10.

Platelet Counts in Severely Thrombopenic Samples

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Introduction

Platelets play a major role in haemostasis and therefore numerical deficiency or defects in their function may lead to bleeding.

'One thrombocyte said to his mate:
Come on, let's start to aggregate!
Clinicians always call us a clot –
but we know better, we are not –
not to be understood is our fate'
GVR Born, 1980 [**1**]

The aim of this presentation is to address the issue of platelet counting in severely thrombopenic patients, a clinical situation in which accuracy of the count is of the utmost importance. Over the years many methods of platelet counting have been published indicating the elusiveness of a totally reliable technique.

Platelets were first described as distinct corpuscles and their role in coagulation and thrombosis recognised by the Italian pathologist, Giulio Bizzozero in 1882 [**2**]. It is well recognised that platelets are anucleate cell fragments derived from bone marrow megakaryocytes; that the platelet count in health ranges from 150 – 400 x 10^9 /L; that the mean platelet volume (MPV) in health ranges from 7.7 – 11.2 fL; and that the platelet life-span is about 10 days. In the resting state some 30 % of platelets are sequestered in the spleen.

If the platelet count is severely reduced or if the platelets are functionally defective, bleeding occurs spontaneously into the skin (purpura and ecchymoses), from mucous membranes most commonly epistaxis, menorrhagia, post-partum haemorrhage and protracted bleeding after trauma (or surgery) the last tending to stop with direct pressure. Less commonly, and only with very severe thrombopenia, intracranial, retinal, gastro-intestinal and genito-urinary bleeds may occur.

Examination of a stained peripheral blood film provides a useful estimate of whether the platelet count is normal, high or low. A blood film from EDTA anticoagulated blood should show even dispersion of platelets. Platelet aggregates in EDTA anticoagulated blood are often associated with spuriously low platelet counts. Likewise platelet satellitism (platelets adhering to and encircling neutrophils) induced by IgG or IgM can lead to an inaccurate platelet count. In extreme cases, clumps of neutrophils may be bound together by the adherent platelets. These phenomena have no clinical significance. Abnormalities of platelet morphology can also be observed, e.g. small platelets in the Wiskott-Aldrich syndrome, large platelets in chronic myeloproliferative disease or Bernard-Soulier syndrome or the agranular platelets in grey platelet syndrome.

History of platelet counting

Platelets have proved more difficult to count than red or white cells. Historically techniques for platelet enumeration fell into four groups: (1) direct methods by haemocytometry and microscopy; (2) indirect methods in which red cells were counted directly and their proportion to platelets determined in a stained blood film; (3) semi-automated methods in which counts were performed electronically on platelet rich plasma (PRP): and (4) fully automated electronic methods. For many years the Brecher and Cronkite [**3**] method originally described in 1950 was considered to represent the optimum compromise between accuracy, time and cost and, until comparatively recently, was the reference against which semi-automated and automated methods were compared. In this method the difficulty in distinguishing platelets from other particles is minimized by dilution of blood in 1% ammonium oxalate to produce a clear background followed by the use of phase contrast microscopy.

The first publication describing platelet counting by electronic means was by Bull *et al* [**4**] in 1965 in which whole blood collected into EDTA was either allowed to settle spontaneously or was centrifuged briefly and then the platelets in the supernatant PRP counted using an aperture impedance device equipped with a 70 µm orifice (modified Coulter Counter Model D). This became commercially available as the Coulter Thrombocounter C. Shortly thereafter, totally automated platelet counting became commercially available employing optical systems [**5, 6**] using the principle of reverse darkfield microscopy. This technology was embodied in the Technicon Hemolog and Autocounter.

The next major advance in automated platelet counting was the use of a hydrodynamically focused whole blood aperture impedance counter [**7**], the Clay Adams (later Becton Dickinson) Ultra Flow 100. The improvement in count precision was impressive; the coefficient of variation for whole blood platelet counting was significantly improved over that of other available clinical instruments [**8, 9**]. Optical flowcytometry did not stand still with the development of two angle scatter laser detection devices; low angle scatter (2–3°) defining cell size and high angle scatter (5–15°) refractive index [**10**]. This technology was incorporated into the Bayer H* System haematology analyser. With the use of 2-dimensional platelet analysis, platelets can be distinguished from RBC on the basis of size and refractive index. The platelet refractive index is related linearly to platelet density, which is a measure of the overall concentration of components within a platelet. In spite of all these developments, it was still impossible to develop a reference method for platelet counting. In 1988 [**11**], the International Council for Standardization in Haematology (ICSH) recommended that, in principle, a reference method for platelet counting should be based on a whole blood method because of the biases that occur when preparing platelet rich plasma. Since there did not appear to be any blood counter available which aspirates a known volume of diluted blood through the sensing zone and directly counts platelets in the presence of red cells, it would only be possible to use an instrument capable of

determining the ratio of red cell to platelet count. In the meantime, ICSH, on behalf of the WHO described a recommended method for platelet counting based on chamber counting using phase contrast microscopy [**12**].

In the early 1990s Ault [**13**] and colleagues developed the use of the immunological markers CD41 and CD61 that recognised specific antigens on the platelet surface thus enabling the identification of platelets by flow cytometry. Dickerhof and von Ruecker [**14**] used the same monoclonal antibodies in a ratio method to fluorescent reference particles. These activities set the scene for the creation of an international multicentre study under the auspices of the International Society of Laboratory Haematology (ISLH) to explore the possibility of developing a reference measurement procedure for platelet counting using the two antibodies CD41 and CD61 and based on a red cell to platelet ratio [**15**]. This work led to the publication, by ICSH, of a flow cytometric platelet counting reference method using the RBC/platelet ratio [**16**].

Platelet counting methods

So to summarize the current status, platelet counting can be achieved by
(1) manual phase contrast microscopy,
(2) aperture impedance technology
(3) optical (two angle light scatter and fluorescence) technologies and
(4) immunological flow cytometry.
For assessment of new methods, the manual method is no longer considered satisfactory since it contains too many error prone manipulations. Imprecision is high at low count levels and below platelet counts of 10 x 10^9/L coefficients of variation approaching 30% may be found.

What are the stimuli for improved platelet counting

The reasons for improving platelet counting, particularly in severely thrombopenic patients, stem from current haemato-oncological practice in relation to post-chemotherapy bleeding and prophylactic platelet transfusions. The position is very adequately summarised in the 'Consensus Statement on Platelet Transfusion Therapy' by the Royal College of Physicians Consensus Conference [**17**]:
(1) to allow accurate assessment of bleeding risk and thus decreased use of platelet transfusion
(2) to allow accurate prediction of when platelet transfusions will be needed for an individual patient
(3) to improve management of platelet inventory and permit the use of fresher platelets

The platelet count is vital in assessing the risk of a particular patient developing spontaneous internal bleeding. If there is confidence in the platelet count at low levels, then it is possible to reduce platelet transfusions to those that are clinically necessary. It is necessary to predict with accuracy when and how much pla-

telet transfusion is needed. Platelets for transfusion are a scarce and expensive commodity and Blood Transfusion services struggle to cope with an ever-increasing demand. Urgent consideration must be given to improving the management of the platelet inventory by only permitting the use of platelets in a proper manner.

There are two essential dilemmas:

1. 'Clinicians who utilize platelet thresholds of 10 or 5 x 10^9/L must be aware of the limitations in precision and accuracy of cell counters at this level of platelet count.'
2. 'Accurate counting of low platelet numbers may create difficulties when trying to reduce the threshold below 10 x 10^9/L'

There are two important papers in the scientific literature on the subject. Gmur and colleagues [**18**] felt that a platelet count threshold of 20 x 10^9/L for prophylactic platelet transfusion was based on insufficient data and undertook a prospective study on leukaemic patients to assess if a more restrictive protocol would suffice. In addition to platelet count, the presence of fever, bleeding, coagulation disorders or the intention to perform therapeutic procedures were considered in addition. Some 31 major bleeding episodes occurred on 1.9% of the study days when platelet counts were 10 x 10^9/L or less and on 0.07% of study days when counts were 10-20 x 10^9/L. These workers have suggested therefore that the threshold for prophylactic transfusions can safely be set at 5 x 10^9/L in patients without fever or bleeding manifestations and at 10 x 10^9/L in patients with such signs. For patients with coagulation disorders or anatomical lesions, or for those on heparin, the threshold should be at least 20 x 10^9/L.
The second paper reports on an Italian multicentre study [**19**] on acute leukaemia patients (excluding acute promyelocytic leukaemia). One group was randomly assigned to receive a transfusion when their platelet count fell below 10 x 10^9/L (or 10 – 20 x 10^9/L) in those who were pyrexial, had active bleeding or the need for an invasive procedure. The second group received platelet transfusion when their platelet count was less than 20 x 10^9/L. Patients in the group with a threshold of 10 x 10^9/L platelets received 21.5% fewer platelet transfusions than the patients in the group with a threshold of 20 x 10^9/L platelets ($P = 0.001$). Major bleeding (defined as any bleeding more than petechiae or mucosal or retinal bleeding) occurred in 21.5 and 20 percent of patients, respectively ($P = 0.41$), and on 3.1 and 2.0 percent of the days of hospitalization. It was concluded that the risk of major bleeding during induction chemotherapy in acute myeloid leukemia was similar with platelet-transfusion thresholds of 20 x 10^9 /L and 10 x 10^9/L (or 10 x 10^9 to 20 x 10^9/L when body temperature exceeded 38°C, there was active bleeding, or invasive procedures were needed). Use of the lower threshold reduced platelet use by 21.5%. No information on the type(s) of cell counter used was provided.

Available top of the range platelet counters

The following analysers are currently in use:

SYSMEX	XE-2100 (Impedance and fluorescence)
Abbott	Cell-Dyn 4000 (Impedance, optical and immunological)
Bayer	Advia 120 (Optical)
ABX	Pentra 120 (Impedance)
Beckman Coulter	LH 750 (Impedance)

Impedance platelet counts: Each individual platelet produces a signal proportional to the platelet volume. A platelet volume distribution histogram is produced. Lower and upper thresholds are defined. A log-normal curve is generated from the raw data and usually extrapolated over the range 0–70 fL. Hydrodynamic focusing and pulse editing reduce error and coincidence events are allowed for. A SYSMEX platelet volume histogram is illustrated in **figure 1** from which the platelet parameters are derived. There is a fixed discriminator at 12 fL and moving lower and upper thresholds. The lower threshold excludes small non-platelet particles and the upper threshold very microcytic red cells and RBC fragments.

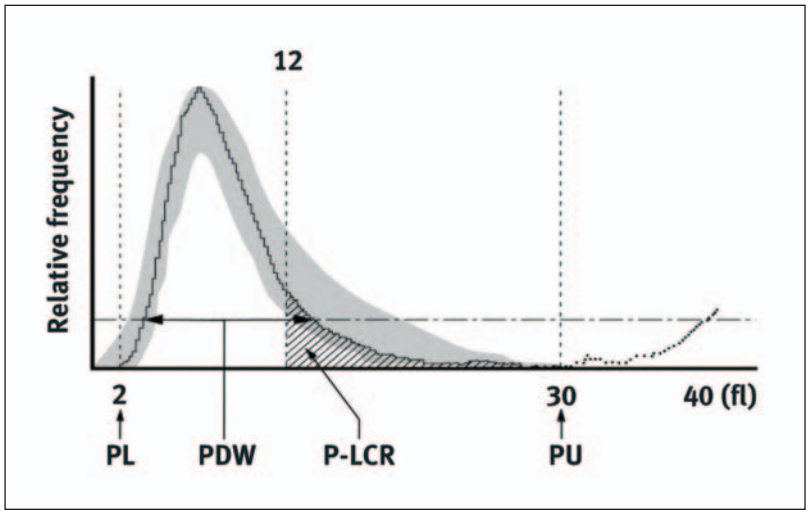

Figure 1
Platelet volume distribution histogram from SYSMEX XE-2100.
PL = lower discriminator for platelet volume distribution;
PDW = platelet distribution width;
P-LCR = platelet-large cell ratio;
PU = Upper discriminator for platelet volume distribution.

Early evaluations of platelet counters revealed count differences between models using similar techniques, particularly at low levels. This reinforced the need for adequate reference measurement procedures.

Optical platelet counts: These are of two types namely (1) two parameter light scatter (forward and side scatter) and (2) combined light scatter (forward scatter) and fluorescence (side fluorescence). In both instances forward scatter indicates particle size and side scatter/side fluorescence indicates particle internal complexity. The SYSMEX optical method employs a fluorescent polymethine dye which stains RNA/DNA in the reticulocyte channel of the XE-2100. **Figure 2** of the RET

scattergram shows the clear separation between erythroid cells and platelets. Again there is a tendency to produce differing counts in the impedance and optical channels particularly at counts below 50 x 10^9/L [**20**].

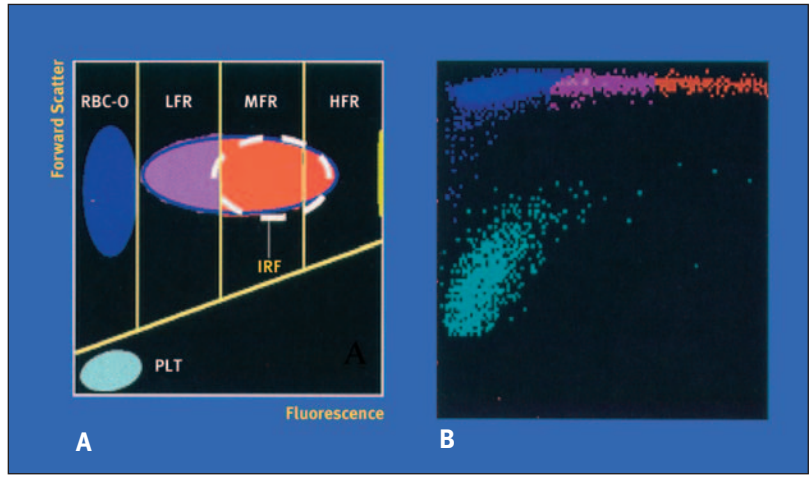

Figure 2
(A) Schematic illustration of reticulocyte (RET) scattergram of the SYSMEX XE-2100 indicating the location of the immature reticulocyte fractions and fluorescent platelets. (B) Illustration of the fluorescent platelet (PLT-O) scattergram.

Immuno-platelet counting: A prime requirement for the RBC/Platelet ratio is unequivocal resolution of platelets from non-platelet particles and from other cells. The antibody must be capable of recognising all platelets. Coincidence (platelet:RBC and RBC:RBC) must be eliminated by selecting optimal dilution. Some 1000 platelet events are counted to ensure accuracy even in thrombopenic samples. A major advantage of this method is its independence from pipetting and diluting errors. An example of immuno-platelet counting is shown in **figure 3**. Immuno-platelet counting is simple, rapid and reliable and is easily transferable to any laboratory with a flow cytometer. There is good correlation with the existing recommended manual method, at normal platelet counts. Superior precision is demonstrated compared to the existing recommended manual method especially in thrombopenic samples.

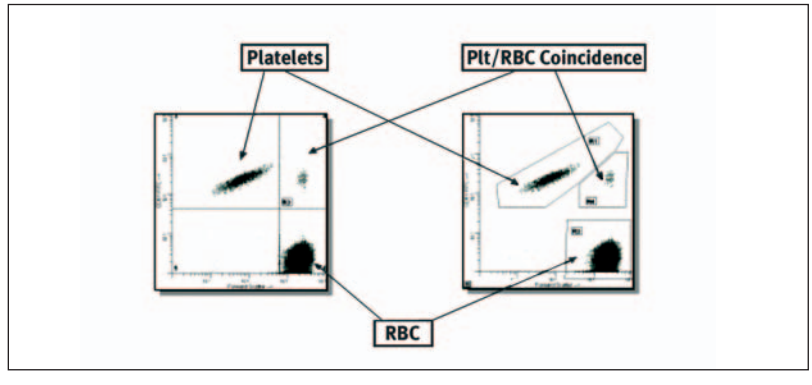

Figure 3
Example of flow cytometry platelet counting: CD61 FITC on the y-axis and forward light scatter on the x-axis.

Current UK multicentre study

There is, within the UK an on-going multicentre study of automated analysers (funded by the National Blood Authority) in patients with thrombopenic counts (below 20 x 10^9/L) following chemotherapy. Comparison of instrument counts will be against the flow cytometry reference method [**15, 16**].

The primary objectives of this study are to determine if there is variation in (1) background count/lower limit of detection and (2) false negative/false positive counts at variable potential threshold values. The results of this study will be reported independently.

Preliminary analysis of 501 chemotherapy samples analysed by the SYSMEX XE-2100 at University College Hospital London compared to the reference flow cytometric method are shown for impedance, optical fluorescence and reported count (dependant on the switching algorithm which selects the most suitable count from both methods) in **figures 4, 5** and **6**. These three methods are all comparable with acceptable correlation values ($r^2 > 0.77$). Previous studies have shown excellent correlation for both methods at normal or higher counts [**20**].

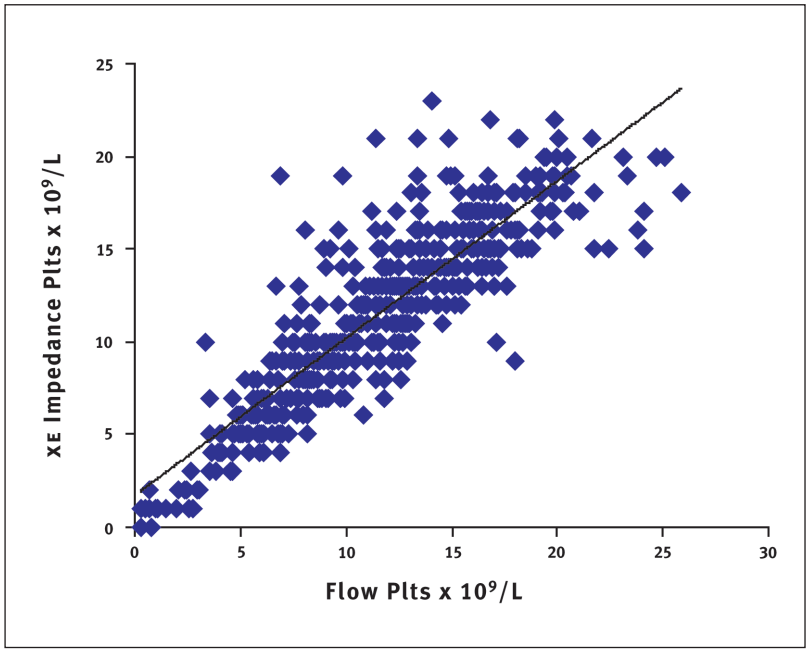

Figure 4
Flow cytometry counts (x-axis) versus XE-2100 impedance (y-axis) counts. y = 0.848x + 1.7228; r^2 = 0.7776.

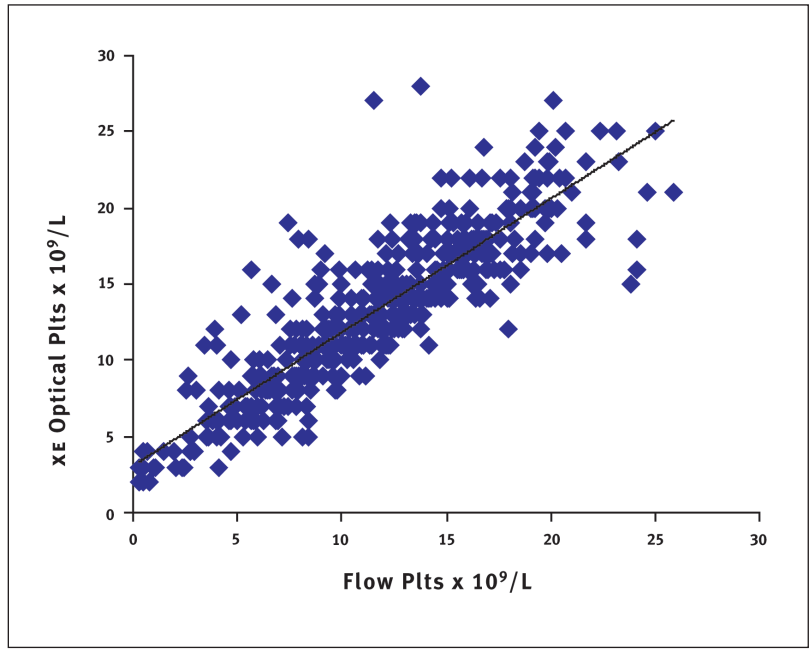

Figure 5
Flow cytometry counts (x-axis) versus XE-2100 optical (y-axis) counts. y = 0.8772x + 3.0537; r^2 = 0.7705.

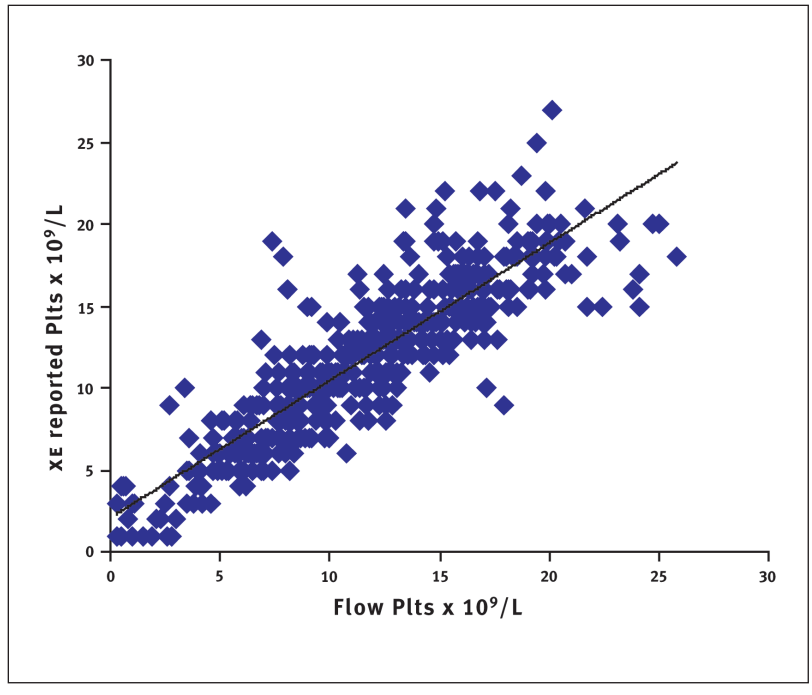

Figure 6
Flow cytometry counts (x-axis) versus XE-2100 reported (y-axis) counts. y = 0.8377x + 2.0711; r^2 = 0.774.

Interferences

RBC fragments	Giant platelets
Microcytic RBC	Platelet clumps
Bacteria	Chylomicrons
Immune complexes	Platelet microparticles
WBC fragments (post chemotherapy)	

Table 1
Potential platelet interfering substances

Table 1 shows a list of potential interfering particles in automated platelet counting. Clearly RBC fragments may cause an increased platelet count and giant platelets may cause a falsely low count but these false counts will be technology dependent. It is in this type of situation that the optical and immuno methods hold an advantage.

Conclusions

This proliferation of methods leaves the laboratory with a number of important questions. Does the increased cost of these additional counting methods represent good value? The answer to this question is affirmative if the laboratory wants to be sure that it is producing the most accurate platelet count. Can rogue results, e.g., platelet clumps, be readily identified? Can the prophylactic platelet transfusion threshold be safely lowered with confidence? This certainly is an important question but do we yet have enough information to provide an answer? Does it all matter? In response to the last question it depends on the individual laboratory requirements. There are also important deficiencies that are seldom addressed. Evaluation protocols are sometimes poorly designed particularly in relation to specimens with low platelet counts and, certainly in the UK, EQA programmes do not address the issue of the very low platelet count with any regularity.

What does the future hold? Answers to these questions must be sought. Will the future lie in reticulated (immature) platelet counts? Would an accurate reticulated platelet count allow a lower prophylactic platelet transfusion threshold with safety? There remain methodological difficulties but most importantly, the potential clinical value of the reticulated platelet count is not widely appreciated. An ISLH Reticulated Platelet Taskforce is in existence and active. Hopefully attitudes will change soon.

References

[1] **Born G.V.R.** (1980)
Personal communication

[2] **Bizzozero G.** (1882)
Über einer neuen Farmbestandteil das Blutes und dessen Rolle bei der Thrombose und der Blutgerinnung. Virchows Archiv fur Pathol. Anatomie und Physiologie 90, 261–232

[3] **Brecher G., Cronkite E.P.** (1950)
Morphology and enumeration of human blood platelets. J Appl Physiol 3, 365

[4] **Bull B.S., Schneiderman M.A., Brecher G.** (1965)
Platelet counts with the Coulter Counter. Amer J Clin Pathol 44, 678–688

[5] **Brittin G.M.** *et al* (1971)
Automated optical counting of blood platelets. Blood 38:422

[6] **Rowan R.M., Allan W, Prescott R.J.** (1972)
Evaluation of an automatic platelet counting system utilizing whole blood. J Clin Pathol 25(3), 218–226

[7] **Haynes J.L.** (1981)
High – resolution particle analysis – Its application to platelet counting and suggestions for further application in blood cell analysis. In Automation in Haematology: What to measure and why? (Eds Ross DW, Brecher G and Wintrobe MM), Springer-Verlag, Berlin, Heidelberg and New York, pp 97–109

[8] **Bacus J.W., Watts S., Trobaugh F.E.** (1980)
Clinical evaluation of a new electrical impedance instrument for counting platelets in whole blood. Amer J Clin Pathol 73, 655

[9] **College of American Pathologists Survey Set H-A Haematology-Clinical Microscopy, Chicago, CAP,** 1979

[10] **Tycko D.H., Metz M.H., Epstein E.A.** *et al* (1985)
Flowcytometric light scattering measurement of red blood cell volume and hemoglobin concentration. Appl. Opt. 24, 1355

[11] **ICSH** (1988)
The assignment of values to fresh blood used for calibrating automated blood cell counters. Clin Lab Haemat 10, 203–212

[**12**] **ICSH** (1988)
Recommended methods for the visual determination of white cell and platelet counts. WHO/LAB/88.3

[**13**] **Ault K.** (1996)
Platelet counting: is there room for improvement. Lab Hemat 2, 139–143

[**14**] **Dickerhof R., von Ruecker A.** (1995)
Enumeration of platelets by multiparameter flow cytometry using platelet specific antibodies and fluorescent reference particles. Clin Lab Haemat 17, 163–172

[**15**] **Harrison P., Ault K.A., Chapman S.,** *et al* (2001)
An interlaboratory study of a candidate reference method for platelet counting. Amer J Clin Pathol 115, 448–459

[**16**] **ICSH** (2001)
Platelet counting by the RBC/platelet ratio method: a reference method. Amer J Clin Pathol 115, 460–464

[**17**] **Norfolk D.R., Ancliffe P.J., Contreras M.** *et al* (1998)
Consensus Statement on Platelet Transfusion Therapy. Royal College of Physicians Consensus Conference. Brit J Haemat 101, 609–617

[**18**] **Gmur J., Burger J., Schanz U.** *et al* (1991)
Safety of stringent prophylactic platelet transfusion policy for patients with acute leukaemia. Lancet, Nov 16 338 (8777) 1223–1226

[**19**] **Rebulla P., Finazzi G., Marangoni F.** *et al* (1997)
The threshold for prophylactic platelet transfusions in adults with acute myeloid leukaemia. Gruppo Italiano Malattie Ematologiche Maligne dell'Adulto. N Engl J Med 337, 1870–1875

[**20**] **Briggs C., Harrison P., Grant D.** *et al* (2000)
New quantitative parameters on a recently introduced automated blood cell counter – the XE-2100. Clin Lab Haemat 22, 345–350