Heterogeneity of Human Platelets. VI.
Correlation of Platelet Function With Platelet Volume

By Simon Karpatkin

Platelet aggregation velocity was measured with an aggregometer in platelet-rich plasma from 34 normal subjects following addition of ADP, collagen, or epinephrine. Platelet volume parameters [mean, mode, megathrombocyte index (large platelets)] were determined with a model B Coulter Counter attached to a P64 Channel Analyzer with electronic recorder and correlated with platelet count. A negative linear relationship was found between platelet count and platelet volume ($r = -0.53$, $p < 0.001$). Platelet aggregation velocity induced with ADP, collagen, or epinephrine (primary wave) was directly proportional to platelet volume and correlated best with megathrombocyte index: ADP, $r = 0.62$, $p < 0.001$; collagen, $r = 0.59$, $p < 0.001$; epinephrine, $r = 0.53$, $p < 0.02$. Platelet aggregation velocity induced with collagen or epinephrine correlated poorly with platelet mean or mode. Platelet aggregation velocity induced with ADP did not correlate with platelet mean or mode volume ($r = 0.27$, $p > 0.1$; $r = 0.22$, $p > 0.1$, respectively). Platelet volume distribution curves of residual nonaggregated platelets revealed a relative absence of larger platelets. Thus, platelet aggregation velocity was proportional to platelet volume, but correlated best with the number of large platelets or megathrombocyte index, particularly with ADP-induced platelet aggregation. It is suggested that the clinical strategy for treatment of platelet disorders would be better directed toward evaluation of platelet size and function rather than number, and that experimental as well as therapeutic procedures for the harvesting of platelets be directed toward a method of collection in which a portion of the megathrombocyte population is not routinely discarded.

The platelet count varies in normal individuals from 150 to $350 \times 10^9$/liter in whole blood, yet platelet counts of $75 \times 10^9$/liter are adequate for the maintenance of hemostasis (i.e., a normal bleeding time). Accordingly, the normal circulating platelet supply is probably 2–4 times greater than that necessary for hemostasis. This observation suggests that either platelet functional capacity is also present in excess or that platelet function is heterogeneous with a subpopulation of platelets responsible for hemostasis in normal individuals. The latter situation could explain the clinical conditions in which patients are apparently free of purpura despite platelet counts in the $15-50 \times 10^9$/liter range. Previous studies had indicated that when a platelet population enriched with megathrombocytes (large-heavy platelets) was exposed to thrombin and epinephrine it released considerably more ADP and ATP than the total platelet population similarly exposed, as well as a subpopulation of platelets enriched with...
small–light platelets. The same finding applied for ATP release when ADP was employed as aggregating agent. Platelet aggregation time, as observed macroscopically, was significantly shorter with large–heavy platelets than with a total platelet population and considerably shorter when compared to a subpopulation enriched with small–light platelets. However, these observations were made on washed platelets which had traveled through a relatively “inert oil” density gradient. Platelet aggregation was monitored crudely with visual macroscopic aggregation, and it could be argued that the small–light platelet fraction was enriched with cellular debris.

With the advent of more quantitative techniques for measuring platelet aggregation and volume without further manipulation of platelet-rich plasma (PRP), it became possible to reexamine (1) whether platelet function is heterogeneous, i.e., whether some platelets aggregate better than other platelets, or (2) whether megathrombocytes are the only functioning platelets. The present report provides data indicating that platelet function is heterogeneous and proportional to platelet volume parameters with ADP-, collagen-, and epinephrine-induced platelet aggregation. With ADP-induced platelet aggregation, megathrombocyte number appears to be the more critical determinant of platelet aggregation.

MATERIALS AND METHODS

Thirty-five healthy laboratory personnel, who had not ingested any drugs for at least 1 wk, were studied. Platelet count, platelet volume distribution, and platelet aggregation were obtained in 34 subjects studied for ADP aggregation, 32 subjects studied for collagen aggregation, and 20 subjects studied for epinephrine aggregation. Blood was collected into sodium citrate (14.3 mM final whole blood concentration) in the laboratory in which the tests were to be performed and kept in tightly capped plastic tubes. Platelet aggregation tests were performed at 37°C, usually within 1 hr of withdrawal of the blood sample, after having been maintained at room temperature for 30 min. Platelet counts were performed manually on whole blood as well as PRP under phase optics as described in detail previously.2-5 Platelet volume distribution measurements were performed on the same sample of PRP prepared from sodium citrate for aggregation studies as described previously.6 A “transistorized” model B Coulter Counter was employed with a 70-μm aperture tube attached to a P-64 Channel Analyzer with automatic electronic recorder. Each window from 1 to 100 was made equal to 0.25 μl following calibration with latex particles. Electronic noise contribution at the lower threshold level was absent to negligible.

A megathrombocyte index of “R10” was arbitrarily defined on the coordinate of the horizontal axis where the 10th percentile on the vertical axis transected the descending limb (right side) of the platelet volume distribution curve. The average R10 window for the 34 subjects tested was found to be 47.0 ± 7.8 (SD), or 17.8 ± 2.0 μl. The mode was obtained from the coordinate on the horizontal axis where a line drawn from the peak of the platelet volume distribution curve

*Similar measurements were made in a previous communication in which EDTA was employed as anticoagulant. Platelets suspended in plasma containing EDTA as anticoagulant gave slightly higher mean values in the order of 17%–33% for these parameters: R10 was 55.1 ± 15.7 or 13.8 ± 3.9 μl; mode was 17.5 ± 5.6 or 4.4 ± 1.4 μl; R50 was 31.2 ± 3.2 or 7.8 ± 0.90 μl; L50 was 8.38 ± 3.2 or 2.10 ± 0.65 μl; and estimated mean was 19.8 ± 2.6 or 5.0 ± 0.65 μl (Fig. 1). An excellent correlation coefficient was obtained with platelet parameters measured from plasma anticoagulated with either EDTA or citrate on blood obtained from the same venepuncture, i.e., R10 = +0.88, p < 0.001, n = 29.
Fig. 1. Mean platelet volume distribution curve and platelet volume parameters obtained from EDTA-anticoagulated platelet-rich plasma of 20 normal subjects. Shaded area: ± 2 SD. Calibration, 1 window = 0.25 cu μm.

The mean platelet volume distribution curve transected the horizontal axis as a perpendicular. The average mode window for the 34 subjects tested was found to be 13.1 ± 2.0, or 3.3 ± 0.5 fl. The mean was estimated by averaging the R50 and L50 horizontal coordinates. The R50 coordinates of the horizontal axis were obtained from the 50th percentile of the vertical axis where it transected the descending limb (right side) of the platelet volume distribution curve. The average R50 window for the 34 subjects tested was found to be 24.0 ± 3.1, or 6.0 ± 0.8 fl. The L50 coordinate of the horizontal axis was similarly obtained from the ascending limb (left side) of the platelet volume distribution curve and was found to be 7.6 ± 0.93, or 1.90 ± 0.23 fl. The average estimated mean window for the 34 subjects was found to be 8.38 ± 3.2, or 4.0 ± 0.45 fl. The above four platelet volume parameters therefore measured gradations of platelet size, with the R10 representing the largest platelet size parameter, the R50 representing an intermediate platelet size parameter, and the mean and mode representing relatively smaller platelet size parameters.

Platelet aggregometry was performed with a Bio Data Aggregometer (Bio Data, Willow Grove, Pa.) at 37°C employing 0.4 ml of PRP. PRP was obtained by centrifuging blood in a desk-top International centrifuge (Boston, Mass.) at 150 g for 5 min at room temperature. PRP was kept at room temperature for 30 min and then equilibrated at 37°C for 3 min prior to testing. Platelet-poor plasma (PPP) was obtained by centrifugation of the remainder of the blood at full speed for 20 min. The following aggregating agents were dissolved in saline in a volume of 0.05 ml: ADP, disodium salt (Sigma Chemical, St. Louis, Mo.) 2.6 x 10^{-6} M and 2.6 x 10^{-8} M (final concentration); connective tissue suspension prepared by the method of Zucker and Borrelli, henceforth referred to as “collagen,” at dilutions of 1:18,000 and 1:36,000 (final concentration); epinephrine hydrochloride (Parke Davis, Detroit, Mich.), 5 x 10^{-5} M and 5 x 10^{-6} M (final concentration).

Platelet aggregation was usually tested within 1 hr of withdrawal of blood from the test subject. All dilutions, additions, and measurements were performed in the same time sequence for each subject tested.

The primary wave of platelet aggregation was quantified by measuring the slope of the initial velocity of the downward sweep of the aggregation curve in units of light transmission/min. A tangent was drawn to the initial slope of the curve. The aggregometer was standardized automatically with PPP on a scale of 0–1000 light transmission units. Average aggregation velocity was determined for each aggregating agent by taking the average velocity of two dilutions of aggregating agent which were employed above. This parameter was determined on undiluted PRP as well as PRP diluted 1:2 with PPP because dilution of PRP with PPP gave higher aggregation velocities for ADP in 16 of 32 subjects (Table 1). The average maximum slope value of the diluted or non-diluted PRP was designated “maximum aggregation velocity.”

Statistical evaluation of data was performed by linear correlation and regression analysis employing the technique of least squares and Student’s t test with a Sharp electronic calculator and program (model 364P-11, Sharp Electronics, Paramus, N.J.).
Table 1. Dilution Enhancement Effect on PRP With ADP-induced Platelet Aggregation

<table>
<thead>
<tr>
<th>Patient Age, Sex</th>
<th>PRP Platelets × 10^9/liter</th>
<th>HCT (vol./100 ml)</th>
<th>Diluted†</th>
<th>Undiluted*</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>1:2</td>
<td>1:4</td>
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<tr>
<td>34, M</td>
<td>510</td>
<td>1650</td>
<td>1800</td>
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<tr>
<td>23, M</td>
<td>475</td>
<td>1200</td>
<td>1250</td>
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<td>46, M</td>
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<td>2000</td>
<td>2200</td>
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<td>31, M</td>
<td>430</td>
<td>1400</td>
<td>1750</td>
<td>1850</td>
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<td>40, M</td>
<td>360</td>
<td>1250</td>
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<td>420</td>
<td>1300</td>
<td>1750</td>
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<td>30, M</td>
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<td>50, M</td>
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<td>1350</td>
<td>1500</td>
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<td>37, M</td>
<td>400</td>
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<td>1900</td>
<td></td>
</tr>
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<td>500</td>
<td>1900</td>
<td>2750</td>
<td></td>
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<td>29, F</td>
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<td>2150</td>
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</tr>
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<td>30, F</td>
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<td>1500</td>
<td>2050</td>
<td></td>
</tr>
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<td>1650</td>
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</tr>
<tr>
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<td>1750</td>
<td>2700</td>
<td>1125</td>
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<tr>
<td>31, F</td>
<td>490</td>
<td>1750</td>
<td>2666</td>
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</table>

*Average aggregation velocity at two concentrations of ADP (2.6 × 10^{-5} M and 2.6 × 10^{-6} M) with undiluted PRP.
†Average aggregation velocity at two concentrations of ADP with PRP diluted as shown with PPP.

RESULTS

Control Studies

There were 35 subjects who were studied for platelet function, volume, and count; 21 were male, average age 37, and 14 were female, average age 33. The average platelet count for the 21 males was 281 × 10^9/liter and for the 14 females 292 × 10^9/liter. The average megathrombocyte index for the females of 49.0 was slightly greater than the average megathrombocyte index for the males, 44.1 (Table 2). Platelet aggregation was also slightly greater in females compared to males for ADP- and collagen-induced aggregation, and considerably greater for females for epinephrine-induced aggregation (Table 2). Maximum average ADP aggregation velocity for females correlated with megathrombocyte index (r = 0.72, p < 0.01); the same applied for males (r = 0.54,
A negative correlation of platelet count with megathrombocyte index was also obtained with EDTA employed as anticoagulant ($r = -0.56, p < 0.001, n = 44$).

$p < 0.05$). No inverse correlation was noted between the subject's hematocrit and maximum aggregation velocity in 25 subjects tested with hematocrits varying from 33.0% to 61.7% ($r = -0.05, p > 0.1$), suggesting that the citrate concentration of plasma had little effect on platelet aggregation velocity.

Figure 2 demonstrates the correlation obtained between the whole blood platelet count and the megathrombocyte index (R10 value). A negative linear correlation of $r = -0.53$ was obtained which was significant at the $p < 0.001$ level ($n = 34$). Negative linear correlations* were also obtained with the three other parameters of platelet volume (Table 3, column 1).

**Correlation of Platelet Aggregation With Platelet Volume**

Figure 3A demonstrates the correlation obtained between ADP-induced maximum average aggregation velocity and megathrombocyte index. A positive linear correlation of $r = 0.62$ was obtained, which was significant at the $p <$

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Platelet Count*</th>
<th>ADP†</th>
<th>Collagen‡</th>
<th>Epinephrine§</th>
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<tr>
<td>Megathrombocyte index (R10)</td>
<td>$r = -0.53$</td>
<td>$r = 0.62$</td>
<td>$r = 0.59$</td>
<td>$r = 0.53$</td>
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<tr>
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<tr>
<td>$R50$</td>
<td>$r = -0.43$</td>
<td>$r = 0.43$</td>
<td>$r = 0.34$</td>
<td>$r = 0.51$</td>
</tr>
<tr>
<td>$p = 0.01$</td>
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<td>$p &lt; 0.05$</td>
<td>$p &lt; 0.02$</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>$r = -0.36$</td>
<td>$r = 0.27$</td>
<td>$r = 0.40$</td>
<td>$r = 0.38$</td>
</tr>
<tr>
<td>$p &lt; 0.05$</td>
<td>NS‡</td>
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<td>$p &lt; 0.1$</td>
<td></td>
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<tr>
<td>Mode</td>
<td>$r = -0.33$</td>
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<td>$r = 0.39$</td>
<td>$r = 0.46$</td>
</tr>
<tr>
<td>$p &lt; 0.05$</td>
<td>NS</td>
<td>$p &lt; 0.05$</td>
<td>$p &lt; 0.05$</td>
<td></td>
</tr>
</tbody>
</table>

*Correlation coefficients of platelet count and volume parameters obtained from blood anticoagulated with sodium citrate.
†Number of pairs is given in parentheses.
‡Correlation coefficients of maximum average platelet aggregation velocity and aggregating agent.
§NS: $p > 0.1$.

* A negative correlation of platelet count with megathrombocyte index was also obtained with EDTA employed as anticoagulant ($r = -0.56, p < 0.001, n = 44$).
Fig. 3. Correlation between maximum average aggregation velocity and megathrombocyte index. (A) ADP-induced aggregation (maximum average of $2.6 \times 10^{-5}$ M and $2.6 \times 10^{-6}$ M). Regression line, $y = 34.74x$. Correlation coefficient, $r = 0.62$, has $p < 0.001$; $n = 34$. (B) Collagen-induced aggregation (maximum average of 1:18,000 and 1:36,000 dilution). Regression line, $y = 30.24x$. Correlation coefficient, $r = 0.59$, has $p < 0.001$; $n = 32$. (C) Epinephrine-induced primary wave aggregation (maximum average of $5 \times 10^{-5}$ M and $5 \times 10^{-6}$ M). Regression line, $y = 6.26x$. Correlation coefficient, $r = 0.53$, has $p < 0.02$; $n = 20$.

0.001 level ($n = 34$). Analysis of data by the method of least squares revealed a directly proportional relationship with the straight line $y = 34.74x$ going through the origin. A poorer correlation was obtained when maximum average aggregation velocity was correlated with the R50 value ($r = 0.43$, $p < 0.02$) and no correlation was obtained when maximum average aggregation velocity was correlated with mode platelet volume ($r = 0.22$, $p > 0.1$) or mean platelet volume ($r = 0.27$, $p > 0.1$) (Table 2).

Figure 3B demonstrates the correlation obtained between collagen-induced maximum average aggregation velocity and megathrombocyte index. A positive linear correlation of $r = 0.59$ was obtained, which was significant at the $p < 0.001$ level ($n = 32$). Analysis of data by the method of least squares revealed a directly proportional relationship with the straight line $y = 30.24x$ going through the origin. A poorer correlation was obtained when maximum average aggregation velocity was correlated with the R50 value ($r = 0.34$, $p < 0.05$), when maximum average aggregation velocity was correlated with mode platelet volume ($r = 0.39$, $p < 0.05$), or when maximum average aggregation velocity was correlated with mean platelet volume ($r = 0.40$, $p < 0.01$) (Table 2).

Figure 3C demonstrates the correlation obtained between the maximum average velocity of the primary wave of epinephrine-induced aggregation and megathrombocyte index. A positive linear correlation of $r = 0.53$ was obtained, which was significant at the $p < 0.02$ level ($n = 20$). Analysis of data by the method of least squares revealed a directly proportional relationship with the straight line $y = 6.26x$ going through the origin. A similar correlation was obtained when primary wave maximum average aggregation velocity was correlated with the R50 value ($r = 0.51$, $p < 0.02$). A poorer correlation was obtained when primary wave maximum average aggregation velocity was compared to the mode platelet volume ($r = 0.46$, $p < 0.05$) or when primary wave maximum average aggregation velocity was compared to the mean platelet volume ($r = 0.38$, $p < 0.1$) (Table 2). No correlation was obtained with the secondary wave of platelet aggregation and megathrombocyte index ($r = 0.16$, $p > 0.01$).
Figure 4 demonstrates the residual platelet volume distribution curve of PRP after aggregation with collagen and compares this to the original PRP prior to addition of collagen. The platelets remaining in suspension in the cuvette (allowed to settle) after higher and lower concentrations of collagen and cessation of aggregation (approximately 4 minutes) represented approximately 5% and 10%, respectively, of the initial PRP platelet count. The remaining "un-aggregated" platelets varied inversely with increasing concentration of aggregating agent (four experiments, data not shown). Similarly, with ADP-induced platelet aggregation the platelets remaining in suspension in the cuvette after addition of the higher and lower concentrations of aggregating agent represented 10% and 25%, respectively, of the initial PRP count and varied with increasing dilution of aggregating agent (four experiments, data not shown).

**DISCUSSION**

These data clearly indicate heterogeneity of platelet function with respect to aggregation velocity, with heterogeneity being a function of platelet volume. With epinephrine- or collagen-induced platelet aggregation four different parameters of platelet volume [R10 (megathrombocyte index), R50, mean, and mode] were all proportional to aggregation velocity. With ADP-induced aggregation, platelet aggregation velocity was related more to the number of large platelets (megathrombocyte volume and R50) than all platelet volume parameters, since no significant correlation was noted with mean or mode. It is of interest in this regard that with collagen and epinephrine there was usually a better correlation of aggregation velocity with megathrombocyte volume than with mean or mode volume. Statistically, it is highly unlikely that these correlations may be due to measurement artifacts, since all three major correlations were directly proportional relationships. Indeed, since some larger platelets are obviously entrapped in the buffy coat following conventional methods of PRP preparation, it is very likely that a correlation which could include the total...
platelet population of whole blood might provide an even better relationship. However, such a correlation is not feasible, since methods are not available for the isolation of a complete population of unwashed nonresuspended platelets.*

These results are consistent with our original observations on washed isolated platelet subpopulations separated on the basis of density, wherein heavy-large platelets were shown to aggregate better than light–small platelets,4 as well as recent observations made by Kraytman,16 who has demonstrated that large canine platelets are preferentially trapped (utilized) following extracorporeal circulation of whole blood through a column of glass beads, and with similar observations by Mannucci and Sharp,11 made with platelet volume distribution curves before and after addition of aggregating agent, which are confirmed in this paper. It is obvious that in general more small than large platelets remain nonaggregated after addition of an aggregating agent. This finding should be interpreted with caution, however, since it is possible that the aggregation process may lead to the release of platelet fragments which do not aggregate but are large enough to be detected by the Coulter Counter.

The data obtained also indicate a negative linear relationship between platelet count and platelet volume, and established for a normal population of individuals those observations recently reported by O’Brien,13 who has also noted a negative linear relationship between platelet count and mean platelet volume in an assortment of patients.

It should be stressed that these data describe phenomena in vitro rather than platelet function in vivo. However, numerous clinical studies exist which associate bleeding tendencies with impairment of platelet aggregation in vitro,14–17 and platelet aggregometry has become a standard technique for measuring platelet “function.” Furthermore, platelet volume as well as megathrombocyte enumeration has become a useful clinical tool for evaluating platelet disorders.18–24 Therefore, these correlations are of value.

Whether functional heterogeneity is a function of platelet senescence with loss of membrane components25–30 and/or a reflection of heterogeneous platelet production31,32 remains to be established. It is of interest in this regard that young rabbit platelets adhere more readily to collagen than do older platelets.33 Indeed, newly formed platelets have been claimed to be the only functional platelets which appear to be sequestered by the spleen for the first 36–60 hr after production.3 Of further interest is the recent observation that megathrombocytes have also been shown to be preferentially sequestered by the spleen.34

The above observations, if applicable in vivo, could help explain the often noted clinical observation of the absence of purpura in the presence of profound thrombocytopenia (15–50 × 10^9/liter range) in disorders of increased platelet turnover with increased megathrombocytes. They may also be of value in the treatment of patients with platelet transfusions with respect to the harvesting of a total platelet population (i.e., recovery of heavy–large platelets trapped in the buffy coat8) as well as the transfusion of platelets with greater

*For example, experiments with rabbit blood anticoagulated with EDTA revealed whole blood platelet yields of 45%–78% in the initial preparation of PRP. This yield could be increased by 15%–30% following resuspension and centrifugation of the remaining red blood cells and buffy coat in isotonic media. These additionally recovered platelets were enriched with megathrombocytes.
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functional capacity. Finally, it is suggested that the strategy of treatment for platelet disorders might better be directed toward evaluation of platelet volume and function rather than platelet number.

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