Review

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Reticulated platelets – clinical application and future perspectives

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Abstract: Reticulated platelets are immature platelets freshly released from the bone marrow into the circulation and contain vestigial amounts of ribonucleic acid. Thus, they can serve as an indicator for the activity of thrombopoiesis. Despite the current lack of a standardized reference method, two types of hematology analyzers have incorporated a fully automated measurement of reticulated platelets. The “immature platelet fraction” (IPF; Sysmex XE-/XN-series) has some clinical utility in the differential diagnosis of thrombocytopenia. This is less clear for “reticulated platelets” (retPLT; Abbott CELL-DYN Sapphire/Alinity HQ). The usefulness of these parameters in the prediction of platelet recovery after chemotherapy or stem cell transplantation and as a decision aid for platelet transfusions has not been unequivocally confirmed. Recent findings have shown an association of reticulated platelets with an adverse risk in patients with coronary artery disease and stroke as well as resistance to anti-platelet therapy. Furthermore, a role of reticulated platelets for the prediction of sepsis was indicated. However, validation in larger prospective trials is necessary to establish the clinical benefit of reticulated platelets in these conditions. This review gives an overview of the available analytical methods and summarizes the current knowledge regarding the clinical application of reticulated platelets.

Keywords: immature platelets; IPF; reticulated platelets.

Brief summary: Reticulated platelets represent the most immature platelets recently released from the bone marrow into the circulation and indicate the activity of megakaryopoiesis. Their measurement may have clinical utility to assess the etiology of thrombocytopenia. Whereas an international reference has yet to be defined, two fully automated methods to measure reticulated platelets are commercially available.

Formation of platelets by megakaryopoiesis

Platelets represent the final stage of megakaryopoiesis and constitute an integral element of hemostasis. In the process of megakaryopoiesis pluripotent hematopoietic stem cells in the bone marrow proliferate and differentiate to become megakaryoblasts and eventually megakaryocytes. Megakaryocytes then form proplatelet projections that fragment into new platelets. In healthy humans roughly 1×10^{11} platelets are produced per day, whereas this rate can be accelerated up to 10-fold in conditions of increased demand [1]. While the platelet count varies greatly between healthy individuals ($150\text{--}400 \times 10^9/\text{L}$), the platelet count in any one individual is more stable throughout life [2]. Furthermore, an inverse relationship between platelet count and mean platelet volume (MPV) exists during steady-state conditions, keeping the total platelet mass (i.e. platelet count adjusted for MPV) constant [2, 3]. Similar to reticulocytes, the newly generated platelets still contain residual ribonucleic acid (RNA) and are thus termed reticulated platelets [4]. Whereas mature platelets persist in the circulation for 7–10 days, reticulated platelets have a much shorter lifespan of <1 day [5]. Thereby, these newly released platelets reflect the activity of megakaryopoiesis.

Measurement of reticulated platelets by flow cytometry

In 1969 Ingram and Coopersmith discovered in a canine model that newly released platelets following acute blood loss contain coarse condensations of residual RNA,

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which can be stained supravitaly with methylene blue and counted microscopically [6]. Kienast and Schmitz were the first to introduce flow cytometric measurement of reticulated platelets based on thiazole orange staining [7]. However, this flow cytometric determination of reticulated platelets by research protocols is time-consuming and requires considerable expertise. Standardization remained difficult even when the same dyes and protocols were used and the reference range for reticulated platelets of healthy individuals, as measured by custom made flow cytometry, has not been clearly defined [8–11]. Multiple factors contributing to this analytical issue were identified: the type and concentration of fluorescent dye, incubation time and temperature, fixation, RNase treatment and the flow cytometric data analysis [9, 11–14]. One of the major obstacles is the non-RNA specific binding of fluorescent dyes due to uptake in alpha dense granules, resulting in high background staining [9, 10, 15]. As the content of these dense granules is dependent on platelet size this problem may partly be solved by optimizing the assay conditions, i.e. degranulation by synthetic thrombin receptor ligand, and by applying a two-dimensional gating process [9, 10].

Automated determination of reticulated platelets

The first fully automated method to measure reticulated platelets based on flow cytometric analysis was established with the R-3000 reticulocyte analyzer (Toa Medical, later Sysmex Corporation, Kobe, Japan) equipped with special software using auramine O as fluorescence dye and a 488 nm argon laser [16, 17]. These early studies recognized a strong positive correlation between the percentage of reticulated platelets and the fraction of large platelets as well as for large platelets and MPV [17]. Sysmex later integrated the measurement of reticulated platelets as a parameter called “immature platelet fraction” into the XE-2100 and XE-5000 hematology analyzers (Sysmex Corporation, Kobe, Japan) within the reticulocyte count (RET/PLT channel) [18]. The later instruments operate a 633 nm semiconductor diode laser and use a proprietary fluorescence dye containing polymethine, which passes the cell membrane and stains residual RNA in reticulated platelets. In the scatterplot displaying cell size (forward light scatter) vs. RNA content (fluorescence) immature platelets are defined by a fixed gate as platelets with large cell size and the highest fluorescence intensity. IPF is given as the percentage of thus defined immature

platelets of the total platelet count (IPF) as well as absolute immature platelet count (absolute IPF). This method has been shown to produce reliable results in a number of studies and reference values for healthy individuals with a mean IPF of 3.4% (range 1.1%–6.1%) were established [18–23]. The latest Sysmex XN-series of hematology analyzers determines the IPF within a dedicated fluorescence platelet assay (PLT-F) using a proprietary oxazine fluorescence dye [24]. Again, the scatterplot of cell size (forward scatter) vs. RNA content (fluorescence intensity) is applied to quantify the IPF (Figure 1) [24]. For the Sysmex XN system reference values for healthy individuals with a median IPF of 2.6% (range 1.0%–7.3%) were established [25]. Hematological conditions pose a special challenge for correct counting of platelets. It was noted that fragments of apoptotic blood cells (e.g. in leukemic blood samples) might interfere with the IPF measurement and give falsely high results due to staining with the reticulocyte stain in the Sysmex XE-series [24, 26, 27]. These artifacts seem to be limited with the XN-series analyzers [24, 28]. The oxazine fluorescence dye used with the new PLT-F channel of the Sysmex XN-series strongly stains intra-platelet structures as mitochondria and cytosolic RNA, but plasma membranes of platelets, erythrocytes and red

Figure 1: Scatterplot of reticulated thrombocyte measurement by Sysmex XN automated hematology analyzer.

Sysmex XN-series determines immature platelet fraction within a dedicated platelet fluorescence assay (PLT-F channel) by forward light scatter vs. sideward fluorescence. FSC, forward scatter; IPF, immature platelet fraction; PLT-F, platelets; RBC, red blood cells; SFL, sideward fluorescence; WBC, white blood cells. Reprinted from Sysmex Europe GmbH with permission.

cell fragments are only slightly labeled [29]. In contrast, the polymethine fluorescence dye applied with the RET/PLT-channel by the predecessor Sysmex XE-series analyzers stains not only platelets, but also plasma membranes of erythrocytes effectively [29]. These improved staining properties of the oxazine fluorescence dye enable a better separation of immature platelets from other populations of similar size (e.g. red cell “ghosts”) in the scatterplot of cell size (forward light scatter) vs. RNA content (fluorescence intensity). The XN-series also shows improved precision of platelet counts below $50 \times 10^9/\text{L}$ and better correlation ($r^2 \geq 0.80$) with the flow cytometric reference method for platelet enumeration due to a 5 times larger sample volume analyzed [24, 27, 28, 30–32]. This progress in the XN-series may account for the moderate correlation of IPF between the XE-2100 and the XN-2000 ($r^2 \geq 0.60$) [24, 27] and potentially improves the utility of IPF especially in hematological patients with very low platelet counts and in clinical settings with interference of aberrant cell populations and debris (e.g. chemotherapy, thrombotic-thrombocytopenic purpura).

Abbott also introduced a method to quantify reticulated platelets, called “reticulated platelet”, based on RNA staining as integral part of the reticulocyte assay with the CELL DYN-Sapphire (Abbott Diagnostics, Santa Clara, CA, USA) [33]. This assay uses a 488 nm laser and the proprietary RNA fluorescence dye CD4K530 [34] to separate platelets and red blood cells by an embedded multi-dimensional analysis of three angles of light scatter (0° , 70° and 90°) and fluorescence intensity. First a variable gate adjusted to the red cell population is applied to identify the platelet population. Within the platelet gate, mature and reticulated platelets are discriminated by fluorescence intensity vs. 7° intermediate angle scatter. This algorithm allows correcting for size-dependent background fluorescence of platelets as already suggested earlier [9]. The gating strategy is depicted by Hoffmann et al. [33]. Reticulated platelets are given as a fraction of the total platelet count (retPLT) as well as absolute count of reticulated platelets (absolute retPLT). For healthy individuals reference values with a mean retPLT between 1.4% and 2.2% (reference range 0.4% to 2.8%–6.0%) were established [33, 35–38]. The recently released Abbott Alinity HQ hematology analyzer determines reticulated platelets (%rP) by a multidimensional analysis of light scatter (axial light loss, polarized side scatter, depolarized side scatter and four intermediate angles of light scatter) and fluorescence intensity [39]. This enables a reliable separation of platelets and red blood cells. However, a performance evaluation of the Abbott Alinity HQ regarding reticulated platelets is lacking thus far.

As for other hematological parameters all automated analyzers use whole blood samples anticoagulated with ethylenediaminetetraacetic acid (EDTA- K_2) for the measurement of reticulated platelets (IPF and retPLT). After blood collection stability of IPF (Sysmex XE-2100) ranges between 24 and 48 h [18, 40] and of retPLT (Abbott CELL DYN-Sapphire) between <6 h [38] and at least 26 h [33], provided samples are kept at room temperature. When blood samples are kept at 4°C , IPF increases linearly within the first 24 h, which can be corrected for by a simple algorithm [41–43]. With the Sysmex XN stability of platelet enumeration in the PLT-F channel decreases over time when samples are stored at room temperature (mean difference -6.6% and -14.4% after 48 h and 72 h) [27].

Clinical applications of reticulated platelets

Differential diagnosis of thrombocytopenia

First the clinical application of reticulated platelets focused on the diagnostic work-up of thrombocytopenia of unknown etiology. While bone marrow biopsy still remains the gold standard to estimate the activity of megakaryopoiesis, the fully automated measurement of reticulated platelets enables a fast and non-invasive evaluation of thrombopoiesis and can be carried without specialized hematological expertise. As thrombocytopenia due to peripheral consumption (e.g. immune thrombocytopenia [ITP], microangiopathic disease) results in compensatory increased megakaryopoiesis, a consecutive elevation of reticulated platelets is expected. In contrast, decreased amounts of reticulated platelets due to low megakaryopoietic activity are anticipated in bone marrow failure (BMF; e.g. myelodysplastic syndrome [MDS], aplastic anemia [AA], neoplastic disease, chemotherapy-induced thrombocytopenia [CIT]). A number of studies reported markedly elevated reticulated platelets by custom-made flow cytometry methods [7, 44, 45] as well as by automated hematology analyzers [18, 19, 21, 22, 38, 46–49] in patients with ITP (median IPF 7.7–25.2%, median retPLT 9.1%). However, multiple studies also described increased levels of reticulated platelets in patients with MDS and AA [22, 47, 50–53]. In the largest cohort of patients with AA ($n = 51$) the level of immature platelets was moderately elevated to a mean IPF of 3.5% (range 0.6%–12.9%; healthy individuals: mean IPF of 1.2%, range 0.3–5.9%, $n = 2039$) as measured by Sysmex XE-2100 (Figure 2) [22]. This increase of immature platelets

Figure 2: Immature platelet fraction in healthy individuals and patients with aplastic anemia [22].

Immature platelet fraction in patients with immune thrombocytopenia ($n=150$, mean IPF of 7.7%, range 1.0%–33.8%) is significantly higher than in healthy individuals ($n=2039$, mean IPF of 1.2%, range 0.3–5.9%) and patients with aplastic anemia ($n=51$, mean IPF of 3.5%, range 0.6%–12.9%). AA, aplastic anemia; IPF, immature platelet fraction (Sysmex); ITP, immune thrombocytopenia; normal, healthy individuals. Reprinted from Korean J Lab Med 2010;30:455 with permission.

in AA was less pronounced and significantly different ($p < 0.0001$) from patients with ITP (mean IPF of 7.7%, range 1.0%–33.8%; $n=150$) [22]. Despite considerable overlap regarding the level of immature platelets in both patient groups, the authors were able to define a cut-off value of $\text{IPF} \geq 7.3\%$ to differentiate patients with ITP from those with AA with reasonable sensitivity and specificity of 54.0% and 92.2%, respectively [22]. Higher IPF in BMF and thus a lower discriminatory power was found in another prospective study [52]. A smaller study recently described an improved discrimination between BMF due to AA ($n=18$) or CIT ($n=10$) and ITP ($n=47$) with the Sysmex XN-1000 applying a cut-off of $\text{IPF} > 5.8\%$ (sensitivity 85.1%, specificity 89.3%) [54]. Only one study investigated the role of automated measurement by the Abbott CELL-DYN Sapphire to distinguish between ITP and BMF (AA, MDS, acute myeloid leukemia) [52]. The levels of reticulated platelets showed considerable overlap between both groups and had no discriminatory power at all, whereas in a side-by-side analysis of the same samples IPF (Sysmex XE-5000) had some diagnostic relevance [52]. The authors suggested that this lack of discriminatory power with the Abbott CELL-DYN Sapphire was likely related to the dynamic threshold applied for the correction of size-dependent background fluorescence [11, 52]. The adjustment for platelet size by the Abbott CELL-DYN Sapphire algorithm thus rather

seems to be a disadvantage of this analytical method compared to the strategy without correction for platelet size as used by Sysmex. There are only a few reports on the role of reticulated platelets in MDS. When reticulated platelets were determined by custom-made flow cytometry, comparable levels ($p=0.28$) in patients with MDS (mean 8%, range 4%–17%, 95% confidence interval [CI]: 2–13%, $n=6$) and healthy individuals (mean 5%, range 0%–26%, 95% CI: 4%–6%, $n=71$) were reported, whereas patients with ITP had markedly increased levels (mean 23%, range 5%–58%, 95% CI: 14%–32%, $p < 0.0001$) [44]. In contrast, a number of studies using automated measurement of immature platelets described normal or increased levels as compared to healthy individuals [50–53]. Sugimori et al. reported a mean IPF of 8.80% in a larger cohort of MDS patients ($n=51$) as compared to 2.07% ($p < 0.01$) in healthy individuals ($n=170$) as measured with the Sysmex XE-2100 (Figure 3) [51]. The authors noted an increased $\text{IPF} > 10\%$ in 16 out of 51 MDS patients, while 19 patients had IPF levels within the range of healthy individuals [51]. Whereas there was an inverse correlation between platelet count and IPF in healthy individuals and ITP, this was not reproduced in this report focusing on MDS [51]. The authors further speculated that a prominent dysmegakaryopoiesis indicated by a high IPF may be linked to a poor prognosis in MDS [50, 51, 53], but this has not been independently confirmed in larger cohorts of patients.

Figure 3: Immature platelet fraction in healthy individuals and patients with myelodysplastic syndrome [51].

Immature platelet fraction in healthy individuals and patients with thrombocytopenia. IPF values as determined by Sysmex XE-2100 are plotted for healthy individuals (mean IPF of 2.07%, $\text{SD} \pm 1.06\%$), patients with myelodysplastic syndrome (mean IPF of 8.80%, $\text{SD} \pm 8.09\%$) and patients with immune thrombocytopenia (mean IPF of 18.1%, $\text{SD} \pm 11.5\%$). IPF, immature platelet fraction (Sysmex); ITP, immune thrombocytopenia; MDS, myelodysplastic syndrome; SD, standard deviation. Reprinted from [51] with permission.

A reciprocal relationship of low platelet counts and increased levels of immature platelets irrespective of the cause of thrombocytopenia was noted by in the number of reports [22, 38, 47, 48, 52, 55, 56]. It was hypothesized that in severe thrombocytopenia a shift might take place towards even more immature forms of reticulated platelets, which are released into the circulation [52]. Thus, the lifespan of these reticulated platelets would be increased, comparable to a phenomenon called “reticulocyte shift” in anemia [52, 57, 58]. This reciprocal interaction restrains the usefulness of reticulated platelets in the differential diagnosis of thrombocytopenia. Moreover, enhanced platelet turnover due to peripheral consumption of platelets caused by autoantibodies is only one component in the multi-causative pathogenesis of ITP. However, platelet turnover may also be normal or even decreased [59–61]. In ITP autoantibodies, including anti-glycoprotein IIb/III and anti-glycoprotein Ib/IX antibodies, interfere with the platelet production by megakaryocytes in the bone marrow and result in decreased thrombopoiesis [62, 63]. These pathophysiological facts limit the utility of reticulated platelets to distinguish ITP from other causes of thrombocytopenia (i.e. AA, MDS).

Recovery of thrombopoiesis after cytostatic therapy and transfusion management

Intensive cytostatic therapy to treat hematological malignancies and several types of solid tumors as well as hematopoietic progenitor stem cell transplantation (HPSCT) can induce pronounced transient cytopenia and necessitate prophylactic transfusion support. In order to predict recovery of thrombopoiesis and reduce unnecessary platelet transfusions several studies investigated the usefulness of reticulated platelets determined by custom flow cytometry [64–67] and automated analyzers [20, 55, 56, 68–74]. In general, these studies observed a rise of reticulated platelets several days before platelet recovery. Although a number of studies used fully automated measurement of IPF, these studies differed in their definition regarding the prediction of platelet recovery (platelet count $>20\text{--}30 \times 10^9/\text{L}$ within a median of 1–7 days), investigated heterogeneous patient cohorts (autologous/allogeneic HPSCT) and thus identified different thresholds of IPF (3.5%, 5.3%, $>5.8\%$, $>6.2\%$, $>7\%$, $>10\%$, $>12.1\%$) [20, 55, 68–73]. A consistent cut-off or increment of IPF to reliably predict platelet recovery is therefore still lacking. This is underscored by a systematic analysis of 37 courses of CIT/HPSCT, which demonstrated a poor capacity for both IPF (area under the curve [AUC] = 0.57, $p = \text{n.s.}$) and retPLT

(AUC = 0.57, $p = \text{n.s.}$) as well as for absolute IPF (AUC = 0.68, $p < 0.0001$) and absolute retPLT (AUC = 0.67, $p = 0.0002$) to predict platelet recovery within the following 2 days [56]. Another recent analysis of 44 allogeneic HPSCT patients demonstrated comparably poor prediction rates of 68.2% for platelet recovery ($\geq 20 \times 10^9/\text{L}$) by IPF, whereas other platelet parameters as PDW, MPV and platelet larger cell ratio (P-LCR) showed better prediction rates of 88.6%, 93.2% and 93.2%, respectively [74]. The median time benefit (i.e. the time between first rise of the prediction parameter and platelet recovery) of 4.0, 4.5 and 5.0 days for PDW, MPV and P-LCR, respectively, was also significantly longer than for IPF (1.5 days) [74]. However, the authors noted a large inter-individual variation between the rise of the aforementioned parameters and platelet recovery and concluded, that IPF was not clinically meaningful to predict platelet recovery to $>20.0 \times 10^9/\text{L}$, which is the critical decision-making level for platelet transfusion [74]. Although earlier studies suggested that reticulated platelets may be used as a parameter to optimize transfusion policies for platelet concentrates in patients undergoing intensive chemotherapy or HPSCT [20, 55, 71, 75], appropriate prospective trials have not been set-up yet. The large inter-individual variation between rise of IPF and platelet recovery and the fact, that IPF significantly drops after transfusion with platelet concentrates, strongly interfere with such strategies in patients who frequently receive platelet transfusions [20, 56, 69, 74, 76].

Acute coronary artery disease

Recently the role of reticulated platelets in acute coronary artery disease has been investigated. Immature platelets have increased cell volume and are metabolically more active than mature platelets [77, 78]. They have a greater prothrombotic potential due to higher levels of intracellular thromboxane A2 as well as increased levels of procoagulant surface proteins such as P-selectin and glycoprotein Ib and IIb/IIIa receptors [79–81]. In patients with unstable angina as well as non-ST-segment elevation myocardial infarction (non-STEMI) and STEMI significantly elevated levels of reticulated platelets were detected as compared to healthy volunteers by custom flow cytometry [82]. These results were later confirmed in a number of studies using the Sysmex XE-2100 automated analyzer [81, 83–85]. Conflicting with this, recent studies regarding patients presenting to the emergency department with acute chest pain did not find any significant difference in the IPF between patients with and without acute coronary syndrome (ACS) [86, 87]. However, this discrepancy may

be related to the comparison group of patients with chest pain for reasons other than ACS, which might include individuals with elevated IPF due to various other medical conditions (e.g. infection, inflammation) [88]. Immature platelets may serve as a prognostic marker for the outcome of patients with ACS. High levels immature platelets (IPF > 6.2%) within 24 h of hospitalization were shown to be an independent risk factor (odds ratio [OR] = 2.42, 95% CI: 1.08–5.43, $p = 0.032$) for in-hospital mortality in patients with ACS after adjusting for other covariables (age, diabetes mellitus, heart failure, ST-segment deviation, troponin level) [85]. Three studies investigated the utility of immature platelets in patients with ACS to assess long-term morbidity and mortality. Cesari et al. demonstrated that increased IPF (IPF $\geq 3.1\%$) within 24–48 h following percutaneous coronary intervention (PCI) for ACS (STEMI, non-STEMI/unstable angina) was able to predict cardiovascular mortality after 1 year follow-up (OR = 4.15, 95% CI: 1.24–13.91, $p = 0.02$) in a multivariate model adjusted for Global Registry of Acute Coronary Events (GRACE) risk score [89]. Ibrahim et al. observed that an absolute IPF $\geq 7, 6 \times 10^6/L$ in patients with ACS and stable ischemic heart disease (SIHD) was associated with an increased risk to experience a major adverse cardiovascular event (MACE) in a follow-up period of 31 months (hazard ratio [HR] 4.65, 95% CI: 1.78–12.16, $p < 0.002$) [90]. In agreement with this, Freynhofer et al. reported that IPF (cut-off of IPF $\geq 3.35\%$) distinguished significantly between those patients with and without MACE at 6 months follow-up ($p = 0.021$) in a large observational study of 486 patients with ACS and SIHD, who underwent PCI [91]. However, an independent benefit of IPF beyond that of classical risk factors to identify ACS patients with an increased likelihood for an unfavorable outcome has to be confirmed by further studies before IPF may become relevant for routine clinical practice.

Antiplatelet therapy

Reticulated platelets have also been investigated in response to anti-platelet therapy. Acetylsalicylic acid (ASS) is commonly used as a secondary prophylaxis in cardiovascular disease and transient ischemic attack/acute ischemic stroke (TIA/AIS). By the means of selective acetylation, it is an irreversible inhibitor of cyclooxygenase-1 (COX-1) and COX-2 and prevents the production of procoagulant thromboxane A₂ (TxA₂). This irreversible inhibition of COX-1 in anucleate platelets has a sustained effect, as it renders platelets unable to synthesize TxA₂ and therefore to aggregate for their entire lifespan

(7–10 days) [92]. Similarly, the thienopyridines ticlopidine, clopidogrel and prasugrel permanently inhibit platelet activation by adenosine-diphosphate (ADP) via irreversible binding to the P2Y₁₂-receptor. In-vivo the circulating platelet pool partly recovers the ability to produce TxA₂ due to newly produced platelets as early as 4 h after ASS ingestion and full aggregation was demonstrated in in-vitro experiments when only 2.5% ASS-free platelets were present [93]. Consistent with this, a higher platelet turnover, as indicated by higher levels of reticulated platelets, was associated with resistance to antiplatelet therapy with ASS [94]. Higher levels of reticulated platelets also correlated with resistance to anti-platelet therapy with clopidogrel [95], prasugrel [96, 97] and to dual antiplatelet therapy with ASS/thienopyridine [80, 98–101]. Hypo-responsiveness to clopidogrel (aggregation >50% in response to ADP) in healthy individuals was associated with high numbers immature platelets (upper tertile) and an IPF $\geq 3.6\%$ identified subjects with an impaired response to clopidogrel with a sensitivity and specificity of 85.7% and 81.8%, respectively [95]. A significant relationship between resistance to combined antiplatelet therapy with ASS/clopidogrel and reticulated platelets (retPLT and IPF, respectively), was confirmed in patients with diabetes mellitus type-2 [102], SIHD and ACS [98–100]. However, during the maintenance treatment with clopidogrel, prasugrel or ticagrelor reticulated platelets did not correlate with an impaired antiplatelet response [99, 103–106]. Recent studies indicated, that beyond the effect of increased platelet turnover intrinsic properties of immature platelets contribute to the impaired antiplatelet response to thienopyridines [107, 108]. In summary, the studies evaluating the role of reticulated platelets in response to anti-platelet therapy have shown inconsistent results and warrant further research.

Ischemic stroke

TIA and AIS result from embolic or thrombotic occlusion of cerebral vessels and may cause a focal cerebral ischemic injury. Thus far there are only limited studies regarding the role of reticulated platelets in the incidence, pathophysiology and outcome of ischemic cerebrovascular disease [109]. While two preliminary studies reported an increased percentage of reticulated platelets following cardioembolic ischemic stroke [110, 111], McCabe et al. did not find any significant increase in reticulated platelets in patients with AIS or TIA compared with healthy individuals [112]. The small number of patients in the earlier studies and the methodological differences in the

custom flow cytometry analysis may account for these divergent results. A recent study using automated measurement of IPF by Sysmex XE-2100 revealed a significantly higher IPF in symptomatic carotid stenosis patients early (at ≤ 4 weeks) (IPF = 5.78%, $p < 0.001$) as well as late (≥ 3 months) (IPF = 5.11%, $p = 0.01$) after TIA/AIS than in asymptomatic patients (IPF = 3.48%) [113]. In order to elucidate the role of reticulated platelets in ischemic cerebrovascular disease clearly further research is required.

Infection

Thrombocytopenia is often seen in patients who develop severe infection and sepsis. While the cause of thrombocytopenia in sepsis may be multifactorial, increased platelet consumption due to an uncontrolled procoagulant response with thrombin formation and septic coagulopathy play a main role [114, 115]. In this respect it was recognized that reticulated platelets provide meaningful information for the diagnosis and follow-up of patients with sepsis [116–122]. Increased levels of IPF were found to significantly correlate with infection as determined by positive blood cultures (positive blood culture: mean IPF

4.86%; negative blood culture: mean IPF 1.79%, $p < 0.0001$) even if patients were non-thrombocytopenic, indicating subliminal platelet consumption [116]. IPF increased before the onset of sepsis by a median of 2 days [117, 119, 121], was independent from other coagulation parameters [118, 120] and predicted mortality [119, 122]. The diagnostic performance of IPF (AUC = 0.82 [121], AUC = 0.87 [120]) in the discrimination of septic patients from non-septic patients was comparable with current biomarkers of infection such as c-reactive protein (CRP; AUC = 0.81 [121], AUC = 0.94 [120]) and procalcitonin (AUC = 0.92) [120]. Of note, the prognostic efficiency of absolute IPF count to identify patients 2 days before sepsis onset was superior to that of CRP by multivariate analysis [121]. Whereas absolute IPF count displayed a gradual increase from 6 days before the onset of sepsis, the kinetics of CRP fluctuated strongly [121]. Discrepant results were found when IPF was used to assess the severity of sepsis [118, 120]. While IPF correlated with the severity of sepsis in patients treated in an intensive care unit [118], this was not the case in another study that included general ward patients [120]. Larger prospective studies are warranted to confirm these preliminary data and evaluate IPF as a biomarker for the early prediction of sepsis.

Table 1: Clinical applications for reticulated platelets.

Condition	Objective	Reference
Thrombocytopenia	Differentiate hypo- from hyperproliferative thrombocytopenia	[7, 18, 19, 21, 22, 38, 44–54, 123]
	Predicting platelet recovery after CIT/HPST	[20, 55, 56, 64–74]
	Transfusion management	[20, 55, 56, 69, 71, 74, 75]
	Assessing treatment response to thrombopoietic drugs	[124]
Acute coronary syndrome	Predicting outcome	[85, 89–91]
	Monitoring of treatment response to antiplatelet therapy	[80, 94–108, 125]
Ischemic stroke	Predicting outcome	[110–113]
Infection	Prediction and diagnosis of sepsis	[116–122]
Miscellaneous conditions with preliminary data		
Myeloproliferative diseases	Assessing platelet turnover in ET	[126]
	Surrogate for JAK2 V617F mutation in PV and ET	[127–129]
	Prognostic biomarker in MF	[130]
Myelodysplastic syndrome	Prognostic biomarker	[50, 51, 53]
Thrombotic thrombocytopenic purpura	Diagnosis and monitoring of treatment response	[131–133]
Disseminated intravascular coagulation	Monitoring severity and predicting mortality	[134]
Heparin-induced thrombocytopenia	Predicting presence of anti-platelet factor 4/heparin complexes	[135]
Diabetes mellitus	Predicting cardiovascular complications	[136]
Pregnancy	Diagnostic biomarker for preeclampsia/HELLP syndrome	[137–139]
Liver disease	Predictive marker for esophageal varices	[140]
Dengue fever	Predicting platelet recovery in dengue fever	[141]

CIT, chemotherapy induced thrombocytopenia; ET, essential thrombocythemia; HELLP syndrome, hypertension/elevated liver enzymes/low platelets syndrome; HPST, hematopoietic progenitor stem cell transplantation; MF, primary myelofibrosis; PV, polycythemia vera.

Other hematological and non-hematological conditions

Additionally, the utility of immature platelets was assessed in a number of other hematological and non-hematological diseases (Table 1). The scientific evidence for these conditions however is still very limited as most of these findings are described only in a small number of patients and by a single report.

Limitations to the use of reticulated platelets

The lack of a standardized reference method to determine reticulated platelets has hindered a broader clinical

application until now. Only a single flow cytometry study applied the ICSH reference method to enumerate platelets with the aim to develop a future reference method for reticulated platelets [142, 143]. Therefore, the performance of the aforementioned automated analyzers cannot be independently validated. Two studies showed only a modest correlation of $r^2=0.38$ between the parameters for reticulated platelets (IPF and retPLT) when Sysmex XE-2100/XE-5000 and Abbott CELL-DYN Sapphire were compared directly (Figure 4) [35, 38]. This lack of agreement seems to be related to the different gating algorithm applied by the manufactures, which leads to two slightly differently defined populations of reticulated platelets [38]. Whereas in a study of Meintker et al. there was only weak correlation ($r^2=0.25$) of both IPF (Sysmex XE-5000) and retPLT (Abbott CELL-DYN Sapphire) with other parameters indicative for increased thrombopoiesis as MPV [2], Hoffmann et al. showed a modest, but significant ($p < 0.001$) negative correlation (Spearman's $\rho = -0.399$) of retPLT and MPV in healthy individuals [33, 38]. In conclusion the parameters IPF and retPLT are not interchangeable and reference ranges as well as measurement values cannot simply be translated. Furthermore, most of the clinical studies cited above, only used the well-established IPF measurement by Sysmex analyzers. Comparable data for retPLT as determined by Abbott CELL-DYN Sapphire and Alinity HQ are lacking. In summary, a standardized reference method for the measurement of reticulated platelets is urgently needed.

Figure 4: Comparison of IPF (Sysmex XE-5000) and retPLT (Abbott CELL-DYN Sapphire) [38]. (A) Correlation. (B) Bland-Altman plot. Reprinted from [38] with permission.

Conclusion and future perspectives

Whereas IPF and retPLT are markedly increased in hyperproliferative thrombocytopenia such as ITP, hypoproliferative conditions (e.g. AA, MDS, CIT) frequently also show moderately elevated levels as a reciprocal relationship between reticulated platelets and platelet count irrespective of the cause of thrombocytopenia exists. Thus, the discriminatory power of IPF measurements is only moderate. Improved detection algorithms of the XN analyzers may lead to higher clinical utility, but this should be proven in larger prospective studies, defining IPF thresholds that take patients' current platelet numbers into account.

Although an IPF-guided transfusion management would be highly desirable to avoid unnecessary platelet transfusions, considerable inter-individual variations between the time period of increasing IPF and platelet recovery has thus far impeded this concept.

Preliminary studies indicated clinical utility of IPF and retPLT for the risk assessment of coronary artery

disease and stroke as well as for monitoring of anti-platelet therapy, though validation in larger randomized studies and demonstration of added value compared to conventional parameters are warranted before this might be introduced into clinical practice. Apart from this, the role of reticulated platelets has recently been investigated in several other conditions. The evidence in the majority of these settings is still very limited and additional research is necessary to confirm these findings.

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