentration of 5 µM TRAP was added to induce activation. * = P value <.05, ** = P value <.01, non-significant relations are not shown. TO: Thiazole Orange, BD FACS Aria III, Thiazole Orange: 488–550 nm, SSC: Side Scatter, MFI: mean fluorescence intensity, unst.: unstained, sap.: saponin, CAD: coronary artery disease, CD62P: *P*-Selectin, TRAP: thrombin receptor activation peptide.

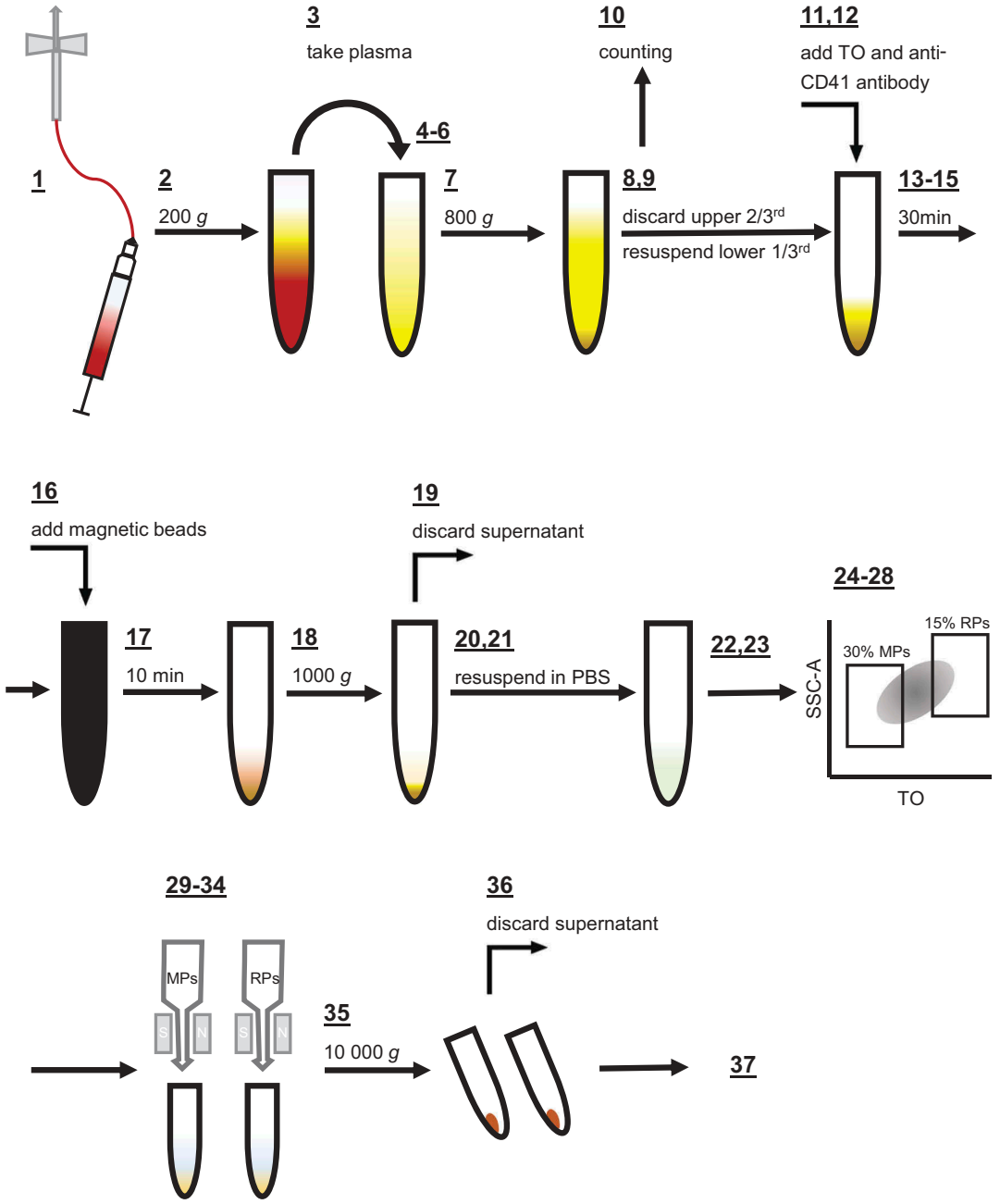

Figure 3. Schematic overview of the isolation method. Steps 1 to 37 are indicated as bold and underlined characters. RPs: reticulated platelets, MPs: mature platelets, SSC-A: side scatter, TO: Thiazole Orange, × *g*: times gravity, S and N: south and north magnetic poles.

CRITICAL Only separate up to 10×10^6 platelets per column in order to not overload the column.

(33) Wash the columns three times with 500 µL buffer, always waiting until the column is dry before applying the buffer.
(34) Remove column from the magnetic field and place it on a sterile RNase-free pre-chilled collection tube. Elute platelets with 1 mL of buffer by gently pushing the plunger. If using more than one column, increase the elution volume to a maximum of 1.2 mL. (For 2 RNA^{high} columns, elute each sample in 600 µL buffer and collect the elution in the same tube.)
(35) Centrifuge the samples at 12 000 × *g* for 10 min at 4°C. Keep the centrifuge at 4°C and place samples on ice.
(36) Carefully remove the supernatant with a 200 µL pipette tip without touching the pellet.
(37) The pellet is now ready for downstream applications.

Discussion

We describe a reliable standardized validated method to detect, sort and collect reticulated platelets (RPs) from peripheral blood allowing downstream applications in order to investigate their biology. RPs are large, RNA-rich, prothrombotic and hyperactive platelets [1–4]. They are known to be elevated in high-risk populations such as diabetics and smokers as well as in patients with coronary artery disease and acute coronary syndromes [2,5–7].

Over the last years, high levels of RPs in peripheral blood have been shown to be associated with an insufficient antiplatelet response to thienopyridines and aspirin after coronary artery percutaneous interventions, making them an important drug target [4,8]. Moreover, elevated RPs in peripheral blood are strong predictors of mortality and adverse cardiovascular events in patients with coronary artery disease as well as in other pathological settings such as severe sepsis and after major surgery. [7,9,10] However, the reason for these correlations as well as of the hyperactive phenotype of RPs was unknown until recently. Our novel method enabled us to compare the transcriptome of RPs with that of mature platelets for the first time. Interestingly, we detected a significant enrichment of pro-thrombotic mRNA in RPs compared to mature platelets providing the first biological explanation of RPs hyper-reactive nature [11].

The limited knowledge concerning the biology of RPs is mainly attributable to the absence of standardized isolation and sorting methods. In general, platelet isolation is extremely challenging as these anuclear cells are prone to adhesion and activation. To avoid unspecific activation, the addition of several inhibitory substances is recommended [12]. In addition, depending on the downstream application, cell fixation is often not possible, as fixation agents are known to inhibit the activity of retro-transcriptase. No specific marker is known for RPs and the gold standard method to study them is based on cell size and measurement of the RNA-content with the fluorescent dye Thiazole Orange (TO) [2,13–17]. With this regard, several different approaches with distinct TO concentrations have been used often starting from whole blood instead of PRP [16,18,19]. Of importance, these methods have been used only for flow cytometry so far without testing their feasibility for platelet sorting. We demonstrate with our RNase experiments that staining of RPs with TO, if used at a correct concentration and incubation time, is highly RNA specific (Figure 2, s. supplemental file for RNase experiment methods) and allows correct and reproducible staining and isolation of these cells (Figure 1). Moreover, we propose a new column-based magnetic cell isolation method (graphical summary in Figure 3) to improve platelet recovery after sorting and to avoid unnecessary high-speed centrifugation, which may injure the platelets and promote unspecific activation. At the same time, using platelet-specific CD61-MicroBeads-antibodies we gain an additive checkpoint for positive selection avoiding contamination with other cell types.

However, TO-staining still bears several limitations as its specificity is time- and concentration-dependent. In particular, prolonged staining time and excessively high concentrations can promote unspecific labeling to platelet alpha granules, which affects RNA-specificity and alters the results [20,21]. For these reasons, concentration and staining time should be carefully titrated and always verified with RNase experiments (Figure 2). With our method, we proved that TO-signal is RNA-specific (Figure 2A-C) and stable for up to 4 h (Figure 2D). Moreover, platelet activation assays using thrombin receptor activation peptide (TRAP) proved that the sorted platelets are viable and still able to react upon stimulation (Figure 2I). Of note, TRAP-induced activation showed a significant higher expression of the activation marker *P*-Selectin (CD62P) in RPs compared to MPs, confirming the hyperreactive nature of the isolated RPs.

Hille et al. [22] proposed a new method for staining and sorting of RPs based on the fluorescent dye SYTO^{TM} 13. Despite some very interesting aspects of this approach, information about the specificity of SYTO^{TM} 13 to bind RNA is lacking, because RNase-treatment experiments are not shown. Moreover, the provided data do not allow a direct comparison of the SYTO^{TM} 13 method with the TO method due to several reasons: 1) In their experiments, the authors used uncommonly low concentrations of TO of only about 1/10 of the established and suggested TO concentration in literature to detect platelet RNA [2,15–17] and 2) the authors used unusually long incubation times for TO of up to 90 min, which does not adhere to previous experiences in literature [2,16,17] and which is known to affect the RNA specificity of TO.

Recently, Angénieux et al. [23] investigated the reliability of HLA I/MHC I cell surface expression with flow cytometry as a marker of RPs. The authors reported that a higher expression of HLA I/MHC correlated with an increased TO staining, proposing this marker as a new valuable parameter to identify RPs in patients with thrombocytopenia. However, like TO, HLA I/MHC I is not specific for RPs as it is also expressed in lower amounts in older platelets. In addition, it was not tested as a marker to guide platelet sorting.

Conclusion

We herein present a structured and reproducible method to detect, isolate and collect RPs in peripheral blood by RNA-specific staining with TO and subsequent magnetic labeling allowing deep biological investigation of these cells.

Declarations and Disclosure of Conflict of Interest

The authors declare no competing financial interests. This study obtained approval from the Ethics Committee of the Technical University Munich, Ismaninger Straße 22, 81675 Munich, Germany. All participants have given written informed consent for this study. The financial support from the German society of cardiology (grant DGK, Forschungsstipendium, DGK102018 to D.B.) and the European society of cardiology (ESC first contact initiative grant 2018 to D.B.) is gratefully acknowledged.

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ORCID

Melissa Klug http://orcid.org/0000-0001-5193-2770
Donato Santovito http://orcid.org/0000-0003-1811-4746
Dario Bongiovanni http://orcid.org/0000-0002-4162-1482

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3.2 Publication 2: Mass Cytometry of Platelets from PRP

Citation

The article titled "Mass cytometry of platelet-rich plasma: a new approach to analyze platelet surface expression and reactivity" was published online at Taylor & Francis on 27 December 2021 (www.tandfonline.com).

Full Citation:
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3.2.1 Summary and Author Contributions

Summary

In this publication, we propose a new methodological pipeline to perform mass cytometry of platelets with and without activation stimuli. Assessing platelet receptor expression at single cell level with high resolution is possible using mass cytometry by time of flight (CyTOF). Whereas previous CyTOF research mainly focused on leukocyte subgroup analysis, we use it to decipher platelet heterogeneity and are the first to use platelets from PRP. Published protocols until now only allow platelet analysis from whole blood, and require samples to be measured immediately after staining. With our approach, we offer a standardized technique that allows the preparation, staining, acquisition and analysis of platelets from PRP with high resolution. The study performed includes the validation of a common freezing technique on platelets after staining which simplifies platelet measurement due to the possibility of sample storage in -80°C until acquisition. Moreover, our standardized method to measure platelet reactivity includes the usage of different external stimuli and offers a reproducible protocol for a subsequent bioinformatic investigation of the data. Using platelets from PRP

allows the acquisition of a high cell number that is required for deep bioinformatic analysis of different platelet subgroups. We use our previously designed freely available analysis pipeline optimized for platelets to assess differential expression between differently treated platelets and investigate overall platelet heterogeneity. Our protocol can be further used to investigate platelet heterogeneity using CyTOF in several disease settings.

Author Contribution

In the following M.K. refers to Melissa Klug, the author of this doctoral thesis. Together with D.B. and I.B., M.K. designed the research. Additionally, M.K. wrote the initial draft of the paper, while D.B. and I.B. added their thoughts. All experiments were carried out by M.K., who guided her master student K.K. and MD student J.H. to perform experiments alongside. G.V. and M.K. took turns in recruiting healthy donors and collecting their blood. M.K. created all the figures besides Figure 1, which was done by K.K under the guidance of M.K. After learning from M.R. who acquired the fresh and frozen samples, M.K. operated the CyTOF alone and acquired all other CyTOF data presented in the paper with K.K or J.H helping in the preparation of the samples. Normalization, gating, transformation, viSNE and differential expression analysis was carried out by M.K. All other bioinformatic analyses were carried out by K.K., O.L, J.B. and M.L. Finally, M.K. discussed the results with D.B. and I.B. who gave further input for interpretation and reviewed the paper alongside M.S., J.R., G.C., K.L. and M.L..

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Journal title: Platelets
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Year of publication: *
Name: Melissa Klug
Street address: Ismaninger Strasse 22
Town: Munich
Postcode/ZIP code: 81675
Country: Germany
Email: melissa.klug@tum.de
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Mass cytometry of platelet-rich plasma: a new approach to analyze platelet surface expression and reactivity

Melissa Klug [1,2,3], Kilian Kirmes [1], Jiaying Han [1], Olga Lazareva [3], Marc Rosenbaum [4,5], Giacomo Viggiani [1], Moritz von Scheidt [2,6], Jürgen Ruland [4,5], Jan Baumbach [7], Gianluigi Condorelli [8], Karl-Ludwig Laugwitz [1,2], Markus List [3], Isabell Bernlochner [1,2], & Dario Bongiovanni [1,2,8]

[1]*Department of Internal Medicine I, School of Medicine, University Hospital Rechts der Isar, Technical University of Munich, Munich, Germany,* [2]*German Center for Cardiovascular Research (DZHK), Partner Site Munich Heart Alliance, Munich, Germany,* [3]*Chair of Experimental Bioinformatics, Technical University of Munich, Freising, Germany,* [4]*School of Medicine, Institute of Clinical Chemistry and Pathobiochemistry, Technical University of Munich, Munich, Germany,* [5]*TranslaTUM, Center for Translational Cancer Research, Technical University of Munich, Munich, Germany,* [6]*Department of Cardiology, Deutsches Herzzentrum München, Munich, Germany,* [7]*Chair of Computational Systems Biology, University of Hamburg, Hamburg, Germany, and* [8]*Department of Cardiovascular Medicine, Humanitas Clinical and Research Center Irccs and Humanitas University, Rozzano, Italy*

Abstract

Mass cytometry (CyTOF) is a new technology that allows the investigation of protein expression at single cell level with high resolution. While several protocols are available to investigate leukocyte expression, platelet staining and analysis with CyTOF have been described only from whole blood. Moreover, available protocols do not allow sample storage but require fresh samples to be obtained, processed, and measured immediately. We provide a structured and reproducible method to stain platelets from platelet-rich plasma to study thrombocyte protein expression and reactivity using mass cytometry. With our method, it is possible to acquire a large number of events allowing deep bioinformatic investigation of platelet expression heterogeneity. Integrated in our protocol is also a previously established freezing protocol that allows the storage of stained samples and to delay their measurement. Finally, we provide a structured workflow using different platelet stimulators and a freely available bioinformatic pipeline to analyze platelet expression. Our protocol unlocks the potential of CyTOF analysis for studying platelet biology in health and disease.

Keywords

Mass cytometry, platelet heterogeneity, platelet reactivity, platelets

History

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Method

Materials

Biological Materials

Twelve to eighteen milliliters of human peripheral blood. Caution: all experiments involving human subjects must comply with the declaration of Helsinki and be carried out following national and institutional guidelines. Written informed consent must be obtained.

Reagents and Consumables

- Safety-Multifly needle 21 G with tube 80 mm, DEHP-free - Sarstedt Catalog #: 85.1638.203
- 3 mL S-Monovette® tubes, Citrate 3.2% (1:10), 66 × 11 mm, green EU code, paper label, 50/inner box sterile – Sarstedt Catalog #: 05.1165.001
- 15 mL conical tubes – Thermo Fisher Scientific Catalog #: 10136120
- 15 mL conical tubes – Eppendorf Catalog #: 0030122151
- 5 mL round-bottom polypropylene tubes – Thermo Fisher Scientific Catalog #: 10314791
- 5 mL tubes with cell strainer – Corning Catalog #: 10585801
- Adenosine 5′-diphosphatase, Apyrase from potatoes (10 U/100 µL in PBS stock) – Sigma-Aldrich Catalog #: A6535-100UN
- CRP-XL (collagen-related peptide) – Cambcol Laboratories Catalog #: CRP-XL
- Thrombin receptor activating peptide (TRAP-6-Amide) – Bachem Catalog #: 4031274
- Prostaglandin E1 (C20H34O5 1 mg/mL DMSO stock, 0.1 mg/mL in PBS) – EMD Millipore Corp. Catalog #: 538903
- EDTA ($C_{10}H_{14}N_2Na_2O_8$ * 2 H_2O, 0.5 M, pH 8.0) – Invitrogen Catalog #: 5575020
- Citric Acid ($HOC(COOH)(CH_2COOH)_2$, 1 M stock) – Sigma Aldrich Catalog #: 251275
- HEPES ($C_8H_{18}N_2O_4S$, 1 M buffer solution, pH 7.2–7.5) – Thermo Fisher Scientific Catalog #: 15630080
- Ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid ($[-CH_2OCH_2CH_2N(CH_2CO_2H)_2]_2$; EGTA, 0.5 M stock, pH 8) – Sigma-Aldrich Catalog #: E3889
- PBS (Ca2+ and Mg2+ free; pH 7.4) – Gibco Catalog #: 10010023
- FBS (Fetal Bovine Serum) - Sigma Aldrich - Catalog #: F7524
- DMSO (dimethyl sulfoxide) - Sigma Aldrich - Catalog #: D2650
- AB Stabilizer, PBS base - Boca Scientific- Catalog #: 131050
- Maxpar® Cell Staining Buffer (CSB) - Fluidigm Catalog #: 201068
- Maxpar® Fix and PermBuffer - Fluidigm Catalog #: 201067

Correspondence: Isabell Bernlochner Klinikum Rechts der Isar, Klinikum Rechts der Isar, Technische Universität München, Ismaninger Str. 22, Ismaninger Str. 22, 81675, München, Germany
Isabell.bernlochner@mri.tum.de; bongiovanni@tum.de

- Maxpar® Cell Acquisition Solution (CAS) - Fluidigm Sciences - Catalog #: 201240
- Maxpar® X8 Multimetal Labeling Kit - Fluidigm - Catalog #: 201300
- Cell-ID™ Intercalator-Ir - Fluidigm - Catalog #: 201192A
- Cell-ID™ Cisplatin - Fluidigm - Catalog #: 201064
- EQ Four Element Calibration Beads - Fluidigm - Catalog #: 201078

Antibodies

- CD42a (GR-P)- 141Pr - Thermo Fisher Scientific - Catalog #: MA1-91023
- CD62P (KO-2-7)-161Dy- Thermo Fisher Scientific - Catalog #: MA1-81809
- GPVI (HY101)-175Lu - Thermo Fisher Scientific - Catalog #: 14–9813-81
- PAC1 (Activated GPIIbIIIa/αIIbβ3)-155Gd - BD Biosciences - Catalog #: 340535
- PAR1 (ATAP2) −147Sm - Thermo Fisher Scientific - Catalog #: 35–2200
- PEAR (492621)-174Yb - Novusbio - Catalog#: MAB4527
- CD107a/LAMP1 (H4A3)-151Eu - Fluidigm Sciences - Catalog #: 3151002B
- CD154/CD40L (24-31)-168Er - Fluidigm Sciences - Catalog #: 3168006B
- CD29 (TS2/16)156Gd - Fluidigm Sciences - Catalog #: 3156007B
- CD3 (UCHT1)-170Er - Fluidigm Sciences - Catalog #: 3170001B
- CD31/PECAM-1 (WM59)-145Nd - Fluidigm Sciences - Catalog #: 3145004B
- CD36 (5–271)152Sm - Fluidigm Sciences - Catalog #: 3152007B
- CD40 (5C3)-142 Nd - Fluidigm Sciences - Catalog #: 3142010B
- CD41 (HIP8)-89Y - Fluidigm Sciences - Catalog #: 3089004B
- CD42b (HIP1)-144Nd - Fluidigm Sciences - Catalog #: 3144020B
- CD47 (CC2C6)209B - Fluidigm Sciences - Catalog #: 3209004B
- CD61 (VI-PL2)-146Nd - Fluidigm Sciences - Catalog #: 3146011B
- CD63 (H5C6)-150Nd - Fluidigm Sciences - Catalog #: 3150021B
- CD69 (FN50)162Dy - Fluidigm Sciences - Catalog #: 3162001B
- CD9 (SN4 C33A2)-171Yb - Fluidigm Sciences - Catalog #: 3171009B

Equipment

- Helios CyTOF system – Fluidigm
- Rotating shaker – Heidolph Instruments Catalog #: 544–41200-00
- Heraeus Megafuge 1.0 R - Thermo Fisher Scientific Catalog #: 75003041

Procedure

Panel Design

We chose a set of 21 antibodies, most of them known to be expressed on the platelet surface and involved in several platelet aggregation and adhesion processes. Additionally, known platelet activation markers are included as well as negative markers to exclude contamination of other cell types.

Non commercially available antibodies were conjugated in-house, strictly following the step-wise protocol by Maxpar (Maxpar Antibody Labeling User Guide). All in-house conjugated antibodies were IgG antibodies except for PAC1, which is an IgM antibody that has been previously tested to be suitable for the Maxpar antibody labeling process without losing its functionality to specifically bind the activated alphaIIbetaIII complex[1]. In-house conjugated antibodies need to be titrated after preparation before use.

Platelet Rich Plasma

Platelet-rich-plasma is generated as described in detail before by Bernlochner et al[2] and shown in Figure 1. Briefly, whole blood is centrifuged without brake at 200 x g within 30 min after collection before Apyrase (0.6 U ADPase/mL) and PGE1 (1 μM prostaglandin E1) are added to the plasma. 30 min incubation at 37°C is followed by an addition of 5 mM EDTA and 1 mM citric acid. After a second round of centrifugation without brake at 800 x g, the pellet is resuspended in the lower ⅓ of the plasma in 0.6 U Apyrase/mL, 20 mM of HEPES and 1 mM EGTA .

CRITICAL: When investigating PAC1, EDTA should be avoided as high dosages denatures the integrin complex GPIIb/IIIa. However, without EDTA the samples may show a low activation (low expression of activation markers). It is critical to verify this step and to be consistent with the entire experimental pipeline.

Viability Staining

(1) Carefully add 600 μL PRP and 400 μL PBS into a LoBind tube to reach an approx. concentration of 1×10^8 platelets per mL.
(2) Stain cells for 5 min with 1 μL per 1 mL sample Cell-ID Cisplatin (5 μM final concentration) at room temperature to distinguish live from dead cells.
(3) Stop staining by adding 5 mL Maxpar Cell staining buffer (CSB) (5 times the volume of the cell suspension).
(4) Centrifuge suspension immediately at 300 g for 5 min (with brake) and discard supernatant carefully. From here on, all centrifugation steps are carried out with brake.

CRITICAL: Discard the supernatant with a pipet and not a vacuum pump to avoid platelet activation

(5) Resuspend the cells in 50 μL CSB buffer (approx. 1–3 x 10^6 platelets in total volume).

Platelet Stimulation

(6) Stimulate previously resuspended platelets from step 5 in-vitro for 2 min by adding 10 μM TRAP (7.7 μL of 130 μM stock), or 2 μg/mL CRP-XL (2.8 μL of 0.07 μg/μl stock).

OPTIONAL: Alternatively, ADP can be added at this step, for example 5 μM or 20 μM ADP, as described before[3,4].

(7) Also other known platelet stimulators can be used at this point. An appropriate concentration should be determined experimentally beforehand.

CRITICAL: Wait 2 minutes before adding the antibody cocktail to ensure proper platelet stimulation.

(8) This protocol can be used to find different expression profiles of platelet subgroups and the overall platelet population in several settings. See Figure 2 for the analysis of the different platelet activators in 4 healthy donors. As expected, the activation marker expression increases after stimulation. Furthermore, other differences between baseline and stimulated samples in healthy donors are shown. For example, according to previous studies, the expression of CD42a and CD42b receptors goes down after TRAP stimulation, while CD42a is also lower expressed after CRP-XL simulation[1,5].

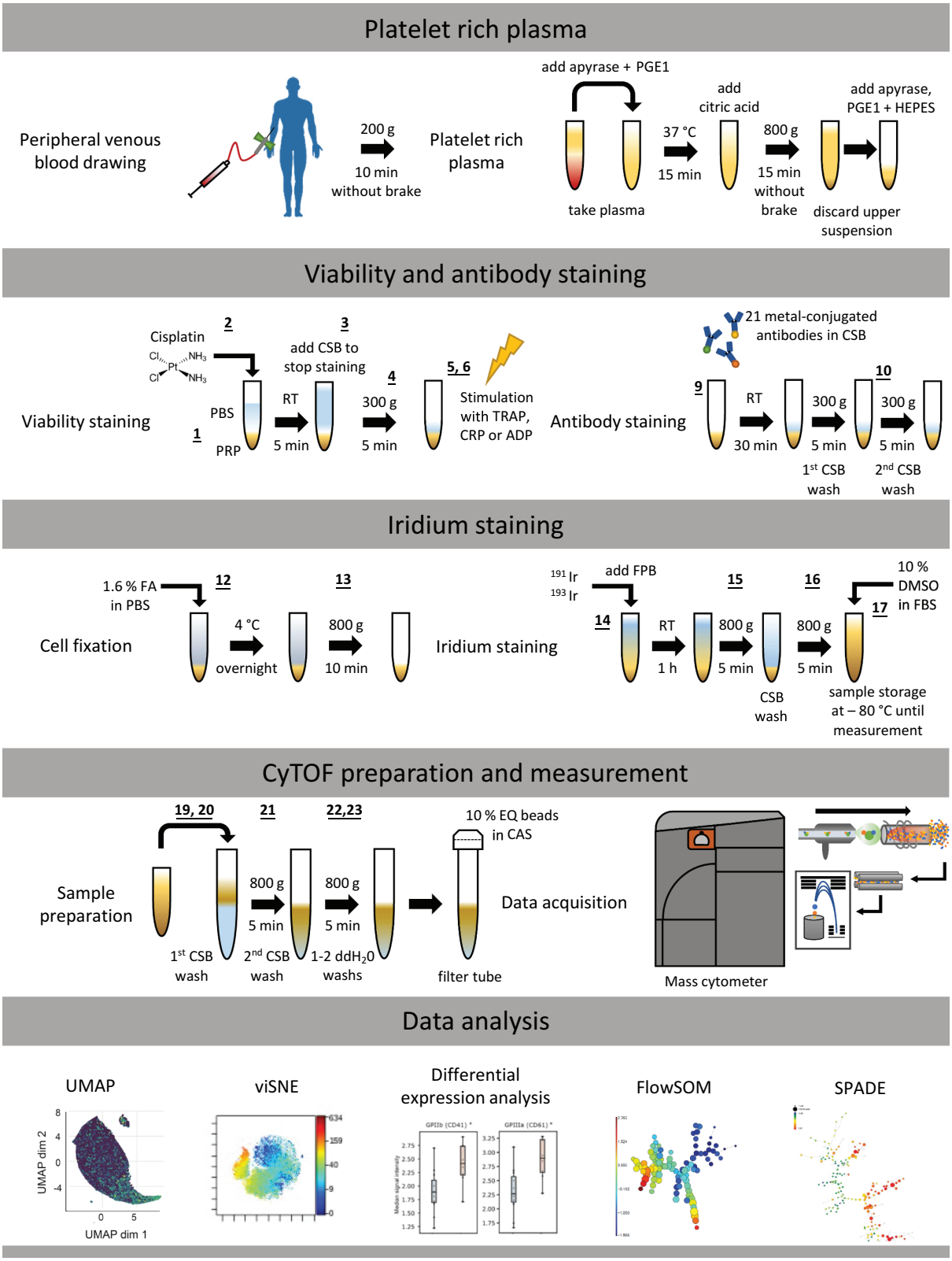

Figure 1. Graphical abstract. Schematic illustration of the CyTOF workflow. Steps 1 to 23 are indicated as bold and underlined characters. If not indicated differently, all centrifugation steps are carried out at RT with brake. × *g*: times gravity, TRAP: thrombin receptor-activating peptide, CRP-XL : collagen-related peptide, ADP: adenosine diphosphate, CAS: cell acquisition solution.

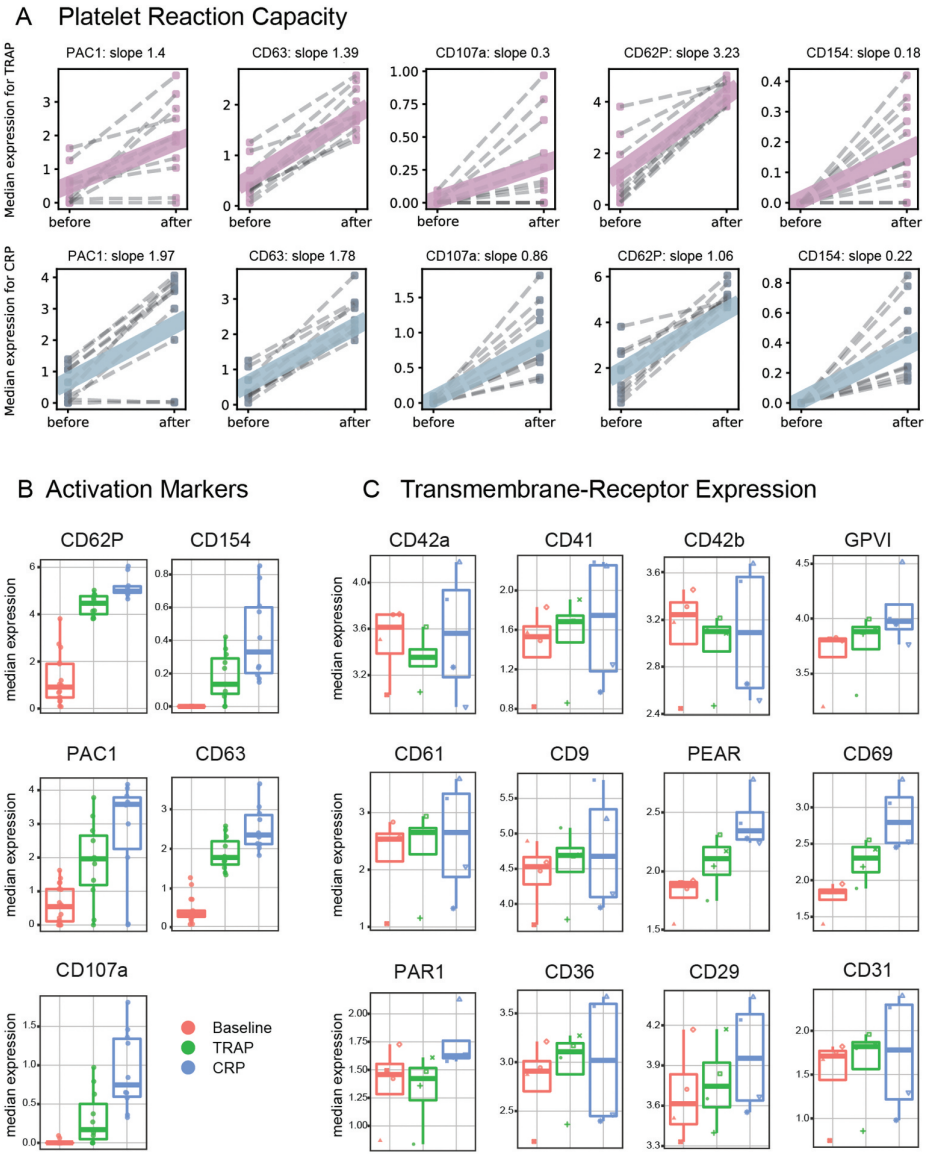

Figure 2. A. Platelet reaction capacity. Median signal intensity increase of activation marker expression after 10 µM TRAP- (red, first row) or 2 µg/mL CRP-XL- stimulation (blue, second row) compared to non-stimulated platelets (baseline). Linear model analysis detected a higher expression of activation markers after CRP-XL stimulation. (α: signal increment slope coefficient; ns: non-significant. N = 11 healthy donors for TRAP, N = 10 healthy donors for CRP-XL. B. Activation marker expression in baseline (red, N = 17), TRAP stimulated (green, N = 11) and CRP-XL stimulated (blue, N = 10) samples as well as C. relevant transmembrane receptor expression. The horizontal line within the box plot represents the median, the top and bottom the interquartile range (Q1–Q3), whisker bars indicate the largest observation that is less than or equal to the upper inner fence (UIF = Q3 + 1.5 × IQR) or the smallest observation that is greater than or equal to the lower inner fence (LIF = Q1–1.5 × IQR) and each dot represents the mean expression of one sample. N = 4 healthy donors.

Antibody Staining and Wash

(9) Add 50 µL antibody cocktail (cocktail is mixed according to the table in Appendix A of the "Maxpar Cell Surface Staining with Fresh Fix" protocol). Cells are stained for 30 min at room temperature.

OPTIONAL: Prepare the antibody mixture beforehand like previously described[6]. Signal loss and antibody aggregates can occur, therefore previously published protocols should be followed [7].

(10) Wash cells twice by adding 2 mL Maxpar CSB buffer to the suspension and centrifuged at 300 g for 5 min and the supernatant is carefully aspirated.

(11) The cell pellet gets resuspended by firstly adding EGTA to a final concentration of 1 mM and secondly adding 200 µL PBS buffer.

CRITICAL: Ensure the complete disruption of the pellet.

Cell Fixation

(12) Prepare fresh 1.6% formaldehyde solution and add 1 mL to the cell suspension and incubate the cells overnight at 4°C.

OPTIONAL: Incubation can also take place for 10 minutes at RT. Both incubation periods (short and overnight) are

recommended by the state-of-the-art MaxPar protocol (Maxpar Cell Surface Staining with Fresh Fix) guideline.

(13) Centrifuge at 800 g for 10 min (with break) and discard the supernatant carefully.

Iridium Staining

(14) Add 1 mL Maxpar Fix and Perm buffer and Cell-ID™ Intercalator-Ir—125 µM (1:1000) and incubate the suspension for 1 h at room temperature.

OPTIONAL: Iridium staining can also be performed for up to 48 h at 4°C before data acquisition.

(15) Centrifuge the cells at 800 g for 5 min and discard the supernatant carefully.
(16) Resuspend the cells in 2 mL Maxpar CSB buffer and centrifuge the cells again for 5 min at 800 g and again discard the supernatant.

Cell Freezing

(17) The cells get resuspended in 1 mL 10% DMSO/ Fetal bovine serum (FBS). Samples can be stored at – 80°C until measurement.
(18) The freezing process at this point does not alter signal profiles. Please see Figure 4 for the comparative analysis of fresh versus frozen samples of two healthy donors.

CRITICAL: Freezing process may cause cell loss which makes it less suitable for methods using whole blood as these samples only have a low platelet count. By using PRP more than enough platelets are present even after several rounds of washing after thawing the samples.

Sample Preparation before Acquisition

(19) Thaw the samples on ice and centrifuge them down using a bench-top centrifuge for 10 sec before continuing.
(20) 1 mL of the sample is used and 2 mL CSB buffer is added into a 5 ml polypropylene tube. The suspension is centrifuged at 800 g for 5 min and the supernatant is discarded. Repeat this wash step once.
(21) In the third wash step add 2 mL ddH2O/MilliQ water and centrifuge the sample at 800 g for 5 min and discard the supernatant.
(22) A fourth wash step with water can be performed if the signal at the machine shows artifacts or contamination.
(23) The sample is left pelleted at 4°C until measurement.

CyTOF Measurement

(24) Resuspend the pelleted sample in a 10% EQ beads in CAS (cell acquisition solution) solution in an approximate dilution of 1x 10^6 platelets/mL.
(25) Analyze samples on a Helios Mass Cytometer (Fluidigm Corporation, San Francisco, CA). We use the following parameters: lower convolution threshold 400, event length 10–150, sigma 3, signal subtraction 0.

OPTIONAL: Adjust sample acquisition parameters according to the experiment.

(26) Acquire up to 500,000 events per sample depending on the required bioinformatic analysis at a rate of 200–350 events/sec. The acquisition of additional events is possible.

CRITICAL: In mass cytometry, higher cell numbers than in flow cytometry are needed for accurate performance of dimensionality reduction methods. Only with a high number of acquired events, meaningful platelet subclusters have the chance to be discovered.

(27) After acquisition, normalize data using the Fluidigm software.

CyTOF Analysis

(28) Transfer normalized FCS data to your platform of interest.
(29) We recommend gating according to the Maxpar state-of-the-art (Approach to Bivariate Analysis of Data Acquired Using the Maxpar Direct Immune Profiling Assay) protocol. The recommended gaussian parameter clean-up procedure assures exclusion of noise, technical artifacts and ion fusion events. On top, CD41 and CD61 positive events are used to discriminate for platelets only as previously described[8].

CRITICAL: At this step it is important that enough cells have been acquired and that the washing process has been performed according to this protocol. Otherwise, high technical noise can be present which leads to the exclusion of many events and a high amount of cell loss.

(30) Files need to be transformed before any further analysis. Kaluza, FlowJo, Cytobank (https://premium.cytobank.org/cytobank/), OMIQ (https://www.omiq.ai/) or the R catalyst package (https://github.com/HelenaLC/CATALYST) can be used to transform data for subsequent analysis.

OPTIONAL: Compensation might need to be applied. Therefore, create a compensation matrix using catalyst as described previously[9]. The acquisition of single-stained samples is necessary for this step.

(31) For dimensionality reduction methods, Cytobank, OMIQ or R (catalyst, diffcyt) are preferable sources. Besides many others, these platforms offer algorithms like viSNE, diffusion map, FlowSOM and UMAP[10,11]. See Figure 3 for an exemplary viSNE analysis of one healthy donor.

CRITICAL: In the absence of bioinformatic knowledge, oftentimes clustering is performed ill-suited for platelets and other cell types. Most importantly, to assess differential expression of functional markers across clusters, they cannot be used for the clustering process itself[12]. As typical lineage markers are lacking within the platelet populations, all platelet surface markers not associated with platelet activation are used for the generation of clusters. Markers used previously to gate for platelets (in this study CD41 and CD61) also should not be used for the clustering. If activation markers are taken into account, differences in terms of different “Islands” within the viSNE plots will only be visible between stimulated and non-stimulated samples. More robust and reliable results are achieved when following the best practice guidelines presented in this paper.
(32) Differential expression analysis can be performed using the R packages diffcyt which is also available as a user-friendly shiny app (https://exbio.wzw.tum.de/cyanus/) with the possibility to use many differential expression analysis methods[13].

Discussion

We present a structured and reliable method to stain and analyze platelets from PRP with CyTOF. This technique enables to record the expression of up to 40 markers at once which severely

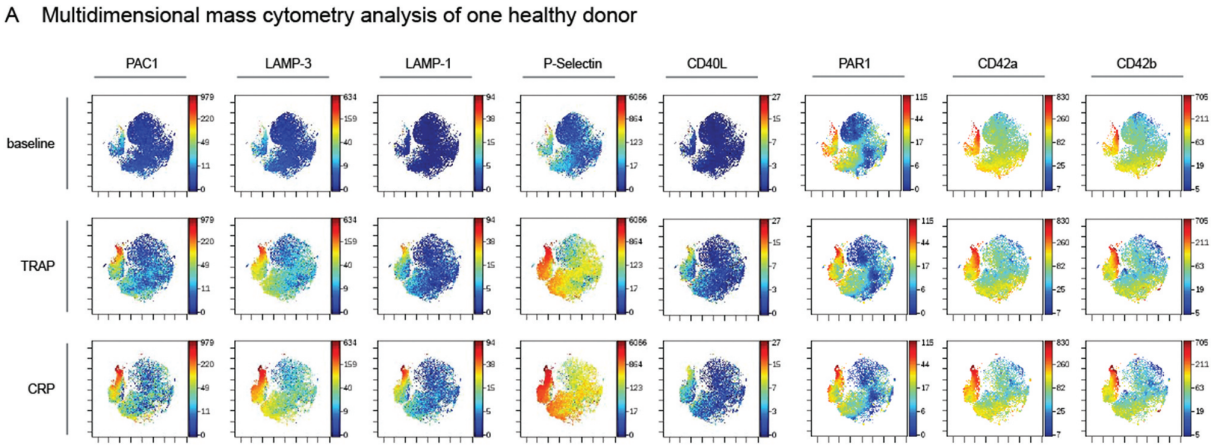

Figure 3. Multidimensional analysis of platelet subpopulations. viSNE plots of one exemplary donor are shown. Samples were stained with a metal-labeled antibody cocktail containing 21 markers. Color intensity refers to antigen expression (low [blue] or high [red]) and each dot represents a single platelet. viSNE = visual stochastic neighbor embedding.

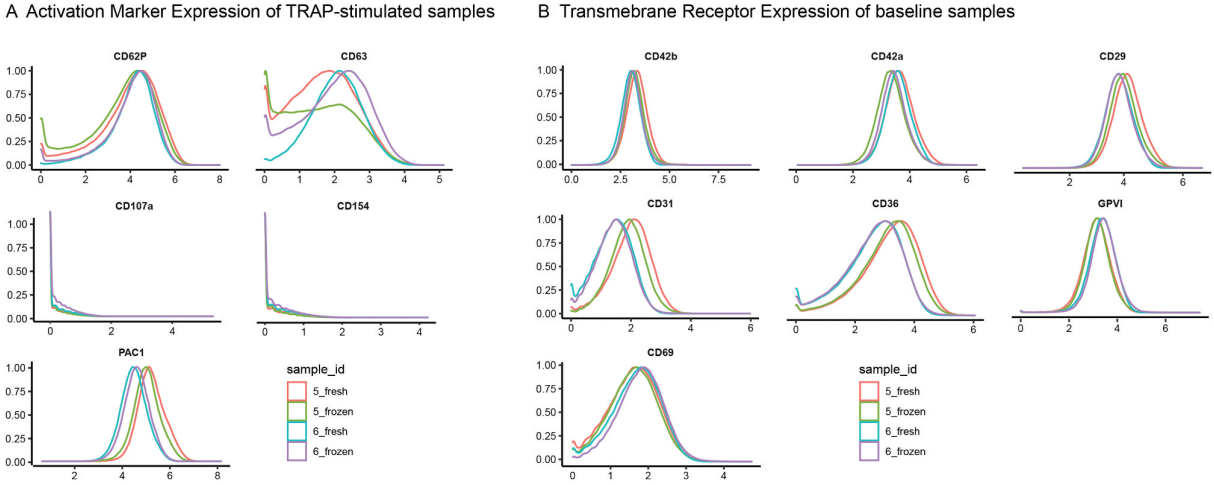

Figure 4. Normalized smoothed densities of marker expression (arcsinh transformed) of A. Activation markers of TRAP-stimulated samples and B. Transmembrane receptor expression of baseline samples. Plots show the density curve for each of the four samples (2 fresh, 2 frozen, n = 2).

improves simultaneous investigation of platelet protein expression heterogeneity.

PRP generation is a common technique in platelet research to concentrate platelets from peripheral blood minimizing the risk of contamination from other cells. It has been proven suitable for platelet research including reactivity studies [14–17]. Recently, PRP has also been used for the first time in mass cytometry studies examining platelet reactivity and investigating platelet subtypes during SarsCoV2 infection and after BNT162b2 vaccination[8,18]. PRP generation from whole blood has been described by several groups before; with the use of the different platelet inhibitors it is quick, simple and reliable as it allows to avoid cell contamination and to maintain thrombocyte reactivity. The use of PRP in mass cytometry allows to save rather expensive CyTOF-antibodies in a high-concentrated platelet solution. On the contrary, staining whole blood, which is characterized by a low platelet concentration wastes cell material and antibodies leading to fewer recorded platelet events in longer time of measurement[1]. With our method along with following the state-of-the-art clean-up protocol using gaussian parameters (Maxpar protocol), it is possible to acquire 300,000 to 500,000 events which enables the investigation of extremely small platelet subgroups of the entire platelet population [19,20]. This is of particular importance as platelets are, in comparison to other cells, a rather homogeneous group. Thus it is necessary to acquire a high number of cells in order to achieve the desirable power to perform suitable bioinformatic analyses.

Nevertheless, CyTOF allows for the first time investigation of platelet heterogeneity. When investigating disorders that require the analysis of large platelets like macrothrombocytopenia, a protocol that uses whole blood might be beneficial. Nonetheless, the use of PRP is the method of choice for several other cardiovascular diseases. We compared the pre-processing of our protocol to whole blood data from Spurgeon et al.[6]. and concluded that by using PRP instead of whole blood less cells are lost in the gating process which improves efficiency and quality (Supplementary Figures 1 and 2). While CyTOF protocols and workflows are usually made for other cell types, our protocol has been designed and optimized for platelets only and allows other

researchers to further investigate platelet heterogeneity with high power and resolution.

For CyTOF analysis in general and platelet analysis in particular, we have developed the freely available analysis pipeline CYANUS[13] and an adaption which is built on analysis scripts previously employed in two CyTOF studies[8,18]. The analysis pipeline is based on diffcyt,[21,22] and presents an alternative to commercially available tools. Additionally, it offers the possibility to perform differential expression analysis between groups or conditions and introduces a novel analysis method CyEMD (a model-free approach using the earth mover's distance). This method was specifically tailored for the characteristics of platelet markers which can show extreme zero-inflation that biases previously existing methods[13].

CYANUS, which is included in this protocol, has been proven extremely useful to compare fresh versus frozen platelet samples and to rule out that the freeze-thawing process impairs the protein expression signal (Figure 4).

The presented freezing process offers the possibility to delay the measurement and to accumulate samples, reducing the batch effect by acquiring all samples in the same run[23]. This approach has been proven useful in several studies using other cell types [6,24]. In our experience, freezing in DMSO/FBS lowers the chances of cell clots that could alter and complicate sample analysis. Therefore, acquisition time can possibly be decreased using DMSO/FBS.

We acknowledge that CyTOF protocols for platelets, originally developed for other cell types, still require further improvements. The proposed antibody panel (Supplemental table 1) requires expansion. However, to date, only a small number of platelet antibodies is commercially available and applicable for CyTOF and the validation of suitable antibodies is resource demanding. Of equal importance would be the extension of the platelet antibody panel to intracellular markers. Although they would probably require an additional cell permeabilization step, they would expand the possibilities to discover further, yet unknown platelet subgroups.

Conclusion

We present a structured and reproducible method to prepare, stain, and acquire platelets from platelet-rich-plasma as well as to decipher and analyze platelet expression heterogeneity using a Helios mass cytometer. Our described method provides a tool that allows to detect and further characterize unknown platelet subgroups among the heterogeneous human platelet pool.

Acknowledgements

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Author Contributions

M.K., D.B. and I.B. designed the research and wrote the paper. M.K., K. K. and J.H. performed the experiments and made the figures. K.K., O.L., M.L. and J.B. did the bioinformatic analyses. G.V. recruited the donors. M.K. and M.R. acquired the data at the CyTOF. M.S., J.R., G.C., K.L., M.L., I.B. and D.B. interpreted the results and reviewed the paper.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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ORCID

Melissa Klug http://orcid.org/0000-0001-5193-2770
Kilian Kirmes http://orcid.org/0000-0001-6940-0983
Jiaying Han http://orcid.org/0000-0003-4158-4826
Olga Lazareva http://orcid.org/0000-0001-9546-7807
Marc Rosenbaum http://orcid.org/0000-0001-6834-385X
Giacomo Viggiani http://orcid.org/0000-0003-2843-4188
Moritz von Scheidt http://orcid.org/0000-0001-7159-8271
Jürgen Ruland http://orcid.org/0000-0002-8381-3597
Jan Baumbach http://orcid.org/0000-0002-0282-0462
Gianluigi Condorelli http://orcid.org/0000-0003-0481-6843
Karl-Ludwig Laugwitz http://orcid.org/0000-0003-4948-4846
Markus List http://orcid.org/0000-0002-0941-4168
Isabell Bernlochner http://orcid.org/0000-0002-0981-839X
Dario Bongiovanni http://orcid.org/0000-0002-4162-1482

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3.3 Publication 3: SARS-CoV-2 Infection is Associated with a Pro-Thrombotic Platelet Phenotype

Citation

The article titled "SARS-CoV-2 infection is associated with a pro-thrombotic platelet phenotype" was published at Nature Cell Death & Disease on 5 January 2021.

Full Citation:
"Bongiovanni, D., Klug, M., Lazareva, O. et al. SARS-CoV-2 infection is associated with a pro-thrombotic platelet phenotype. Cell Death Dis 12, 50 (2021). https://doi.org/10.1038/s41419-020-03333-9."

3.3.1 Summary and Author Contributions

Summary

As increased cardiovascular events and abnormal coagulation parameters were reported during SARS-CoV-2 infection, we aimed to investigate platelets from COVID-19 patients. In this study, we compared platelet transmembrane receptor and adhesion marker expression from 8 hospitalized COVID-19 patients to 11 healthy donors. Therefore, we investigated platelets at baseline level and after in vitro TRAP-stimulation using mass cytometry by time of flight (CyTOF). Our customized antibody panel consisting of 21 surface proteins allowed the largest investigation of platelets using CyTOF to date. In line with the clinical observations, we detected an increased reactivity of platelets from COVID-19 patients as they expressed activation markers P-Selectin and LAMP-3, as well as several adhesion markers involved in platelet-platelet or platelet-leukocyte interactions significantly higher than platelets from healthy donors. Although a higher expression of activation markers in COVID-19 patients was also detected after TRAP-stimulation, the overall platelet reaction capacity was lower than in the healthy donors. This impaired reaction capacity as well as the high baseline reactivity of platelets in COVID-19 patients suggest a hyperreactive platelet phenotype during SARS-CoV-2 infection. Our findings are in compliance with other researchers aiming to

investigate benefits of antiplatelet therapy in COVID-19 patients.

Author Contribution

The author of this dissertation, M.K, conceived of and designed the experiments with D.B. and I.B.. With guided help of master student M.B, M.K. conducted the experiments while S.U. acquired the data at the CyTOF at the Medical Facility in Ulm. M.K. preprocessed and analyzed the data, while O.L. and M.L. conducted the major bioinformatic analysis upon consultation with M.K. and D.B.. S.W. and Ma.L. collected the samples and recruited the patients. Along with D.B. C.S. G.C. K.L., M.K. interpreted the results. Additionally, M.K. wrote the manuscript together with D.B., while O.L and M.L. added the bioinformatic methods part. All authors revised the manuscript.

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Bongiovanni et al. *Cell Death and Disease* (2021)12:50
https://doi.org/10.1038/s41419-020-03333-9

Cell Death & Disease

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SARS-CoV-2 infection is associated with a pro-thrombotic platelet phenotype

Dario Bongiovanni[1,2,3], Melissa Klug[1,2,4], Olga Lazareva[4], Simon Weidlich[5], Marina Biasi[1], Simona Ursu[6], Sarah Warth[6], Christian Buske[6,7], Marina Lukas[5], Christoph D. Spinner[5], Moritz von Scheidt[2,8], Gianluigi Condorelli[3], Jan Baumbach[4], Karl-Ludwig Laugwitz[1,2], Markus List[4] and Isabell Bernlochner[1,2]

Abstract

Novel coronavirus disease 2019 (COVID-19) is associated with a hypercoagulable state, characterized by abnormal coagulation parameters and by increased incidence of cardiovascular complications. With this study, we aimed to investigate the activation state and the expression of transmembrane proteins in platelets of hospitalized COVID-19 patients. We investigated transmembrane proteins expression with a customized mass cytometry panel of 21 antibodies. Platelets of 8 hospitalized COVID-19 patients not requiring intensive care support and without pre-existing conditions were compared to platelets of healthy controls (11 donors) with and without in vitro stimulation with thrombin receptor-activating peptide (TRAP). Mass cytometry of non-stimulated platelets detected an increased surface expression of activation markers P-Selectin (0.67 vs. 1.87 median signal intensity for controls vs. patients, $p = 0.0015$) and LAMP-3 (CD63, 0.37 vs. 0.81, $p = 0.0004$), the GPIIb/IIIa complex (4.58 vs. 5.03, $p < 0.0001$) and other adhesion molecules involved in platelet activation and platelet–leukocyte interactions. Upon TRAP stimulation, mass cytometry detected a higher expression of P-selectin in COVID-19 samples compared to controls ($p < 0.0001$). However, we observed a significantly reduced capacity of COVID-19 platelets to increase the expression of activation markers LAMP-3 and P-Selectin upon stimulation with TRAP. We detected a hyperactivated phenotype in platelets during SARS-CoV-2 infection, consisting of highly expressed platelet activation markers, which might contribute to the hypercoagulopathy observed in COVID-19. In addition, several transmembrane proteins were more highly expressed compared to healthy controls. These findings support research projects investigating antithrombotic and antiplatelet treatment regimes in COVID-19 patients, and provide new insights on the phenotypical platelet expression during SARS-CoV-2 infection.

Introduction

Despite severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)'s worldwide spread, little is known about the pathophysiological mechanisms leading to multiorgan damage in coronavirus disease 2019 (COVID-19). A hypercoagulable state with increased incidence of cardiovascular complications and venous thrombotic events has been reported in several studies[1–7]. Abnormal coagulation parameters are observed in hospitalized patients and are associated with poor prognosis[8–10]. Interestingly, two studies reported in COVID-19 alterations in platelet transcriptome and proteome, and an increased platelet reactivity[11,12]. A recent study described the presence microvascular thrombi in lung, heart and kidney containing neutrophil extracellular traps (NETs) in severe SARS-CoV-2 infection, as well as circulant neutrophil-platelet aggregates and immunothrombotic dysregulation,

Correspondence: Dario Bongiovanni (bongiovanni@tum.de) or Isabell Bernlochner (Isabell.bernlochner@tum.de)
[1]Department of Internal Medicine I, School of Medicine, University hospital rechts der Isar, Technical University of Munich, Munich, Germany
[2]German Center for Cardiovascular Research (DZHK), Partner Site Munich Heart Alliance, Munich, Germany
Full list of author information is available at the end of the article
These authors contributed equally: Dario Bongiovanni, Melissa Klug
These authors jointly supervised this work: Markus List, Isabell Bernlochner
Edited by A. Stephanou

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which changes with disease severity[13]. Recently, a computational system's medicine platform identified as new drug target several proteins involved in the coagulation cascade[14,15]. However, the role of platelet activation and changes of transmembrane receptor expression in COVID-19-induced coagulopathy still needs to be further investigated.

Platelets not only play a pivotal role in vascular hemostasis but are also involved in immune response, tumor progression, and other inflammatory processes[16]. They are activated during sepsis and in septic shock, and antiplatelet therapy has been suggested as a novel strategy to prevent organ damage[17]. In fact, in the presence of severe infections or cytokine storms[18,19], platelet hyperreactivity may be responsible for major cardiovascular adverse events[20]. Viral infections are known to be associated with coagulation disorders[21]. Interestingly, an increased incidence of acute coronary syndrome has been observed after influenza infection[22], suggesting that viral diseases could trigger platelet activation leading to cardiovascular complications. Moreover, viral-induced coagulopathies have been already observed in SARS-CoV-1 infection including thrombocytosis, disseminated intravascular coagulation, and thromboembolism[23,24]. In this study, we investigated the expression of platelet transmembrane receptors and adhesion molecules at baseline level and after in vitro platelet stimulation in hospitalized COVID-19 patients without pre-existing conditions and in healthy donors using mass cytometry by time of flight (CyTOF). Here we present the largest existing CyTOF panel of platelet antibodies specifically developed to investigate platelet activation and adhesion (Table 1).

Table 1 Mass cytometry panel.

Antigen	Common name	Biological function
CD107a	LAMP-1	Cell adhesion, activation marker
CD141	Thrombomodulin	Thrombin-binding protein
CD154	CD40L, CD40 ligand	Regulation of platelet–leukocyte interactions
CD29	Integrin subunit β1	Fibronectin and collagen receptor subunit
CD3	TCR–CD3 complex	Adaptive immune response, negative control
CD31	PECAM-1	Cell adhesion
CD36	GPIV	Thrombospondin receptor, cell adhesion
CD40	TNFRSF5	Mediates immune and inflammatory responses
CD41	Integrin αIIb, GPIIb	α-Unit of fibrinogen receptor
CD42a	GPIX	Von Willebrand factor receptor unit
CD42b	GPIbα	Von Willebrand factor receptor unit
CD47	MER6	adhesion receptor for THBS1 on platelets
CD61	Integrin β3, GPIIIa	β-Unit of fibrinogen receptor
CD62P	P-Selectin	Cell adhesion, activation marker
CD63	LAMP-3	Cell adhesion, platelet activation marker
CD69	CLEC2C	Signal transmission in NKCs and platelets
CD9	Tetraspanin-29	Cell adhesion, integrin binding
F2R	Par1	Thrombin receptor
GPVI	Platelet glycoprotein 6	Collagen receptor
GPIIbIIIa	GPIIb/GPIIIa complex	GPIIb/GPIIIa complex-specific antibody
PEAR1	JEDI	Platelet endothelial aggregation receptor

Methods

Data and code availability

All mass cytometry data have been made available at flowrepository.org and can be accessed at repository ID FR-FCM-Z2MT. The scripts used in this analysis have been deposited at github.com and can be accessed at https://github.com/biomedbigdata/SARS-CoV-2-platelets-analysis.

Study design and participants

SARS-CoV-2-infected patients hospitalized at the Klinikum recht der Isar, Munich, Germany, between March and May 2020 with symptomatic COVID-19 not requiring intensive care unit admission and without known pre-existing conditions were recruited in our study and compared to an asymptomatic control cohort of healthy donors. Inclusion criteria for the COVID-19 group were a symptomatic (dyspnea) SARS-CoV-2 infection confirmed by a positive reverse-transcription PCR assay from any respiratory specimen or IgM antibodies in peripheral blood, age between >18 and <70 years, and written informed consent.

Exclusion criteria were known platelet dysfunctions, relevant thrombocytopenia (<100 G/l) or thrombocytosis (>500 G/l), impaired renal function (glomerular filtration rate < 60ml/min), hemoglobin < 10g/dl, leukocytes < 1 G/l, any known pre-existing condition except arterial hypertension, any medication except antihypertensive drugs, and a history of hematological neoplasia including active lymphoma, mental impairment, or pregnancy.

Blood samples were collected from patients within the first 36 h after admission. As a control group, we recruited a healthy and asymptomatic cohort of donors. All healthy donors were tested negative for SARS-CoV-2 IgG and

IgM, were followed up, and did not develop any symptoms in the weeks following the recruitment. Throughout the entire study design, patients' samples were handled together with control samples. The study complied to the Declaration of Helsinki, was approved by the local ethics committee (approval numbers 147/20 and 352/18), and all participants provided written informed consent.

Sample collection and preparation

Peripheral venous blood was collected in citrate tubes and immediately processed to produce platelet-rich plasma (PRP) as described before[25,26]. CyTOF staining assay was performed according to the manufacturer's protocols. Briefly, 600 µl PRP previously inhibited by a mixture of 0.6 U Apyrase/ml, 20 mM of HEPES, and 1 mM EGTA was diluted in phosphate-buffered saline (PBS) pursuant to the gold standard protocol for mass cytometry (Fluidigm, San Francisco, CA, USA) to a final concentration of 10^5 platelets/µl. The PRP was stained with 5 µM Cell-ID™ Cisplatin (Fluidigm) for 5 min and then washed with 5 ml MaxPar Cell Staining Buffer (Fluidigm). After centrifugation, cells were resuspended in 50 µl Cell Staining Buffer. Two samples were prepared from each donor: one baseline sample (non-stimulated platelets) and one sample stimulated with 10 µM thrombin receptor-activating peptide (TRAP). TRAP addition was followed by a 2 min incubation at room temperature. In the same cell suspensions platelets were stained with 50 µl of the custom-made CyTOF-antibody panel in Cell Staining Buffer for 30 min (containing anti-CD3-170Er, anti-CD9-171Yb, anti-CD29-156Yb, anti-CD31-145Nd, anti-CD36-152Nd, anti-CD40-142Nd, anti-CD41-89Y, anti-CD42a-141Pr, anti-CD42b-144Nd, anti-CD47-209Bi, anti-CD61-146Nd, anti-CD62P-161Dy, anti-CD63-150Nd, anti-CD69-162Dy, anti-CD107a-151Eu, anti-CD141-166Er, anti-CD154-168Er, anti-GPVI-175Lu, anti-GPIIb/GPIIIa complex-155Gd, anti-Par1-147Sm, and anti-PEAR-147Sm; see Supplemental Material for antibody information). After washing twice with 2 ml Cell Staining Buffer at 300 g for 5 min, cells were fixed overnight at 4 °C in 1 ml of 1.6% Formaldehyde. After fixation, cells were pelleted at 800 × *g* for 10 min, the supernatant was aspirated and removed. Then, cells were gently vortexed and resuspended in the residual volume (~50 µl) and incubated with 125 nM Iridium in 1 ml MaxPar Fix and Perm Buffer (Fluidigm) for 1 h following the manufacturer's protocol (Fluidigm). Afterwards, they were centrifuged at 800 × *g* for 5 min, then washed with 2 ml Cell Staining Buffer at 800 g for 5 min, then frozen in 10% dimethyl sulfoxide (DMSO) in fetal bovine serum until acquisition[27]. After thawing the samples, they were washed twice with Cell Staining Buffer and once with water at 800 × *g* for 5 min to eliminate DMSO remnants. Cells were then handled according to the manufacturer's protocol. Prior to measurement, cells were diluted to a final concentration of 10^3 platelets/µl before addition of EQ calibration beads. Cells were measured using a Helios mass cytometer (Fluidigm). Throughout the study, patients' samples were measured with at least one control sample to reduce batch effect. In total, 476,756 ± 151,746 events were acquired at a rate of 300–500 events per second. Experiments were carried out by the same scientist and antibodies were from the same lot. See Supplemental Material for a complete reagent list.

Mass cytometry

CyTOF allows multidimensional relative protein quantification for single-cell datasets and we adapted it for platelets using a customized mass cytometry panel of 21 antibodies (Table 1). For custom-made antibody conjugations, 100 mg of carrier-free antibody was coupled to metal-labeled X8 polymer according to the manufacturer's instructions (Fluidigm). Briefly, using the MaxPAR antibody conjugation kit (Fluidigm) following the manufacturer's recommended protocol, six antibodies were conjugated to isotopically enriched lanthanide metals. After labeling, the antibodies were stored in an antibody stabilization buffer (Boca Scientific, Westwood, MA, USA) at 4 °C. The other antibodies were pre-conjugated, CyTOF-ready, and commercially available (Fluidigm Sciences). Please see the Supplemental Materials for the reagent list. All custom-conjugated antibodies were validated with calibration beads. In detail, 0.5 µl of the conjugated antibody was added to one drop of beads and incubated for 15 min. After two washing steps with 1.5 ml PBS at 300 × *g* for 10 min, the mixture was washed twice with de-ionized water at 300 × *g* for 10 min, and resuspended in 200 µl water until acquisition.

CyTOF processing

After acquisition, samples were cleaned up according to the latest standard of data pre-gating (Fluidigm) using the Cytobank™ software (www.cytobank.org, Beckman-Coulter, Brea, CA, USA)[28]. To avoid leukocyte contamination, we gated the acquired events for platelet-specific markers: only CD41 (GPIIb)- and CD61-(GPIIIa) positive events were selected for further analysis and defined as platelets (Supplemental Fig. I). CD3 marker was included in the panel as an additional negative control (Supplemental Fig. II).

Computational analysis

All models were built and assessed using the statsmodels v0.11.1 python package[29]. CyTOF data were processed using Cytobank and analyzed using R 4.0 (R Development Core Team, 2005) and Python 3.6 (Python 3 Reference Manual, 2009). For visualization of activation markers in reduced dimensions, we performed uniform manifold

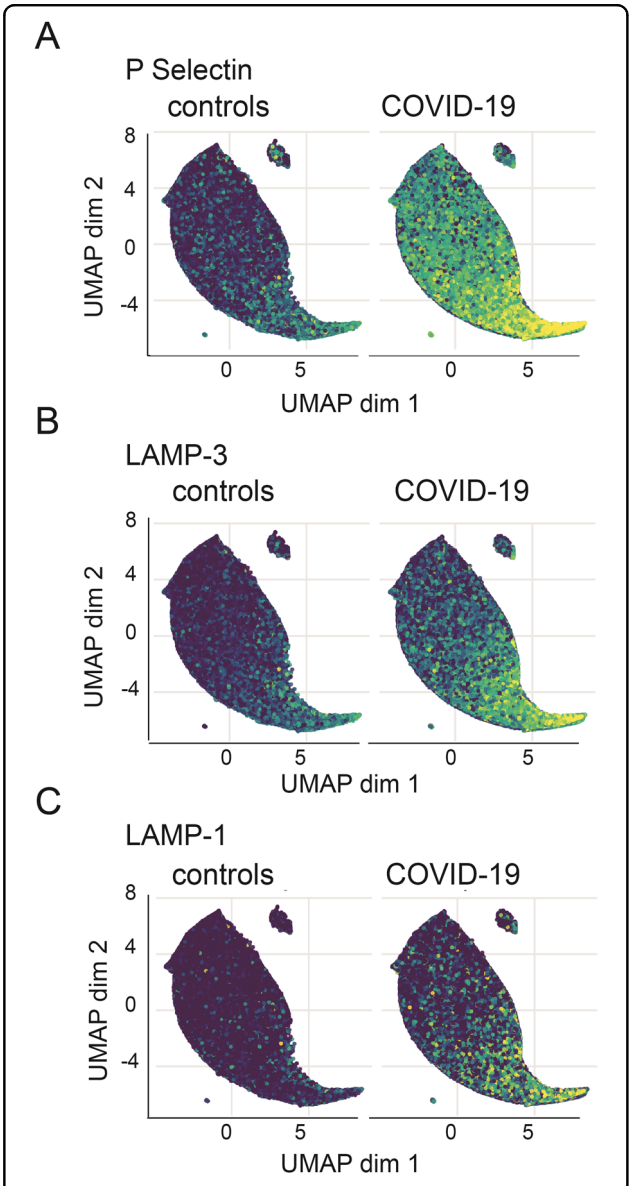

Fig. 1 Activation marker expression in non-stimulated platelets. Uniform manifold approximation and projection (UMAP) after equal random sampling from each sample and scaled, arcsinh-transformed expression [0–1] for each activation marker colored according to the expression level: **A** P-Selectin, **B** LAMP-3, and **C** LAMP-1, $N = 8$ COVID-19 patients, 11 healthy donors.

approximation and projection based on 16 markers (excluding the activation markers) using the CATALYST v1.12.1 R package. To account for differences in coverage between samples, we randomly sampled the minimum number of events acquired (41,525 events per sample, Fig. 1). Following standard practice for differential marker expression in CyTOF[30], we built mixed-effect linear models for the TRAP-stimulated and non-stimulated sample groups, respectively. We considered the sample-wise median signal intensity as dependent variable, disease status as fixed and patient IDs as random effect, i.e., each patient has a different intercept. Furthermore, we built a linear model for all samples (TRAP-stimulated and non-stimulated) with an interaction term to assess whether activation is significantly affected by disease status. *P*-values of model coefficients were corrected for multiple hypothesis testing using the Benjamini–Hochberg method (false discovery rate < 0.05)[31].

Differential analysis of overall marker expression

Statistical significance is evaluated based on regression analysis. To estimate whether the sample-wise median expression of a marker is significantly different between COVID-19 patients and healthy controls, the following linear mixed-effect model was used:

$$Y_{ij} = \beta_{0j} + \beta_{cj} x_{ci} + \gamma_i + \epsilon_{ij} \quad (1)$$

where Y_{ij} is the median expression of the j-th marker for i-th patient, x_{ci} is a binary variable indicating if a patient i belongs to case or control group, and $\gamma_i \sim N(0, \sigma_i^2)$ is a random intercept for each patient. The latter allows us to disentangle within-sample and within-group variance.

Slope coefficients β_{cj} were tested for significance of the linear relationship between the independent variable x_c and the dependent variable Y:

$$H_0 : \beta_{cj} = 0 \quad (2)$$
$$H_1 : \beta_{cj} \neq 0$$

Difference in TRAP stimulation effect for COVID-19 patients

To analyze if a higher expression of activation markers in COVID-19 quiescent platelets is coupled with a reduced capacity to react upon activation stimuli, we compared slope coefficients for the covariate that corresponds to activation for cases and controls separately. We used the following model for healthy controls:

$$Y_{hj} = \beta_{0j} + \beta_{aj} x_{ah} + \gamma_h + \epsilon_{hj} \quad (3)$$

where h belongs to a set of indices for all healthy controls and x_{ah} is a binary variable that indicates if a sample h was TRAP-stimulated or not. We computed a set of slopes β_{aj} that show an average linear increase in expression after activation for every marker j.

A similar model was used for COVID-19 patients:

$$Y_{pj} = \beta_{0j} + \beta_{aj} x_{ap} + \gamma_p + \epsilon_{pj} \quad (4)$$

where p belongs to a set of indices for all COVID-19 patients. Although we can compare β_a coefficients from the

model (3) and model (4) directly, we cannot conclude if the difference between slope coefficients is statistically significant. To evaluate if there is a statistically significant difference in the reaction to TRAP stimulation between patients and controls, we used a single model with an interaction effect term. Significance of the Interaction effect means that activation status and patient condition (disease or control) combined have a significantly larger effect on median signal intensity as compared to the sum of the individual factors alone. Formally, this results in the following model:

$$Y_{ij} = \beta_0 + \beta_a x_{aij} + \beta_c x_{cij} + \beta_{intij} x_{intij} + \gamma_i + \epsilon_{ij} \quad (5)$$

where x_{int} is an interaction term defined as $x_a \times x_c$. The slope coefficient for the interaction β_{int} was then tested for statistical significance as shown in Eq. (2).

Clustering analysis: FlowSOM algorithm

Automated clustering analysis was done using the FlowSOM algorithm[32]. After gating (Supplemental Fig. I), data were compensated, transformed with an estimated logical transformation, and scaled. Cells were assigned to a 10×10 self-organizing map and then metaclustering in 12 clusters was performed. The number of clusters was selected based on relative change in area under the cumulative distribution function curve that indicated that cells stratification in more than 12 clusters cannot improve the clustering results. For each cluster-marker pair, a p-value was computed using a linear mixed-effect model:

$$Y_{ij} = \beta_0 + \beta_{cj} x_{ci} + \gamma_i + \epsilon_{ij} \quad (6)$$

where Y_{ij} is the median expression of the j-th marker for i-th cluster, x_{ci} is a binary variable indicating if cell population Y_{ij} belongs to case or control group, and $\gamma_i \sim N(0, \sigma_i^2)$ is a random intercept for each cell subpopulation. Slope coefficients β_{cj}were tested for significance of the linear relationship between the independent variable x_c and the dependent variable Y.

Results

Study population characteristics

Eight hospitalized symptomatic COVID-19 patients without pre-existing conditions requiring oxygen support were recruited and compared to a cohort of 11 asymptomatic healthy donors, tested negative for SARS-CoV-2 (mean age COVID-19: 51.4 ± 11.7, controls: 44.7 ± 13.0, $p = 0.27$; male COVID-19 62.5%, controls: 45% $p = 0.49$). Seven patients showed typical COVID-19 pulmonary lesions in chest computed tomography. Patients were admitted through the emergency department and moved to normal wards due to dyspnea. During the hospitalization, one patient was transferred to an intermediated care unit (2 days after blood collection) for a few hours, for the purpose of monitoring due to respiratory deterioration. No patient required assisted ventilation and all were discharged in good condition (average hospitalization 9.5 ± 6.3 days). No major adverse events (bleeding and thromboembolic events) were reported. All admitted patients were not under regular medication, except one with two antihypertensive medications: amlodipine and valsartan. For a detailed description of the study population, see the Supplementary Table 1.

Platelet surface receptor and adhesion molecule expression in non-stimulated platelets

Compared to healthy controls, non-stimulated platelets of COVID-19 patients showed a significantly higher spontaneous expression of specific platelet activation markers (Fig. 1), such as P-Selectin (0.67 vs. 1.87 median signal intensity for healthy donors vs. patients, $p = 0.0015$) and LAMP-3 (0.37 vs. 0.81 median signal intensity, $p = 0.0004$, Fig. 2a), as well as the the GPIIb/IIIa complex (4.58 vs. 5.03 median signal intensity, $p < 0.0001$). In addition, we detected a higher spontaneous expression of some constitutive receptors and adhesion molecules involved in platelet activation and aggregation in COVID-19 platelets, such as the transmembrane integrins GPIIb ($p = 0.0001$) and GPIIIa ($p < 0.0001$), as well as the glycoproteins GPIbα ($p = 0.0086$) and GPIX ($p = 0.0126$, Fig. 2b). The expression level of all other activation markers and adhesion molecules are shown in Supplemental Fig. II.

Diseased platelet reactivity after TRAP stimulation

To further investigate platelet reactivity, we stimulated the collected platelets with 10 µM TRAP. Upon TRAP stimulation, mass cytometry also detected a significantly higher expression of the platelet activation marker P-selectin in samples of COVID-19 patients compared to healthy controls ($p = 0.0176$), but LAMP-3 did not show significant differences ($p = 0.40$, Fig. 3a). Interestingly, the GPIIb/GPIIIa complex remained upregulated in COVID-19 patients after TRAP stimulation ($p < 0.0001$). Similar to non-stimulated platelets, we also observed a higher expression level for the integrins GPIIb ($p < 0.0001$), GPIIIa ($p = 0.0009$), as well as for the glycoproteins GPIbα in TRAP-stimulated platelets compared to healthy controls ($p < 0.0001$, Fig. 3b). In Table 2, we provide a complete result list of markers tested.

To assess the reaction capacity of platelets upon stimulation, we compared the expression of activation markers before and after stimulation with TRAP. Interestingly, we observed a significantly reduced capacity of COVID-19 platelets to increase the expression of the activation markers LAMP-3 and P-Selectin ($p = 0.04$ and $p = 0.04$, respectively) upon stimulation (Fig. 4).

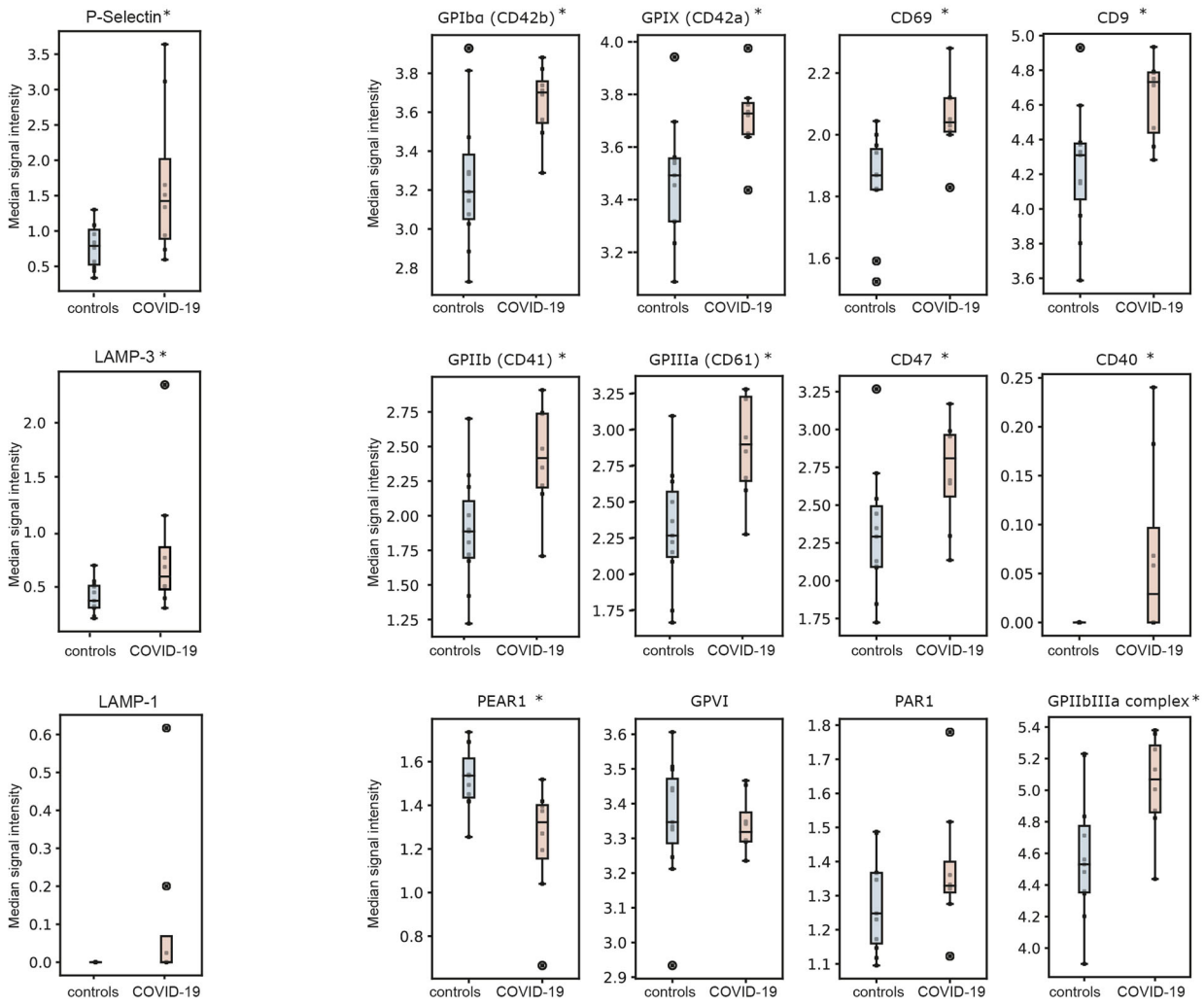

Fig. 2 Marker expression in non-stimulated platelets. Median signal intensity of activation markers (**A**) and relevant transmembrane proteins (**B**) in non-stimulated platelets. COVID-19 patients are plotted in red, whereas controls are plotted in blue. The horizontal line within the box plot represents the median, the top and bottom the interquartile range(Q1–Q3), whisker bars indicate the largest observation that is less than or equal to the upper inner fence (UIF = Q3 + 1.5 × IQR) or the smallest observation that is greater than or equal to the lower inner fence (LIF = Q1–1.5 × IQR) and the circles indicate outliers, if present; *$P < 0.01$. $N = 8$ COVID-19 patients, 11 healthy donors.

Discussion

We analyzed the expression of activation markers and transmembrane receptors in platelets of hospitalized stable COVID-19 patients without pre-existing conditions and without anticoagulants or antiplatelet drugs (except prophylactic low-molecular-weight heparin during hospitalization). As a major result, we detected significant higher levels of the platelet activation markers P-Selectin and LAMP-3 compared to controls, as well as significantly higher levels of the transmembrane proteins GPIIb/GPIIIa complex, GPIbα, GPIX, CD9, and CD40. After TRAP stimulation, platelets of COVID-19 patients showed significantly higher levels of the collagen receptor GPVI, whereas the receptor PEAR1 showed lower levels in COVID-19. These findings indicate the presence of a hyperactivated phenotype of platelets during SARS-CoV-2 infection, which might contribute to the hypercoagulopathy observed in COVID-19 and might influence disease progression. The adhesion protein P-Selectin translocates to the plasma membrane upon activation and regulates platelet–leukocyte interactions resulting in activation of neutrophil integrins and inducing NETs formation[33,34]. Moreover, platelet–leukocyte interaction may trigger the tissue factor expression as recently described in severe COVID-19[35]. P-Selectin expression together with the upregulation of the integrins GPIIb

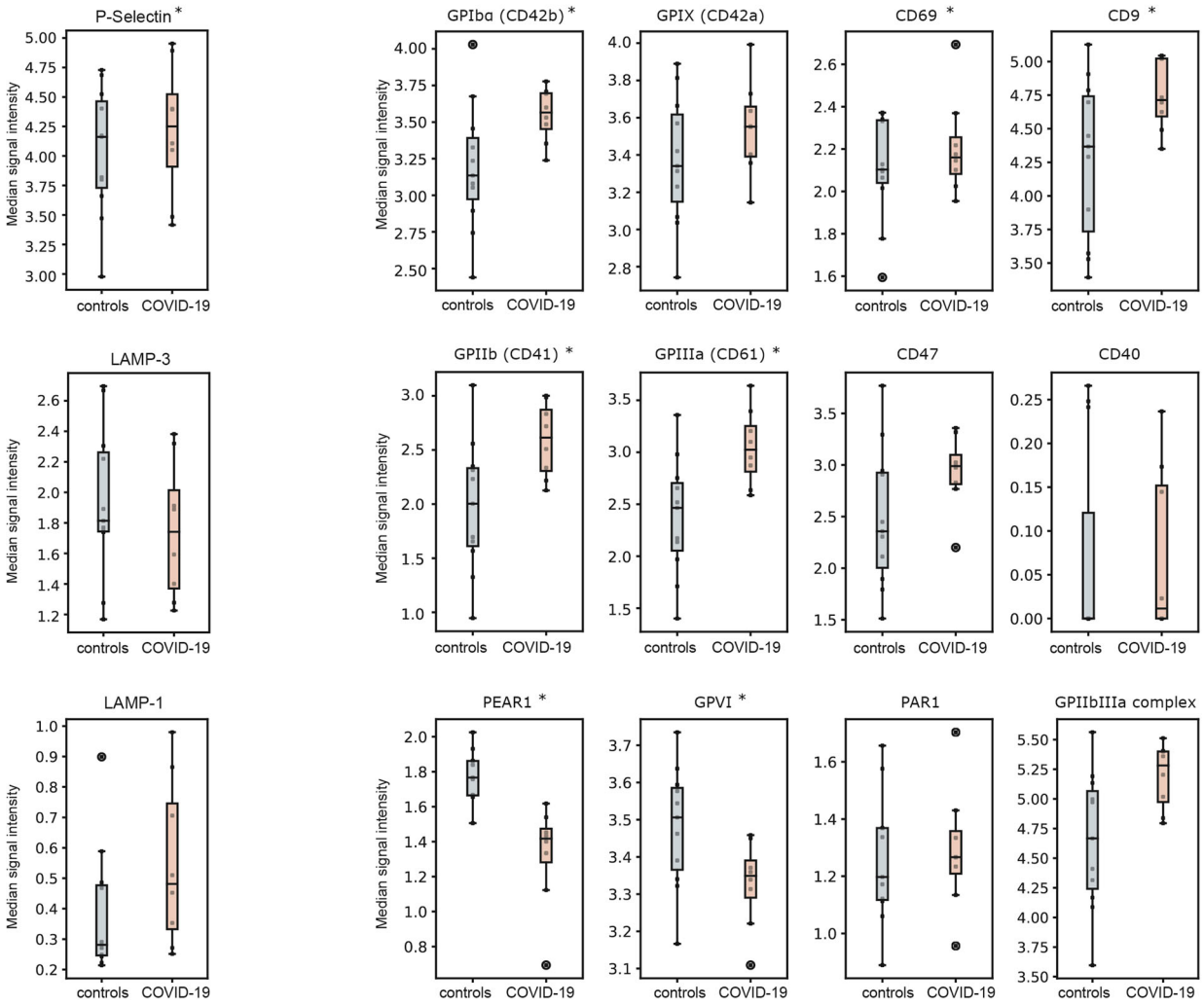

Fig. 3 Marker expression in TRAP-stimulated platelets. Median signal intensity of activation markers (**A**) and relevant transmembrane proteins (**B**) in TRAP-stimulated platelets (10 µM TRAP). COVID-19 patients are plotted in red, whereas controls are plotted in blue. The horizontal line within the box plot represents the median, the top and bottom the interquartile range (Q1–Q3), whisker bars indicate the largest observation that is less than or equal to the upper inner fence (UIF = Q3 + 1.5 × IQR) or the smallest observation that is greater than or equal to the lower inner fence (LIF = Q1–1.5 × IQR) and the circles indicate outliers, if present; *$P < 0.01$. $N = 8$ COVID-19 patients, 11 healthy donors.

(CD41) and GPIIIa (CD61), and the subunits of the von Willebrand receptor GPIbα and GPIX, known to regulate platelet–leukocyte interactions, may contribute to the COVID-19 inflammatory response[33,36]. Consistent with our data, Manne et al.[11] recently reported a higher surface expression of P-Selectin and higher levels of circulating platelet–leukocyte aggregates in COVID-19 patents. Moreover, the study showed a faster platelet aggregation and increased spreading on fibrinogen and collagen in COVID-19 patients compared to controls. The higher surface expression of integrins and adhesion protein detected in our study may provide a first mechanistic explanation to these findings.

To further investigate platelet reactivity in COVID-19, we induced platelet activation with TRAP, which activates platelets by thrombin signaling. After activation, we detected significantly higher levels of platelet activation markers P-Selectin and GPIIb/GPIIIa complex but not LAMP-3 in COVID-19 patients compared to healthy controls. Interestingly, we observed a decreased activation capacity in platelets of COVID-19 patients compared to controls, suggesting that the chronic platelet activation during SARS-CoV-2 correlates with an altered reactivity upon stimuli, which is possibly due to an higher activation level at rest in COVID-19 (Fig. 4)[8,33]. Of note, CD40 ligand (CD154) expression did not provide informative data: signal

Table 2 Median signal intensity and *p*-values of CyTOF panel.

	Non-stimulated			TRAP-stimulated		
	Controls	COVID-19	*p*-Value	Controls	COVID-19	*p*-Value
CD41	1.8856	2.3906	0.0001	1.877	2.5435	<0.0001
CD40	0	0.0308	0.0005	0	0.0612	0.915
CD42b	3.2253	3.6501	0.0086	3.1198	3.4849	<0.0001
CD31	2.2122	2.5058	0.0522	2.1849	2.5	0.2388
CD61	2.3059	2.8594	<0.0001	2.2974	2.9396	0.0009
PAR1	1.2734	1.4094	0.1697	1.2733	1.3221	0.7218
CD63	0.347	0.8061	0.0004	1.9609	2.0531	0.4026
CD107a	0	0.0322	1	0.3622	0.6348	0.2114
CD36	2.928	3.2467	0.4988	2.8899	3.2625	0.2388
GPIIb/GPIIIa complex	4.5825	5.0363	<0.0001	4.5809	5.1493	0.0176
CD29	3.8587	4.0407	0.0761	3.8499	4.0422	0.2077
CD62P	0.6714	1.8705	0.0015	4.0868	4.3855	<0.0001
CD69	1.8559	2.0478	0.0002	2.1615	2.2371	0.0326
CD141	0	0	1	0	0	0.3288
CD154	0	0	1	0.2288	0.2188	0.8033
CD3	0	0	1	0	0	1
CD9	4.2401	4.6766	0.001	4.2612	4.7279	0.0176
PEAR	1.5399	1.2474	0.0039	1.8075	1.41	<0.0001
GPVI	3.3737	3.3506	0.4884	3.4984	3.3675	0.0176
CD47	2.3005	2.7849	0.0015	2.3625	2.9007	0.0555
CD42a	3.4665	3.7047	0.0126	3.3374	3.5062	0.2077

increased after TRAP stimulation but we did not detected any differences among groups (Supplemental Fig. II).

Subgroups investigations using FlowSOM analysis detected some differences in platelet activation patterns between healthy donors and COVID-19 patients (Supplemental Fig. III). However, as shown in Fig. 1, we did not find any defined and distinct subgroups, highlighting the lower heterogeneity of platelets compared to other cells in peripheral blood. Nonetheless, the FlowSOM trees shown in Supplemental Fig. III report a different activation pattern in COVID-19 patients compared to controls involving different platelet subgroups. Further studies are needed, to dissect the role of platelet heterogeneity in COVID-19 platelet activation.

Although the pathophysiological mechanisms behind the high incidence of thromboembolic events in hospitalized COVID-19 patients remain unclear, our data describe with high resolution the presence of activated platelets, which may provide one explanation for COVID-19 coagulopathy and suggests platelet inhibition as a possible therapeutic option in COVID-19 patients. Our data are consistent with previous studies reporting an immunothrombotic dysregulation as a typical marker of SARS-CoV-2 infection[11,13]. However, the key drivers behind platelet activation in COVID-19 remain to be determined. SARS-CoV-2 tropism for thrombocytes has not been proven yet and platelet activation may be induced by infected endothelium as well as by the cytokine storm occurring during SARS-CoV-2 infection[37]. Clinical trials investigating empirically different anticoagulation schemes and antiplatelet therapies are ongoing worldwide, and may provide more insights concerning the clinical relevance of antithrombotic regimes for COVID-19 patients[38].

A strength of our analysis is the simultaneous measurement in a healthy control group, minimizing the risk that the observed higher platelet activation in COVID-19 was due to procedural biases. In addition, we restricted our measurements to stable COVID-19 patients not requiring supported respiration or extracorporeal perfusion, which may induce non-disease associated platelet activation. A further strength of our study consists in the high-resolution achieved by our measurements using

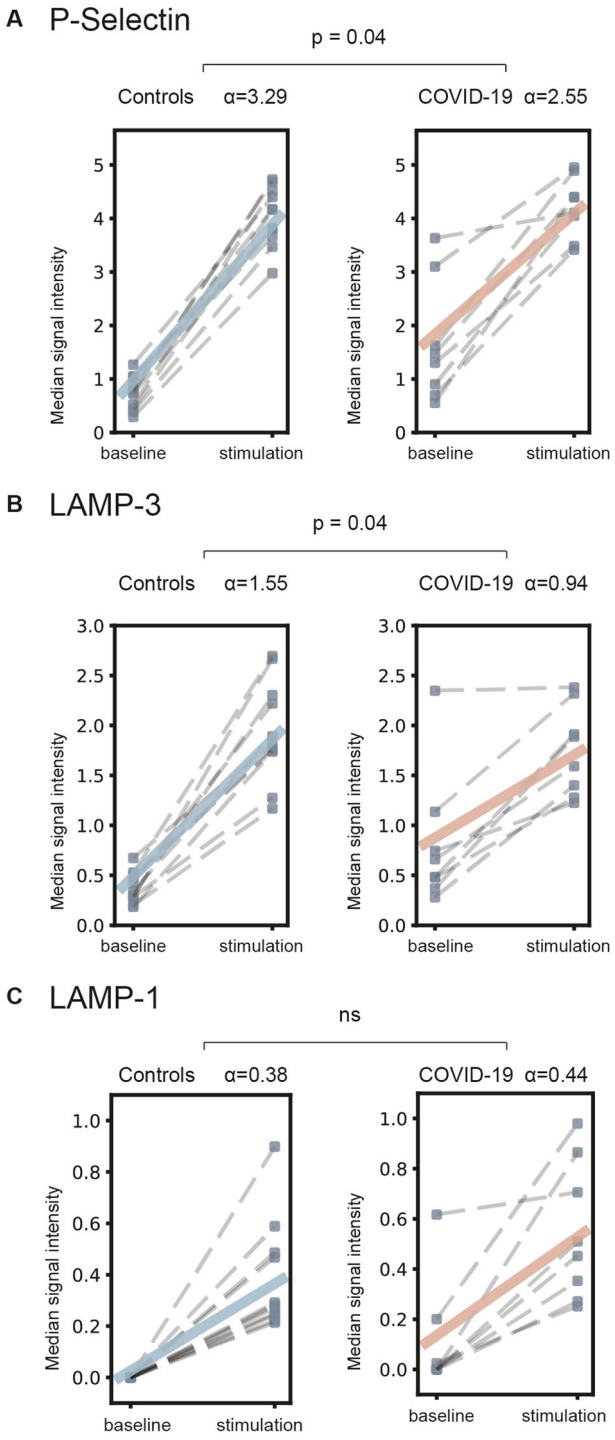

Fig. 4 Platelet reaction capacity. Median signal intensity increase of activation marker expression after TRAP stimulation (10 µM) compared to non-stimulated platelets (baseline). Linear model analysis detected a reduced capacity of COVID-19 patients to increase expression of activation markers LAMP-3 and P-Selectin ($p = 0.04$ and $p = 0.04$) after TRAP stimulation. **A** P-Selectin, **B** LAMP-3, **C** LAMP-1. α: signal increment slope coefficient (for details see Methods); ns: nonsignificant. $N = 8$ COVID-19 patients, 11 healthy donors.

mass cytometry, avoiding the spectral limitation of flow cytometry and allowing the measurements of 21 markers at single-cell level with virtually no overlapping.

Limitations of this study consist in the limited number of patients and in its ex vivo observational nature: our research was limited to the phenotypical observation of platelet surface receptor expressions and we did not assess the pathophysiological mechanisms triggering platelet activation. In fact, other pathways including the cytokine storm and the pro-inflammatory state during SARS-CoV-2 infection may play a relevant role in COVID-19 coagulopathy. Moreover, we did not include patients with non-COVID-19 inflammation and/or other types of viremia (e.g. influenza or other respiratory viruses) as an additional control group. Thus, we cannot quantify the severity of platelet activation in COVID-19 comparing it with other pathological settings. Nevertheless, here we provide the first mass cytometric analysis of platelets in COVID-19 and our results provide the basis for further research regarding pathways of platelet activation in COVID-19 patients as well as for further investigations of platelet biology in other pathological settings.

In conclusion, mass cytometry of COVID-19 patients revealed higher expression levels of platelet activation markers and adhesion proteins compared to healthy controls. These findings provide new insights into COVID-19 coagulopathy and support research projects investigating antithrombotic and antiplatelet treatment regimes in COVID-19.

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Author details
[1]Department of Internal Medicine I, School of Medicine, University hospital rechts der Isar, Technical University of Munich, Munich, Germany. [2]German Center for Cardiovascular Research (DZHK), Partner Site Munich Heart Alliance, Munich, Germany. [3]Department of Cardiovascular Medicine, Humanitas Clinical and Research Center IRCCS and Humanitas University, Rozzano, Milan, Italy. [4]Experimental Bioinformatics, TUM School of Life Sciences Weihenstephan, Technical University of Munich, Munich, Germany. [5]Department of Internal Medicine II, School of Medicine, University hospital rechts der Isar, Technical University of Munich, Munich, Germany. [6]Core Facility Cytometry, Ulm University Medical Faculty, Ulm, Germany. [7]CCC Ulm, Institute of Experimental Cancer Research, University Hospital Ulm, Ulm, Germany. [8]Deutsches Herzzentrum München, Cardiology, Technical University of Munich, Munich, Germany

Conflict of interest
The authors declare that they have no conflict of interest.

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4 Discussion

During the course of my PhD I developed reproducible protocols for the sorting of RPs and for the mass cytometric analysis of platelets from healthy donors. This was of particular importance as these protocols allowed me to characterize the biology of immature platelets for the first time and to decipher the heterogeneity of human platelets further. In the wake of the COVID-19 pandemic, I investigated platelets from COVID-19 patients in comparison to healthy donors using mass cytometry. The main findings of this study were the discovery of a hyper-reactive platelet population and a dysfunctional reactivity pattern of platelets in COVID-19 patients compared to healthy donors.

Here, I focus on the challenges of fluorescence activated cell sorting of RPs, which lay the foundation of RNA extraction and platelet subgroup analysis using RNA-sequencing. Moreover, advantages and limitations of platelet mass cytometry analysis using PRP are discussed. Previously published data on RNA-sequencing and CyTOF analysis from platelets of healthy donors are included to evaluate the functionality of both developed protocols. Finally, also the COVID-19 specific platelet research will be discussed and emphasize the need of platelet CyTOF analysis.

4.1 Sorting

Sorting and the subsequent magnetic-based isolation of RPs in a standardized manner allows the use of downstream applications to examine the biology of RPs. This is of specific clinical interest, as the biological reasons behind the RPs correlation with the prediction of adverse cardiovascular events and mortality are not fully understood [165, 182].

Due to the absence of platelet subtype specific markers, the isolation of RPs is currently only possible by gating platelets according to their RNA amount. The specificity of TO binding to RNA was proven in an experiment part of publication 1 using RNase and saponin. Saponin is used to permeabilize the cell membrane by creating holes [233, 234]. Through these, RNase can enter the cell and digest RNA [31, 235, 236]. Subsequent staining with TO resulted in less to no signal as most RNA has been digested previously. While the use of other nucleic acid dyes like SYTO 13 have been suggested by other researchers, a validation with RNase and a cell permeabilization reagent is still missing [237]. Therefore, the most accurate sorting after RNA content is currently only possible using TO. While other researchers have examined a TO-stained and gated RPs population from whole blood [238, 239, 240], we were the first to sort RPs based on TO-staining. Nonetheless, RNA has also been extracted and sequenced from "RPs" after SYTO13 staining, although validation of RNA specificity is still lacking. In this SYTO13 study, several transcripts involved in platelet activation were found upregulated in "RPs" [176].

In the past, staining with TO has already been shown to be RNA specific in platelets but has been used in several different concentrations [171, 241, 170]. In publication 1, I showed a reproducible method for staining with TO, which usually results in a 2:1 (v/v, TO/PRP) solution. TO staining of whole blood has not been shown to be suitable for platelet isolation, mainly due to the limited platelet amount. While the use of PRP results in a higher available platelet population [242], using only several micro liters of whole blood does not offer enough platelets for separation. As RNA sequencing analysis requires good quality and RNA concentration, around 6 to 10 million platelets need to be sorted. This amount can only be reached using PRP. On the other hand, an advantage of platelet staining in whole blood is the shorter time of the workflow. As platelets are a challenging substrate, the experimental timetable has to be as short as possible. Platelets tend to aggregate upon activation which can occur from the exposure to cold temperature [243, 244, 245], shaking, the application of strong forces, shear stress in general [246, 247, 248] or the attachment to surfaces [249, 250]. Despite the shorter time of the workflow, it has been shown that platelets from whole blood have a higher activation and aggregation rate after exposure to shear stress compared to

platelets from PRP [251].

Moreover, the usage of PRP bears another advantage. During multiple centrifugation rounds, red and white blood cells are already removed from the solution [252]. On the one hand, this reduces platelet activation via other cells in the bloodstream; on the other hand, this facilitates the sorting process. Due to the absence of bigger blood cells disturbing measurement, contamination in the sorted cells is reduced. Additional gating by size excludes cells with a higher forward or side scatter signal. Moreover, the use of DNA stain showed the presence of under 0.5% of cells containing DNA, which emphasizes the purity of the PRP. Nonetheless, some studies point out disadvantages of producing PRP. For example, platelets from PRP were shown to be more activated that platelets derived from buffy-coat [253]. The latter one refers to the layer of blood between plasma and red blood cells following centrifugation, which contains mainly leukocytes and platelets. In our studies, the use of buffy coat derived platelets would not have been beneficial, as we explicitly gate out leukocytes and highly prevent any leukocyte contamination in the final RNA extraction step. Platelet RNA purity is crucial, as out of the total 15 µg/ml RNA in the blood [254], platelet RNA accounts for only <0.5 µg. Hence, even low levels of leukocyte contamination would significantly alter the results. However, at the same time, activation must be kept at a minimum during and after the sorting process.

Additional precautions have to be taken in order to avoid unwanted platelet activation during the generation of PRP [255]. Besides the optimization of timing, platelets need to be inhibited with several substances to keep them detached from one another [256]. In the process of PRP generation, I have optimized the amount and substances for platelet inhibition while preserving platelet reaction capacity. It begins with blood collection into a tube containing the first inhibitory substance: Citrate Dextrose A. While the usage of a tube containing Citrate as well as a combination of platelet antagonists would prevent undesired platelet inhibition more [257], others have found no differences of coagulation between platelets drawn into combinatory tubes and tubes containing only citrate [258]. Further inhibition is achieved by using ADPase which inhibits platelet activation via ADP [259, 260] and the use of prostaglandin E1, a substance known to activate inhibition receptors [83, 261]. The pH of

the platelet solutions were kept at bay using HEPES [262, 263] and citric acid, whereas EGTA and EDTA were used to decrease doublet formation by chelating Ca^{2+} ions in the solution [256, 264]. For the latter, the degree of inhibition highly depends on the applied concentrations and can lead to the inability of platelets to be activated in downstream experiments [265]. Additionally, platelet fixation is only possible when downstream analyses do not require the activity of retro-transcriptase.

In order to assess possible platelet activation occurring during the sorting process, I measured platelet reaction to thrombin in sorted RPs and MPs. As both populations still showed the same ability to react upon stimuli than before sorting, the RPs more than the MPs, an excessive activation during sorting was excluded. However, one limitation is the testing of only one stimulus, thrombin. Other activation pathways like the ADP-mediated one might be exhausted and impaired through the sorting procedure. Nonetheless, even after magnetic based separation, both RP and MPs populations are still capable of activating upon thrombin stimulation. Therefore, the magnetic-based isolation process not only offers an additional exclusion of non-platelet cell types but also does not impair platelet reactivity to any extent and can be used in platelet reactivity studies after sorting.

Using the developed protocol, we investigated the transcriptome of RPs in healthy donors and found several transcripts associated with platelet activation and adhesion upregulated in RPs compared to MPs [169]. Moreover, I found similar transcripts differentially regulated in the platelets of CCS patients. However, these data are still preliminary and remain to be published. Nonetheless our data on healthy donors and CCS patients emphasizes the usability of the developed sorting protocol.

4.2 Mass Cytometry

Mass Cytometry offers the possibility to simultaneously investigate expression of up to more than 40 proteins at once, which allows the investigation of platelet heterogeneity on a deeper level [266, 227]. In comparison to flow cytometry, antibodies are bound to lanthanide metals instead of fluorophores, which narrows the influence of spectral overlap [267]. While spectral

overlap was believed to have no influence at the beginning of mass cytometry research [268, 269], it was soon proven to be detectable [270]. Nonetheless, overlap is present to a lower extent than in flow cytometry. It is mostly found in the neighboring isotope channels of the stained metals or +16 channels away, due to oxidation [270, 271]. However, it can be overcome using a compensation matrix [272].

Current limitations of mass cytometry are the availability of ready-to use antibodies and the limitation of lanthanide heavy-metals. Nonetheless, the spectrum of available metals for coupling is constantly increasing and several researchers have shown that antibodies can also be coupled to silver nanoparticles [273], platinum [274] or organotelluriums [275]. Moreover, imaging mass cytometry emerged, which allows CyTOF analysis from tissue sections [276]. Overall, the CyTOF field is growing, with more researchers using the technique worldwide and the constant improvement of machines, antibodies and labeling options, theoretically allowing the simultaneous analysis of up to 100 markers [277].

In terms of platelet analysis, mass cytometry is still at an early stage, with only one other group worldwide applying MC to detect platelet heterogeneity so far [159]. Compared to our method, Blair et al. used platelets from whole blood and are therefore limited to lower cell numbers, with the ability to acquire only around 30,000 events per sample [159]. Moreover, the clean-up gating process according to Gaussian parameters (method section, Fig.2.2) leads to a significant loss of cells when applied to platelet data from whole blood [225]. Consequently, only few events are available for computational analysis preventing the algorithms to find meaningful clusters [278]. Using PRP, these limitations can be overcome as more than 500,000 events can be acquired from one sample [225, 157]. However, certain use cases might benefit from whole blood analysis. For example when investigating platelet-leukocyte interactions, the formation of thrombi, or diseases that depend on the analysis of big platelets which might be lost during the generation of PRP. In these cases, one needs to balance the amount of whole blood used and the loss of cell and reagent material.

Additionally, our protocol offers the possibility to freeze samples after staining. In this way, several samples can be collected and acquired at the CyTOF in the same run. We validated the freezing protocol previously developed by Sumatoh et al. for platelets, which freezes

the stained samples in 10% DMSO in FBS [279]. Compared with other methods that showed signal loss after freezing, this technique reassures signal stability. Platelets from whole blood have been frozen without a buffer, which increases cell loss and is therefore not recommended for samples with a low cell number [280]. An additional advantage of acquiring frozen samples is the lower abundance of clotting. Especially the use of platelet stimulators like TRAP or collagen lead to rather sticky platelets solutions. This resulted, when fresh cells were acquired directly after staining, in the higher incidence of clots.

Current platelet CyTOF research is united by the problem to identify relevant subgroups and decipher lineage-like platelet types. Due to the absence of typical platelet lineage markers, dimensionality reduction techniques and clustering approaches fail to predict clearly distinct platelet sub-populations [280, 281, 278]. However, CyTOF analysis still offers the possibility to identify platelet groups and clusters that react differently in response to stimuli or under disease conditions [159, 158, 225, 157]. The different reaction capacity of platelets from healthy donors compared to COVID-19 patients will be discussed later (4th part of the discussion) while the differential protein expression between thrombin and collagen-mediated platelets from healthy donors underlies this part.

Stimulation with TRAP and CRP led to differences in surface receptor expressions for several markers. Although they were used in different concentrations (TRAP 10 µM, CRP 2 µg/ml), these concentrations have been shown to lead to similar activation measured by CD62P expression using FC. A reason for the higher PAC1 signal after CRP stimulation could be a stimulus dependent pathway activation. CRP stimulation turns integrins $\alpha 2\beta 1$ into a high affinity state. Subsequently, this results in the formation of platelet-platelet aggregates as αIIb$\beta 3$ fibrinogen receptors are binding with higher affinity to the fibrinogen receptors on other platelets [117].

Single cell proteomics however, may help to discover currently unknown platelet clusters. For example, suitable markers for subgroup detection and in particular RP determining markers could potentially be discovered using single cell proteomics. Moreover, stimulation- or disease-specific alterations of surface expression markers that currently cannot be targeted by CyTOF antibodies could be discovered.

4.3 COVID-19

In the course of the pandemic, data accumulated regarding a burst of thrombotic events during COVID-19 disease progression. We were the first to investigate platelets from COVID-19 patients using CyTOF. In our study, we showed a higher platelet reactivity during mild SARS-CoV-2 infection. Additionally, we detected a reduced capacity of platelets from COVID-19 patients to react upon stimulation. Together with the discovered hyper-coagulable state, these findings highly emphasizes a pre-activated platelet phenotype upon SARS-CoV-2 infection [158].

Our clustering approach showed clusters of platelets behaving differently between case and control as well as between stimulated and non-stimulated samples of the same kind. Mainly due to the absence of platelet-subgroup specific lineage markers, clear subgroups were not characterized. Nonetheless, comparing the subgroups of COVID-19 patients and healthy controls, it became clear that there are different platelet subgroups involved in platelet activation in each of the groups. Adding additional markers to the panel, like intracellular markers, would possibly allow further differentiation between the FlowSOM categorized meta-clusters.

The differential analysis between case and control was based on differences in the median marker expression, as usually done in the CyTOF context [231, 282, 226]. Taking into account all values could partly alter the results but also make them more precise. With the pipeline we have developed after the COVID-19 study, it is possible to calculate differences in marker expression by using the earth-mover's distance (EMD), as described in the method section [226]. As we encounter a high zero inflation for markers CD107a and CD154, our developed CyEMD method could improve the differential expression analysis for at least these two markers. Overall, the bioinformatic tools we used in Publication 3 are integrated into our customized CyTOF data analysis pipeline with the exception of the slope analysis (https://exbio.wzw.tum.de/cyanus/).

Throughout the whole study, I have produced samples of healthy and diseased donors simultaneously to prevent day specific alteration, also called batch effect. As it is widely

known that batch effects can have a high influence on mass cytometry data and oftentimes need to be corrected for either using technical replicates as reference samples [283, 284] or other methods [285, 286], we kept batch variation to a minimum. Thus, we used antibodies from the same lot and measured our data with the same instrument, although it would theoretically be possible to compare CyTOF data from different sites [287]. Additionally, we alternated measurements of case and control, stimulated and baseline samples. Finally, we did not detect any batch effect resulting from different measurement days and therefore did not apply any batch effect correction method.

Current COVID-19 research back up our results as researchers identified higher levels of soluble P-Selectin [288] and a higher abundance of platelet activation and aggregation markers [289, 290] during SARS-CoV-2 infection. While these data could partly explain the occurrence of thrombotic events in COVID-19 patients, further research on other cell types included in thrombus formation is also needed. Other researchers confirmed an increase of thrombotic events after which the platelet reactivity decreased again [291]. Furthermore, increasing platelet thrombus formation has been linked to an abnormal glycosylation on immune complexes against SARS-Co-V-2 spike IgG [292].

Other reasons behind COVID-19 induced platelet activation may include the well-known virus-induced cytokine storm [293]. Alongside endothelial function disparity it triggers an enormous cell activation resulting in an increased platelet-mediated tissue factor production [294, 295]. While a direct interaction of SARS-CoV-2 Spike protein binding to ACE2 receptors on platelets was reported by one single study, this finding requires further validation [296].

More recent studies have picked up on investigating the particular influence of RPs, determined by point-of care systems, during SARS-CoV-2 infection. As reported previously for other diseases, the immature platelet fraction also negatively correlated with COVID-19 disease progression. Higher RPs levels were found in patients with severe disease compared to those with only a mild COVID-19 infection [297].

Despite all these studies supporting our findings, our study bears some limiations. The main limitation is the low sample size of the investigated cohort. Nonetheless, the study

contains enough samples on both sides to perform meaningful statistical analysis and found overall global differences between case and control. Shortly after the discovery of platelet involvement and the existence of cardiovascular events, antiplatelet treatment has become part of the treatment strategy for COVID-19, for example by using therapeutic anticoagulation with heparin [298]. Another study on a large patient cohort showed a lower mortality rate and a decrease in mechanical ventilation time for patients treated with antiplatelet therapy consisting of aspirin alone or in combination with either clopidogrel, ticlopidine, prasugrel or ticagrelor during SARS-CoV-2 infection [299]. More research in this field even led to the suggestion of a provisionary antiplatelet therapy when infected with SARS-CoV-2, which was shown to decrease the in-hospital mortality rate of patients [300].

The lack of comparison to a group of patients suffering from other viral pneumonia diseases like influenza and the therefore unknown specific effect of coronavirus-19, is another limitation of our study. The hyperactive platelet state we observed could theoretically also be due to a higher platelet reactivity appearing during viral infection in general. However, the COVID-19 patients in our study were naïve from treatment and without history of previous medically relevant conditions. We were the first to show an increased expression of important transmembrane receptors as well as activation markers in COVID-19 patients even with a mild disease and without any pre-existing condition. Additionally, there are other studies that included both COVID-19 and influenza patients, which put our results into more context. For example, Zaid et. al showed a higher reactivity of platelets from COVID-19 platelets upon stimulation compared to influenza patients [301]. Additionally, Nicolai et. al discovered an immunothrombotic dysregulation in COVID-19 patients in comparison to non-COVID-19 pneumonia patients [224, 302]. These studies point out once more that COVID-19 disease has its own characteristics and needs to be treated differently to other viral diseases.

All in all, the COVID-19 study has proven the feasibility of the mass cytometry method using platelets from platelet-rich plasma (Publication 2). Additionally, conducting the study on the investigation of platelets in healthy donors undergoing BNT-162b2 vaccination [157] also showed reproducibility of the developed CyTOF method in different settings.

4.4 Conclusion

In the human heterogeneous platelet population, RPs are of particular interest because they correlate with the prediction of adverse cardiovascular events. During the course of my PhD, I developed protocols that enable deep biological characterization of these cells. While the sorting protocol (Publication 1) allows the investigation of the RP transcriptome for the first time, the CyTOF protocol (Publication 2) offers a deep examination of platelet heterogeneity in terms of protein expression. By applying the CyTOF protocol to investigate platelet reactivity in COVID-19 patients (Publication 3), I have proven the feasability of my previously designed CyTOF pipeline. Among others, our COVID-19 project showed a disease-specific platelet heterogeneity with the involvement of certain platelet subgroups during disease progression. The protocols developed during my PhD are the basis for further investigation of the RPs biology and the protein expression diversity of platelets at health and disease.

4.5 Outlook

The possibility to sort RPs and MPs from the entire platelet population as well as the resulting option to perform RNA-sequencing analysis has shed more light on the biology of RPs. In this way, RPs have been shown recently to be hyper-reactive and have a pro-thrombotic influence in healthy but also in severely ill patients, not limited to the cardiovascular field. As the COVID-19 pandemic has proven, platelets play their role during disease progression [303]. RPs in particular correlate with worse outcome and disease severity [297]. More than ever, the pandemic has shown that cardiovascular events are a burden that hospitals face from several patients with different illnesses [304].

In general, further platelet heterogeneity analysis could offer new insights to support a personalized medicine approach. Although it is known that high RP-levels correlate with an insufficient response to antiplatelet treatment, an antithrombotic therapy tailored to RPs has not been investigated yet. Several cardiologic researchers have suggested to tailor standard dual antiplatelet therapy after percutaneous coronary intervention in patients that show elevated levels of RPs. Moreover, it has been speculated that a specific inhibition of RPs could optimize the patient's outcome while simultaneously reducing the burden of bleeding events. In this context, precision medicine based on the phenotypical characterization of patient features in cardiovascular diseases could decrease morbidity and thus lead to a cost reduction in terms of invested resources. However, if such therapeutic procedures can reduce averse events not only in the cardiovascular field but also in other diseases warrants further inverstigation.

Until then, the main short short-term goals are focused on increasing the resolution of platelet heterogeneity characterization. For example, a single-cell whole proteome analysis as well as a single-cell RNA-sequencing analysis would offer deep investigation of the role of platelet heterogeneity in cardiovascular diseases. Nevertheless, these approaches need validated standardized protocols that are currently not yet available.

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Acronyms

ADP	Adenosine Diphosphate
ADPase	Adenosine Diphosphatase
ATP	Adenosine Triphosphate
BSA	Bovine Serum Albumin
Ca^{2+}	Calcium
CAS	Cell Acquisition Solution
CCS	Chronic Coronary Syndrome
CD40L	CD40- Ligand
COVID-19	Coronavirus Disease 2019
CRP-XL	Collagen Related Peptide
CSB	Cell Staining Buffer
CYANUS	Cytometry Analysis Using Shiny
CyTOF	Cytometry by time of flight
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediamine Tetraacetic Acid
EGTA	Ethylene Glycol-bis(β-aminoethyl ether)-N,N,N',N'-Tetraacetic Acid
FACS	Fluorescence Activated Cell Sorting
GP	Glycoprotein
GPCR	G-Protein Coupled Receptor
GTPase	Guanosine Triphosphatase, small G-Protein
HEPES	4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic Acid

HSC	Hematopoietic Stem Cells
ID	Identifyer
IPC	Immature Platelet Count
IPF	Immature Platelet Fraction
LAMP-1	Lysosomal-Associated Membrane Protein 1
LAMP-3	Lysosomal-Associated Membrane Protein 3
MDS	Multi-Dimensional-Scaling
MPs	Mature Platelets
PAR	Protease-Activated Receptors
PBS	Phosphate Buffered Saline
PCI	Percutaneous Coronary Intervention
PCR	Polymerase Chain Reaction
PGE1	Prostaglandin E1
PLC	Phospholipase C
PRP	Platelet-Rich Plasma
PSGL-1	P-Selectin Glycoprotein Ligand-1
RNA	Ribonucleic Acid
RNA-seq	RNA-sequencing
RPs	Reticulated Platelets
RT	Room Temperature
SARS-CoV-2	Severe Acute Respiratory Syndrome Coronavirus 2
SOM	Self Organizing Map
TO	Thiazole Orange
TRAP	Thrombin Receptor Activating Peptide
TXA2	Thromboxane A2
UMAP	Uniform Manifold Approximation and Projection
USA	United States of America
VASP	Vasodilator-Stimulated Phosphoprotein
viSNE	Visual Stochastic Neighbor Embedding
VWF	von Willebrand Factor

List of Publications

Bernlochner I*, **Klug M***, Larasati D, Von Scheidt M, Santovito D, Hristov M, Weber C, Laugwitz KL, Bongiovanni D. Sorting and magnetic-based isolation of reticulated platelets from peripheral blood. Platelets. 2021 Jan 2;32(1):113-119. doi: 10.1080/09537104.2020.1724923. Epub 2020 Feb 11. PMID: 32046562.* equal contribution

Klug M, Kirmes K, Han J, Lazareva O, Rosenbaum M, Viggiani G, von Scheidt M, Ruland J, Baumbach J, Condorelli G, Laugwitz K.-L., List M., Bernlochner I., Bongiovanni D. Mass cytometry of platelet-rich plasma: a new approach to analyze platelet surface expression and reactivity. Platelets. 2021 Dec 27:1-8. doi: 10.1080/09537104.2021.2009453. Epub ahead of print. PMID: 34957922.

Bongiovanni D*, **Klug M***, Lazareva O, Weidlich S, Biasi M, Ursu S, Warth S, Buske C, Lukas M, Spinner CD, Scheidt MV, Condorelli G, Baumbach J, Laugwitz KL, List M, Bernlochner I. SARS-CoV-2 infection is associated with a pro-thrombotic platelet phenotype. Cell Death Dis. 2021 Jan 5;12(1):50. doi: 10.1038/s41419-020-03333-9. PMID: 33414384; PMCID: PMC7790351.*equal contribution

Additionally, I first-authored the following publication:
Klug M*, Lazareva O*, Kirmes K, Rosenbaum M, Lukas M, Weidlich S, Spinner CD, von Scheidt M, Gosetti R, Baumbach J, Ruland J, Condorelli G, Laugwitz KL, List M, Bernlochner I, Bongiovanni D. Platelet Surface Protein Expression and Reactivity upon TRAP Stimulation after BNT162b2 Vaccination. Thromb Haemost. 2021 Aug 13. doi: 10.1055/s-0041-1733934. Epub ahead of print. PMID: 34388849.*equal contribution

In addition to the first author publications, I also published as a contributing author in peer-reviewed journals (not part of this dissertation):

Louadi Z, Elkjaer ML, **Klug M**, Lio CT, Fenn A, Illes Z, Bongiovanni D, Baumbach J, Kacprowski T, List M, Tsoy O. Functional enrichment of alternative splicing events with NEASE reveals insights into tissue identity and diseases. Genome Biol. 2021 Dec 2;22(1):327. doi: 10.1186/s13059-021-02538-1. PMID: 34857024; PMCID: PMC8638120.

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List of Figures

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