Reticulated platelets: analytical aspects and clinical utility

Abstract: Reticulated platelets are immature platelets circulating in blood; they reflect the activity of megakaryopoiesis in the bone marrow. Therefore, they can be used as a non-invasive test in patients with thrombocytopenia in various clinical conditions. The preferred method of analysis is by flow cytometry. However, there is an evident lack of analytical standardization, making it difficult to compare results obtained in different laboratories. Currently, two types of hematology analyzers are on the market offering fully automated measurement of reticulated or immature platelets: the high end analyzers manufactured by Sysmex (XE- and XN-series) and Abbott (CELL-DYN Sapphire). Although the methods are essentially different and cannot be used interchangeably, both have been proven to have clinical utility. Reticulated or immature platelet assays are useful for the differential diagnosis of thrombocytopenia and for monitoring bone marrow recovery after chemotherapy or stem cell transplantation. These assays may aid clinicians in platelet transfusion decisions when recovery from thrombocytopenia is imminent. In addition, preliminary findings indicate that there is a rationale for reticulated or immature platelets for risk stratification in acute coronary syndromes and for monitoring the effect of treatment with antiplatelet drugs in patients with coronary artery diseases. The aim of this paper is to present the present technology available for measuring reticulated platelets as well as an overview of the current status of clinical application. This overview also indicates that more research is needed before reticulated or immature platelet assays can be applied in other clinical conditions than thrombocytopenia and after transplantation.

Keywords: diagnostic use; immature platelets; megakaryopoiesis; reticulated platelets; thrombocytopenia.

Formation and maturation of megakaryocytes

The study of blood platelets or thrombocytes started in the late 19th century, when Wright had developed his variant of the Romanovsky stain that allowed detailed observations of the smallest blood elements [1]. Platelets represent the terminal stage of megakaryopoiesis, a highly complex sequence of events that can be traced back to the generation of the pluripotent hematopoietic stem cell in bone marrow. This pluripotent stem cell proliferates and differentiates via several intermediates into a megakaryoblast and eventually into a megakaryocyte (Figure 1). Megakaryopoiesis is regulated by various growth factors and cytokines, of which thrombopoietin (TPO) is the most important. TPO stimulates the number, the size and the ploidy of megakaryocytic cells and is the key regulator of platelet concentration in the circulation [2]. Once the megakaryoblast stage is reached, the cell loses its proliferative capacity and starts the maturation process. Megakaryopoiesis has a unique way of maturation that does not occur in other cell lines: endomitosis. This is nuclear division without cell division; the cell is multiplying its nuclear material and increases its cytoplasm, all within the same cell. Finally, maturation results in a megakaryocyte that possesses multiple nuclear copies and abundant cytoplasm. The nuclear ploidy of a megakaryocyte is normally between 8N and 64N (median 16N), whereas higher and lower ploidy may occur in various pathological conditions. Depending on the organism’s need of new platelets, endomitosis stops and the formation of platelets commences (Figure 1). First, there is intracytoplasmic formation of channel-like structures composed of lipids, called the membrane demarcation system [3]. These lipids later assemble into membrane bilayers and eventually form the cell membranes of platelets when the megakaryocyte cytoplasm starts to disintegrate. In this phase there is also active protein synthesis and cytoskeletal assembly.
Platelet production by megakaryocytes

Eventually, megakaryocytes form pseudopodia-like extensions protruding into sinuses and release platelets into the extracellular space. How this exactly takes place is not fully elucidated [4–6]. A healthy human produces approximately 1–2 million platelets per second. In response to thrombocytopenia, platelet production can be accelerated by up to a 10-fold increase [7].

Megakaryocyte cytoplasmic volume expands synchronized with nuclear ploidy and this eventually determines the number of platelets that a megakaryocyte will produce. A single megakaryocyte can generate up to 5000 platelets [1, 2]. In steady-state conditions, the platelet production rate is aimed at keeping the total circulating platelet mass (platelet number × mean platelet volume, also called plateletcrit) constant [1]. In conditions of stress, platelets are released from megakaryocytes at an earlier stage, which results in platelets that are larger than normal.

Each individual has its own personal setpoint for platelet count and platelet volume; these are largely under genetic control [8]. As a consequence, intra-individual variations in platelet count are quite small in comparison with the population reference ranges. In the normal population, platelet count is inversely correlated with mean platelet volume (MPV), and consequently the total circulating platelet mass is less variable between individuals than platelet count.

Physiology of reticulated platelets

When platelets are released from the megakaryocyte cytoplasm, they still contain a small amount of RNA. For long it has been thought that this RNA was a vestigial remnant of megakaryocytic RNA, but there are recent indications that platelets are able using this RNA for protein synthesis [9]. Anyway, they represent the youngest platelets in the circulation and are named reticulated platelets (retPLT), in analogy with reticulocytes in erythropoiesis [10]. The concentration of retPLT in bone marrow is on the average 2–3 times higher than in peripheral blood, where they correlate with megakaryocyte numbers [11]. And whereas platelets persist in the circulation for 7–10 days, retPLT have a much shorter lifespan (<1 day). Therefore they can act as a marker of megakaryopoietic activity in the bone marrow [12, 13], which gives retPLT clinical and diagnostic utility.

Reticulated platelet methods – flow cytometry

The initial description of retPLT dates back to 1969, when Ingram and Coopersmith studied a canine model of acute blood loss and observed coarsely punctuated reticulum in platelets after supravital staining of blood with new methylene blue [14]. This technique was initially also used in human blood, but it is obviously not well suited for routine applications.

Hematopoietic stem cell
Myeloid stem cell
Megakaryoblast
Megakaryocytes
Bone marrow
Blood
Platelets
Hematopoietic stem cell
Myeloid stem cell
Megakaryoblast
2N
4N
8N
16N
32N
Megakaryocytes
Endomitosis
Differentiation
Maturation

Figure 1 Schematic representation of megakaryopoiesis. After Kuter [2].
Kienast and Schmitz initiated a breakthrough in the field when they described a flow cytometric technique for analyzing retPLT, based on RNA staining by thiazole orange [15]. In subsequent years, several research groups published their findings in a wide variety of conditions like thrombocytopenia [16–21], thrombocytosis [22, 23], after stem cell transplantation [24–27], hereditary platelet diseases [28, 29], thrombo-embolic disorders [30, 31], kidney disease [32–34], preeclampsia [35], hyperthyroidism [36] and in healthy and thrombocytopenic neonates [37, 38]. The overall conclusion from these studies is that retPLT in blood represent a useful non-invasive marker of megakaryopoietic activity in the bone marrow. However, it also became evident that the flow cytometric assay was prone to methodological variation, which made it difficult to compare results obtained with different assays. For example, the normal reference range was reported to range between 1% and 15% [23, 39]. This wide range can be explained by lack of standardization of the methods. Many factors have been identified that contribute to this analytical issue: the type and concentration of fluorescent dye, incubation time and temperature, fixation, RNase treatment and the flow cytometric data analysis, including gating and threshold settings [17, 18, 39–41].

One of the major problems is that platelets show non-RNA-specific binding of fluorescent dye, resulting in background staining, which is size-dependent [39, 42, 43]. This issue can be solved by optimizing the assay conditions and, in particular, by applying a two-dimensional gating process [39]. Nevertheless, standardization attempts for improving concordance between laboratories were unsuccessful, even when using the same protocol [9].

Recently, new initiatives were undertaken that are aimed at developing a method with the potential to become a future international reference method [44, 45]. However, even if a standardized method would be available, it would carry the disadvantage of not being well suited for routine clinical applications, since flow cytometry requires a great deal of expertise and is hardly available for patient care on a 24/7 basis. For the time being, a surrogate level of standardization is possible, since two manufacturers offer hematology analyzers that are capable of measuring reticulated or immature platelets (Table 1).

### Immature platelet methods – Sysmex hematology analyzers

The first available fully automated method for measuring reticulated platelets was in the R-3000, a dedicated reticulocyte analyzer developed by Toa Medical (later Sysmex) [46, 47]. The method used auramine O as a fluorescent RNA dye and a 488 nm Argon laser. By plotting forward light scatter (representing cell size) against fluorescence (RNA content), reticulated platelets could be distinguished from mature platelets. In normal healthy individuals the mean retPLT count was 0.98%–1.27% and thrombocytopenic patients with a variety of diseases had increased concentrations, in accordance with the flow cytometric results mentioned above [18, 47, 48]. Notably, a strong positive correlation existed between percentage retPLT and the fraction of large platelets, except in patients with aplastic anemia [47]. Furthermore, the large platelet fraction highly correlated with MPV; data on the correlation between retPLT and MPV were not provided, but one can reasonably assume that such correlation was present.

Automated measurement was later integrated into the Sysmex XE-2100 and XE-5000 hematology analyzers, as a part of the reticulocyte determination. This enabled automated quantification of what from then on was called immature platelet fraction (IPF) [49]. As these instruments employ a 633 nm diode laser as the light source, other dyes were needed and a mixture of polymethine and oxazine was selected. As before, a forward scatter versus fluorescence scatterplot defined platelets with the highest fluorescence intensity as immature platelets (Figure 2A). The reference values in a healthy population were clearly higher than with

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Overview of some key characteristics of methods for measuring reticulated or immature platelets.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample preparation</td>
<td>Flow cytometry</td>
</tr>
<tr>
<td>Fluorescent dye</td>
<td>Variable: none, fixation or isolation of platelets</td>
</tr>
<tr>
<td>Incubation time</td>
<td>Variable 15 min–2.5 h</td>
</tr>
<tr>
<td>Precision at normal platelet count, CV</td>
<td>Not specified</td>
</tr>
<tr>
<td>Precision in thrombocytopenia, CV</td>
<td>Not specified</td>
</tr>
<tr>
<td>Reference range,%</td>
<td>Highly variable 1%–15%</td>
</tr>
<tr>
<td>Reference range, 10⁹/L</td>
<td>Mean 3.2</td>
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</table>
the previous R-3000 method: mean 3.4% (range 1.1%–6.1%) [49]. These reference values were later confirmed by others [50–54]. In all studies, patients with idiopathic thrombocytopenia (ITP) had increased IPF; in some patients IPF was even strongly increased, up to 50%–60% [49, 54, 55]. Such extreme values were later recognized to be artifacts due to interference by white blood cell fragments [56].

With the introduction of the XN-series hematology analyzers, Sysmex changed the IPF method. It is now part of the fluorescent platelet assay, which uses a proprietary oxazine-based dye for staining RNA. Still, IPF is derived from the forward scatter (cell size) versus sideward fluorescence (RNA content) scatterplot [56, 57], as shown in Figure 2B. The moderate correlation in IPF between XE-2100 and XN is explained, at least partially, by reduced interference in the XN system [56]. Preliminary data suggest that the XN reference values of IPF are comparable with the XE-2100 method [56].

Reticulated platelet methods – Abbott CELL-DYN Sapphire

Apart from the Sysmex method discussed above, the Abbott CELL-DYN Sapphire is currently the only hematology analyzer capable of measuring retPLT. The assay is an integral part of the reticulocyte assay, which is based on the fluorescent dye CD4K530 [58] that is excited by a 488 nm solid state laser. Three angles of scattered light plus fluorescence are recorded, which allows multi-dimensional separation of platelets and red blood cells. The embedded algorithm defines retPLT in an FL1 versus 7° scatterplot (Figure 2C). This approach enables correcting for the size-dependent background fluorescence of platelets [39]. In healthy individuals, mean retPLT are between 1.4% and 2.2%, and reported reference ranges are 0.4%–2.8% [59], 0.4% to 4.45%–6.0% [60–62] and 1.0%–3.8% [63].

In the CELL-DYN Sapphire, the correlation between retPLT and MPV was investigated in relatively small groups and was described as not significant [64] or only weak [63]. However, in a large group of subjects with normal platelet counts, we found a significant negative correlation between retPLT and MPV [62].

Comparison of reticulated and immature platelet methods

Due to the lack of a standardized reference method for retPLT, assessing the performance of the automated methods is difficult; only a few side-by-side comparisons are available.

Studies where Sysmex XE-2100 IPF was compared with reference flow cytometric retPLT indicated low or moderate correlations [65, 66]. Remarkably, the coefficients of correlation differed between patient groups, from no correlation in healthy individuals to relatively high correlations in patients with thrombocytopenia due to peripheral destruction [66, 67]. One study using the Sysmex XT-2000iV, which uses an identical method as
Hoffmann: Reticulated platelets

XE-2100, demonstrated reasonable correlation between IPF and reference flow cytometry, albeit with a significant systematic bias [68].

Direct comparison between Sysmex XE-2100 or XE-5000 IPF and CELL-DYN Sapphire retPLT resulted in weak or modest correlations [63, 64]. Sapphire was found to have a distinctively narrower reference range than XE-5000, enabling higher sensitivity for separating normal and abnormal patients [63].

These weak correlations and the different relationships between IPF/retPLT and MPV mentioned previously, lead to the conclusion that although both parameters harbor information of platelet turnover, they do reflect different aspects of thrombopoiesis. As a consequence, the two parameters cannot be used interchangeably [63].

Pre-analytical aspects

Obviously, immature or reticulated platelets are normally measured in blood samples collected into K2-EDTA, as for other hematologic parameters. One single study advised that citrate-theophylline-adenosine-dipyridamole solution (CTAD) was to be preferred over EDTA, as IPF measured using a Sysmex XE-5000 remained more stable in blood from patients with chronic ITP [69].

Regarding storage temperature before analysis, most authors agree that XE-2100 IPF is stable for 24–48 h after blood collection, provided the samples are kept at ambient temperature [49, 70, 71]. Others, however, reported stability for 3–8 h only [50, 51]. When blood samples are kept at 4 °C, IPF is rather unstable [69, 72], but the increase in IPF is apparently so predictable that it can be corrected for by a simple algorithm [73].

Stability data on retPLT as measured with CELL-DYN Sapphire range between <6 h [63] and at least 26 h [62].

Clinical utility of reticulated/immature platelets

In the earlier years, retPLT research focused on a possible differential diagnostic aid in patients with thrombocytopenia. Since megakaryopoietic activity is low in patients with bone marrow failure, the assumption was that consequently retPLT would be low, too. In contrast, conditions with peripheral platelet destruction like ITP are characterized by accelerated megakaryopoiesis and hence the retPLT count would be increased. Many studies have now confirmed that retPLT or IPF are valuable in establishing the cause of thrombocytopenia: decreased production can reliably be distinguished from peripheral destruction (see Table 2).

Another well-documented application of retPLT is monitoring the thrombocytopenic phase after chemotherapy and transplantation for hematological malignancies.
Table 2  Clinical conditions where retPLT or IPF can be regarded as established diagnostic or prognostic tools.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Intended goal</th>
<th>References retPLT</th>
<th>References IPF</th>
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</thead>
<tbody>
<tr>
<td>Low platelet count</td>
<td></td>
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<tr>
<td>Thrombocytopenia of unknown etiology</td>
<td>Differentiating hypoproduction from accelerated destruction</td>
<td>[15, 63, 66, 84, 85]</td>
<td>[49, 55, 63, 66, 75]</td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>Predicting platelet recovery</td>
<td>[86, 87]</td>
<td>[76]</td>
</tr>
<tr>
<td>Bone marrow or peripheral stem cell transplantation</td>
<td>Predicting platelet recovery</td>
<td>[17, 88–90]</td>
<td>[91–95]</td>
</tr>
<tr>
<td>Normal or high platelet count</td>
<td>Estimating platelet turnover</td>
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(Table 2). Generally, an increase in retPLT precedes the recovery of platelet count by 2–3 days. This creates the opportunity to defer platelet transfusions that would be given when transfusion decisions are based on platelet counts only. Until present, limited clinical evidence supporting this concept has been reported and there is an evident need for randomized, controlled clinical studies in this field [82, 97, 98]. A highly interesting observation that also needs to be confirmed is that prophylactic transfusions with high IPF platelet concentrates seem to be more effective than low IPF platelets [99].

Researchers in the field of cardiovascular diseases have recently gained interest in retPLT (Table 3). The current literature seems to provide preliminary support for the clinical utility of retPLT or IPF determinations, for risk assessment in acute coronary syndrome [100–104] as well as for monitoring drug treatment of coronary artery disease [78, 79, 105–107]. However, larger and randomized studies are necessary for proving that these concepts are effective and safe in clinical practice. There are also indications that it is preferentially immature platelets that are recruited into arterial thrombi [106] and if confirmed, this finding may have consequences for treatment with antiplatelet drugs [107].

Apart from the above disease states, there are several other conditions where retPLT are supposed to play a role (Table 3). Most of these associations are only described in a single paper or are based on small patient numbers, thus the scientific evidence is still weak. Therefore, additional research studies are absolutely required for obtaining independent confirmation on the utility retPLT or IPF in these settings.

Table 3  Clinical conditions where retPLT or IPF may have potential clinical utility, but further studies are required for confirmation.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Possible application</th>
<th>References retPLT</th>
<th>References IPF</th>
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</thead>
<tbody>
<tr>
<td>Low platelet count without marrow dysfunction</td>
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<td></td>
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<tr>
<td>ITP</td>
<td>Predicting treatment response</td>
<td>[84]</td>
<td></td>
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<tr>
<td>Disseminated intravascular coagulation</td>
<td>Assessing prognosis</td>
<td>[108]</td>
<td></td>
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<tr>
<td>Thrombotic thrombocytopenic purpura</td>
<td>Assessing disease activity and adjusting therapy</td>
<td>[49, 109]</td>
<td></td>
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<tr>
<td>Cyclic thrombocytopenia</td>
<td>Predicting next thrombocytopenia phase</td>
<td>[110]</td>
<td></td>
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<tr>
<td>Myelodysplastic syndrome</td>
<td>Assessing prognosis in cases with aberrant karyotype</td>
<td>[98, 112]</td>
<td></td>
</tr>
<tr>
<td>Normal or high platelet count</td>
<td>Investigate possible linkage with Jak2 mutation</td>
<td>[113]</td>
<td></td>
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<tr>
<td>Essential thrombocytemia and polycythemia vera</td>
<td>Assessing treatment effect</td>
<td>[67]</td>
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<tr>
<td>Therapy with thrombopoietic drugs</td>
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<tr>
<td>Altered platelet function</td>
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<tr>
<td>Acute coronary syndrome</td>
<td>Assessing role of platelet activation in prognosis</td>
<td>[100, 104]</td>
<td>[101–104]</td>
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<td>Drug treatment of coronary artery disease</td>
<td>Predicting treatment response</td>
<td>[78, 106, 107]</td>
<td>[79, 105]</td>
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<tr>
<td>Chronic uremia</td>
<td>Assessing effect of hemodialysis on platelet kinetics</td>
<td>[32]</td>
<td>[114]</td>
</tr>
<tr>
<td>Miscellaneous</td>
<td></td>
<td></td>
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<tr>
<td>Chronic liver disease</td>
<td>Differentiating between hepatitis and cirrhosis</td>
<td>[51]</td>
<td></td>
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<tr>
<td>ICU patients</td>
<td>Predicting development of sepsis</td>
<td>[115]</td>
<td></td>
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<tr>
<td>Neonates</td>
<td>Predicting degree of thrombocytopenia</td>
<td>[116, 117]</td>
<td></td>
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<tr>
<td>Diabetes mellitus</td>
<td>Predicting cardiovascular complications</td>
<td>[81]</td>
<td></td>
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<tr>
<td>Platelet transfusions</td>
<td>Supporting transfusion decisions</td>
<td>[97]</td>
<td>[82, 98, 99]</td>
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</table>
Highlights and conclusions

Summarizing the literature reviewed above, it can be concluded that:

- Reticulated platelets are the most immature platelets in the circulation and they reflect the megakaryopoietic activity in bone marrow
- Reticulated platelets are not necessarily large platelets
- The two commercially available methods (Sysmex IPF and Abbott reticulated platelets) are not interchangeable and each require their own reference values and clinical decision limits
- There is an urgent need for a standardized method that can serve as an international reference for assessing the performance of reticulated/immature platelet methods
- Measuring reticulated/immature platelets is of proven clinically usefulness in patients with thrombocytopenia of unknown etiology and for monitoring bone marrow recovery after chemotherapy and stem cell transplantation
- Reticulated platelets seem to play a role in the etiology of coronary arterial diseases. If confirmed in large-scale trials, measurement of reticulated platelets or IPF may be useful for risk assessment as well as therapy monitoring
- Some other indications look promising, but need further investigation before routine use of reticulated platelet assays is warranted

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