Reference Intervals of Reticulated Platelets and Other Platelet Parameters and Their Associations

Johannes J. M. L. Hoffmann, PhD; Nicole M. A. van den Broek, PhD; Joyce Curvers, PhD

- **Context.**—Reticulated platelets are platelets recently released from the bone marrow, and they can serve as a noninvasive indicator of recent megakaryopoietic activity. Widespread clinical use has been hampered by laborious methods and lack of standardization. Recently, a fully automated method was released on the Abbott CELL-DYN Sapphire hematology analyzer.

- **Objective.**—To establish reference ranges for reticulated platelets. Secondary aims were to investigate associations between reticulated platelets and other platelet parameters like mean platelet volume, plateletcrit, and platelet distribution width.

- **Design.**—Reticulated platelets and other platelet parameters were measured in an unselected cohort of 8089 subjects visiting a primary health care laboratory. The reticulated platelet data were analyzed using the Bhattacharya technique. In addition, a nonparametric method was used in selected subjects with normal platelet counts for providing reference ranges.

- **Results.**—Reticulated platelets ranged from 0.4% to 6.0% or from 1 to 18 × 10^3/L. Reticulated platelets increased significantly with the subjects’ age. Statistically, males had slightly higher values than females, but the differences were negligible. Reticulated platelets were positively correlated with platelet count and negatively with mean platelet volume.

- **Conclusions.**—Reference ranges have been established for reticulated platelets as measured on the CELL-DYN Sapphire hematology analyzer. There were no relevant differences between the sexes, but there was a clear effect of age. An individual’s reticulated platelets are associated with the platelet count as well as mean platelet volume.


---

**MATERIALS AND METHODS**

**Blood Samples**

During 4 weeks in July and August 2011, the laboratory processed all routine blood samples in reticulocyte mode for obtaining the retPLT count. Blood was collected into vacuum tubes containing K₂-EDTA as an anticoagulant (final concentration 1.8 mg/mL; Becton Dickinson, Plymouth, United Kingdom) and the samples were kept at ambient temperature for a maximum of 6

---

Accepted for publication January 10, 2013.

From the Abbott Diagnostics Division, Abbott GmbH & Co KG, Wiesbaden-Delkenheim, Germany (Dr Hoffmann); and Diagnostiek voor U Laboratory, Eindhoven, the Netherlands (Drs van den Broek and Curvers).

Dr Hoffmann is an employee of Abbott Diagnostics. The other authors have no relevant financial interest in the products or companies described in this article.

Reprints: Johannes J. M. L. Hoffmann, PhD, Abbott Diagnostics Division, Scientific Affairs Hematology, Abbott GmbH & Co KG, Max-Planck-Ring 2, D-65205 Wiesbaden-Delkenheim, Germany (e-mail: hans.hoffmann@abbott.com).
hours before the assay was performed. The design of the study was in accordance with institutional ethical guidelines.

Subjects

The laboratory provides diagnostic services to general physicians, to other primary health care professionals, and to facilities for periodic health checks. Consequently, the incidence of disease among the subjects whose blood is analyzed in the laboratory is low. Therefore, we assumed the vast majority of subjects investigated to have platelet parameter values similar to those of the general population.

Methods

The blood samples were measured using 2 CELL-DYN Sapphire hematology analyzers (Abbott), which were calibrated according to the manufacturer’s recommendations using commercial calibration material (CELL-DYN HemCal; Abbott). Daily quality control was performed using CELL-DYN 29 Plus trilevel control blood.

CELL-DYN Sapphire measures retPLTs as a part of the reticulocyte assay. In short, prediluted blood is mixed with the reticulocyte reagent, containing the proprietary fluorescent RNA dye CD4K530. After 47 seconds of incubation, the mixture is transferred to the flow cell, which is illuminated by a 488-nm laser. Up to 50,000 cells are analyzed, and 3 angles of light scatter (0°, 7°, and 90°) as well as fluorescence (530 nm; FL1) are recorded and stored as raw data in a list mode file. This file is used by an embedded algorithm, which first separates platelets and red blood cells on the basis of their 7° and 90° scatter and then creates a retPLT gate in the FL1 versus 7° plot, which allows for correcting the size-dependent background fluorescence. Results are reported within 2 minutes from sampling and retPLTs are expressed as a percentage of the platelets. Figure 1 gives an example of the scatterplot used for the retPLT analysis. The within-day precision of the retPLT assay in fresh human blood ranged from 12.2% (coefficient of variation; n = 10) at an increased level of 4.4% retPLTs to 41.5% at a low-normal level of 1.4% retPLTs. Between-day precision measured with stabilized control material gave similar values.

For investigating retPLT stability, 10 random samples were analyzed immediately after blood collection, then every 2 hours on the same day and every 4 hours the next day. Between the measurements the samples were stored at ambient temperature (recorded range 20.0°C–21.5°C).

CELL-DYN Sapphire calculates MPV from the volume histogram of the platelets as measured in the impedance channel, taking the log-normal distribution into account. Platelet distribution width is also derived from the platelet volume histogram, as the geometric standard deviation multiplied by 10. Plateletcrit (PCT) is calculated by multiplying platelet count and MPV; it represents the volume fraction of platelets in whole blood.

Data Analysis

First, repeat samples and samples with platelet-related flags (such as suspected platelets clumps and discrepancy between the impedance and optical platelet counts) were removed from the data set; 8638 samples remained. Then, demographic information (sex and age of the patients) was retrieved from the laboratory information system and merged with the Sapphire results file, using the sample identification number as the key. The laboratory information system also provided the unique patient identification number, which enabled us to exclude 549 samples from subjects who had been investigated more than once during the study period. Consequently, the data set included only a single sample from each of 8089 unique individuals. This data set was used without any further exclusions in the Bhattacharya11 method for identifying the underlying normal distribution; reference ranges were calculated as mean ± 2 SD. A Bhattacharya macro for Microsoft Excel was kindly made available by Dr Graham R. D. Jones (Department of Chemical Pathology, St Vincent’s Hospital, Sydney, Australia). If the data distribution was found to be non-Gaussian, logarithmic transformation was performed and normality of the resulting data distribution checked again. If there were no statistical differences between females and males, both sexes were taken together.

As an alternative, we investigated only those samples with a platelet count within the laboratory’s reference range (150–400 × 10^9/µL) and applied the Clinical and Laboratory Standards Institute12 nonparametric method for finding the central 95% reference range.

For comparing results between sex and age groups, we used standard statistical methods in the MedCalc software program (version 12.2; MedCalc, Mariakerke, Belgium) as indicated in the text. Statistical significance was defined as P < .05.

RESULTS

Study Subjects

In total, samples from 8089 subjects were included in the study. There were 5216 females and 2873 males. The females had a median age of 50 years (range, 1–102 years), and the males had a median age of 57 years (range, 0–106 years). In the females, the ages were rather regularly divided between 20 and 90 years, with approximately 300 subjects per each 5-year class. In the males, there was a peak at 60 years.

---

Figure 1. CELL-DYN Sapphire reticulocyte dot plot used for calculating reticulated platelets (retPLTs). Mature platelets (PLT) are color coded orange, retPLTs purple, mature red blood cells red, reticulocytes green, and white blood cells blue. The retPLT gate is normally invisible; it is shown here only for the purpose of illustration. Further, the number of events actually measured is higher than the number of dots in the graph, and only about 10% of all events are shown for the purpose of printing speed. Abbreviation: RETC, Reticulocytes dot plot in CELL-DYN Sapphire.

Figure 2. Stability of reticulated platelets (retPLT) at ambient temperature after blood collection, relative to T = 0. Medians and central 95th percentiles are indicated by filled circles and bars, respectively. Statistical significance is indicated with an asterisk (P = .02 by analysis of variance).
Figure 3. Reticulated platelets (retPLT; medians and interquartile ranges) in the different age groups. Group A, <18 years; B, 18–40 years; C, 41–55 years; D, 56–70 years; E, 71–85 years; and F, >85 years.

Figure 4. Reticulated platelets (retPLT; medians and interquartile ranges) in groups arranged according to platelet (PLT) count ($\times 10^3/L$). A, Reticulated platelets as percentage of PLTs. B, Absolute retPLT counts $\times 10^3/L$. Group P1, $\leq$150 (n = 345); P2, 151–200 (n = 1429); P3, 201–250 (n = 2617); P4, 251–300 (n = 2081); P5, 301–350 (n = 986); P6, 351–400 (n = 365); P7, 401–450 (n = 142); P8, >450 (n = 124).

Figure 5. Mean platelet volume (MPV; medians and interquartile ranges) in groups arranged according to platelet (PLT) count (see Figure 4 caption for description of groups).

Figure 6. Reticulated platelets (retPLTs; medians and interquartile ranges) as a function of mean platelet volume (MPV) quintiles. Quintile 1, <7.57 fL; 2, 7.57–8.07 fL; 3, 8.08–8.56 fL; 4, 8.57–9.19 fL; 5, >9.19 fL.
years; all 5-year classes between 25 and 90 comprised at least 120 subjects.

Stability of retPLTs

The stability of retPLTs was investigated using 10 random samples that were kept at ambient temperature for 2 days. Platelet counts were completely stable for at least 30 hours after blood collection (not shown). The retPLT levels were stable up to 26 hours and afterwards significantly increased ($P = .02$ by analysis of variance; Figure 2).

retPLT Reference Range

Initial statistical analysis indicated that the retPLT data were not normally distributed. However, after logarithmic transformation a Gaussian distribution was obtained, and therefore we applied the Bhattacharya method after logarithmic transformation. The reference intervals found with this method are shown in the Table. Differences between sexes were statistically significant, but very small. When expressed as a percentage of platelets, females appeared to have slightly lower retPLT levels than males, as shown in the Table. When expressed as absolute retPLT count, males and females had identical values (median 4.2 x 10$^{3}$/µL; $P = .17$ by Mann-Whitney test).

The 95th percentile method 25 was applied on the 7478 samples with a platelet count within the reference range (150–400 x 10$^{3}$/µL). The references ranges based on percentiles are shown in the Table.

retPLT Correlations

We found a significant correlation between retPLT level and the age of the subjects who had a platelet count in the reference range (Spearman $\rho = 0.156$, $P < .001$). Figure 3 shows that retPLT level increased as a function of age in adult subjects. When subjects with platelet counts outside the reference range were also included, the correlation remained essentially unchanged (not shown).

The association between retPLT level and platelet count is depicted in Figure 4, A; a significant increase in retPLT level with increasing platelet count is evident (Spearman $\rho = 0.242$; $P < .001$). The relation between retPLT level and platelet count is even stronger for the absolute retPLT count (Spearman $\rho = 0.522$, $P < .001$), as shown in Figure 4, B.

The well-known negative association between MPV and platelet count was confirmed in our cohort, as Figure 5 demonstrates, with a Pearson $r = -0.515$ ($P < .001$). We also found that retPLT level was negatively correlated with MPV in a highly significant manner, as depicted in Figure 6 (Spearman $\rho = -0.399$, $P < .001$).

Statistically, the correlation between retPLT level (expressed in %) and PCT was highly significant ($P < .001$), but the coefficient of correlation was rather low: Spearman $\rho = 0.061$. In contrast, the correlation between the absolute retPLT count and PCT was much higher, namely $r = 0.456$ ($P < .001$). Reticulated platelets were not meaningfully associated with the platelet distribution width (results not shown).

MPV, PCT, and Platelet Distribution Width Reference Ranges and Correlations

The other platelet parameters were Gaussian distributed and there were no differences among sexes detected. The reference intervals found are given in the Table.

We also found no substantial correlations between any of these 3 parameters and the subjects’ age, in particular not between MPV and age (not shown). As already indicated in Figures 5 and 6, MPV was highly correlated with platelet count and with retPLT level, respectively. Obviously, PCT was strongly correlated with platelet count (Pearson $r = 0.899$, $P < .001$) and with absolute retPLT count as mentioned above. Platelet distribution width was positively correlated with MPV ($r = 0.301$, $P < .001$) and negatively with platelet count and PCT ($r = -0.156$ and $r = -0.119$, respectively; both $P < .001$); however, in view of the low coefficients of correlation and the high number of observations, a biologic relationship between these parameters is likely absent.

COMMENT

Preanalytic conditions are of vital importance, particularly when measuring cellular parameters. Therefore we first investigated the stability of retPLT level, and found no relevant change up to 26 hours after blood collection when the samples were stored at ambient temperature. Because the retPLT assay of CELL-DYN Sapphire is relatively new, to our knowledge there is no comparative literature yet. Previously it has been reported that the immature platelet fraction as measured on the Sysmex XE-2100 hematology analyzer had rather limited stability, although others found stable immature platelet fraction for 24 hours. One
should realize that the 2 analyzers use completely different methods, which may explain the difference in storage stability.

Reliable reference ranges are essential for interpretation of laboratory results and clinical decision making. Procedures for establishing reference intervals are described in international guidelines and recommendations.22,23 One of the possibilities to establish reference ranges is using patient data and an indirect statistical method, the Bhattacharya24 technique. This is a robust and accepted tool for reference interval determination for analytes with an underlying Gaussian (or log-Gaussian) distribution. It is based on the assumption that the majority of results are unaffected by the condition for which the patient is under investigation.17–19 Our study cohort comprised a large number of unselected subjects who were referred to a primary care laboratory serving the general community, where the incidence of disease is much lower than in a hospital laboratory. Moreover, in the vast majority of subjects no request was made for measuring platelets or platelet parameters. This might be interpreted as a high probability of lack of platelet-related disease. The fact that only 7.6% of the subjects had platelet counts outside the laboratory’s reference range confirmed that our study cohort consisted mainly of persons representative of the general healthy population. Thus, our study cohort was well suited for establishing reference values using the Bhattacharya technique.

The data presented in the Table demonstrate that the reference ranges for retPLTs as found with the Bhattacharya method are practically identical to the nonparametric range in the subjects with normal platelet counts. This holds true for both ways of expressing retPLTs: as a percentage of platelets and as absolute retPLT count. The difference in retPLTs between females and males was statistically significant, but it was actually too small to apply sex-specific ranges. A similar small but irrelevant sex difference or no difference at all has been described for the immature platelet fraction measured on the Sysmex XE-2100.20,21

We demonstrated that retPLTs increase with age. To the best of our knowledge this is a novel finding, which may lead to further insight in how megakaryocyte maturation and platelet production change with age.

Likewise, the negative association between retPLTs and MPV to our knowledge has never been previously reported. This means that retPLTs are higher in small platelets and lower in large ones. This is in contrast with opinions of some authors, who believe that reticulated platelets are large platelets.22,23 Actually, platelet size is determined by megakaryocyte ploidy at the time of platelet production and has no relation with the age of a platelet.24 Given the finding that regulation of platelet production under steady-state conditions is aimed at keeping the total platelet mass constant,24 it is understandable that this can only be achieved when the megakaryocytes produce more smaller or less larger platelets, as is reflected by the retPLTs. This is further corroborated by the finding that more retPLTs are needed to sustain a higher platelet count.

The lack of correlation between age and MPV found here is in contrast with recent data.25 Possible explanations are differences in technology used and the fact that Lippi et al studied hospital outpatients for whom an MPV was available, whereas we investigated an unselected primary care cohort that was representative of the general healthy population.

The main limitation of our study is that the data are only valid for retPLTs measured with the CELL-DYN Sapphire and cannot be automatically extrapolated to other methods for measuring reticulated platelets or immature platelet fraction. The Sapphire method is rather similar to the widely used flow cytometric method with thiazole orange, but it is fully automated and fully standardized regarding incubation time and concentration of the fluorescent RNA dye. Moreover, it uses objective gating as well as correction of the size-dependent background fluorescence by applying the method of Matic et al as shown in Figure 1. The above technology-associated limitation is not only true for retPLTs, but also holds for other platelet parameters, as was recently demonstrated.26 Finally, the theoretical limitation of an indirect method for assessing reference ranges is overcome by the fact that the Bhattacharya technique applied on the unselected cohort gave essentially equivalent results as the nonparametric Clinical and Laboratory Standards Institute method in subjects with normal platelet counts.

In summary, we have established reference ranges for retPLTs measured with the CELL-DYN Sapphire hematology analyzer. There are no relevant differences between the sexes, but there is an effect of the subject’s age. The retPLT reference ranges described here represent an overall picture, which may be refined by taking into account that retPLTs are associated not only with the platelet count, but also with MPV. These reticulated platelet data form the basis for further investigating the clinical utility of this assay in conditions of decreased and increased platelet production.

The authors are indebted to Anouk Bol, MT, and Edwin van Lierop, MT, for their valuable technical assistance.

References

15. Hoffmann et al Reference Intervals of Reticulated Platelets—Hoffmann et al 1639


