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Is it possible to estimate the Morphology Score of Platelets stored for Transfusion by Volume-Determination with an optical Cell Counter?

Optical Cell Sizing and Platelet Morphology

Ist es möglich, den „morphology score" zur Transfusion gelagerter Thrombozyten durch Volumenbestimmung mittels eines optischen Zell-Counters zu schätzen?

Optische Größenbestimmung und Morphologie von Thrombozyten

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Summary:

The optical cell counter Technicon H-1 jr.™ measures platelet size as well als number. The influence of platelet shape on the volume determination is investigated with a view to application to routine quality control of platelet concentrates (PC). Platelets made spherical by EDTA had a higher mean platelet volume (mpv) and a lower platelet distribution width (pdw) compared with citrate anticoagulated platelet rich plasma (PRP). The morphology score and the mpv in stored PC decreased during a 6 day storage period, while the pdw increased. Diffpdw, a parameter related to reversible alterations, also decreased. It correlated better with the morphology score than mpv. It is suggested that volume determination is influenced by platelet morphology, but application to routine quality control is not satisfactory.

Keywords:

Platelet storage – morphology – volume – light scattering

Zusammenfassung:

Der optische Zell-Counter Technicon H-1 jr.™ mißt neben der Thrombozytenzahl auch ihre Größe. Der Einfluß der Plättchenform auf die Volumenbestimmung wird untersucht unter der Fragestellung, ob diese Methode zur routinemäßigen Qualitätskontrolle von Thrombozytenkonzentraten (PC) angewendet werden kann. Thrombozyten, die durch EDTA sphärisch umgeformt wurden, hatten ein höheres mittleres Volumen (mpv) und eine geringere Größenverteilungsweite (pdw) als mit Citrat antikoagulierte plättchenreiche Plasmen (PRP). Unter einer 6tägigen Lagerung von PC fielen „morphology score" und mpv ab, während pdw anstieg. Diffpdw, ein Parameter, der zu reversiblen Änderungen in Beziehung steht, fiel ebenfalls ab und korrelierte besser mit der Morphologie als mpv. Es wird geschlossen, daß die Volumenbestimmung durch die Thrombozytenmorphologie beeinflußt wird, aber zur routinemäßigen Anwendung in der Qualitätskontrolle nicht befriedigt.

Schlüsselwörter:

Thrombozytenlagerung – Morphologie – Volumen – Lichtstreuung

Introduction

The preparation and storage conditions of platelets for transfusion have been improved considerably during the last twenty years. Several ways have been developed to control the quality of stored platelets for research projects. Infused radio-labelled platelets provide techniques for measuring the posttransfusion platelet increment and in-vivo-survival. In-vitro-methods such as the hypotonic stress response (1) and the morphology score (2) are easier and correlate well with the in-vivo-response. However, they are too laborious for routine quality control. Moreover, the estimation of platelet morphology score by microscopy is too unreliable, being subjective. Further attempts have been undertaken by determining the dispersion of platelet volume with aperture-impedance cell counters (3) and recently by using light scattering methods without opening the platelet pack (4, 5). A modified and improved development of this idea seems to get closest to quick and accurate routine quality control but further improvements are necessary to make it reliable (6, 7).

Guidelines for platelet counts, referring also to contaminating white and red cells, have been published (8). These aim at a uniform and precise quality assurance for platelet products throughout the European Community. It would be very helpful to estimate the quality of platelets during the determination of the cell counts. Some modern cell counters are based on light scattering technology, which in principle is applicable to the assessment of platelet morphology. We used a new version of an optical cell counter, the Technicon H-1 jr.™.

We looked at the ability of the cell counter to detect differences in platelet shape that occur during storage by comparing the morphology score (2) with the results of the counter's platelet volume assessment in independently collected samples from a variety of routine platelet concentrates.

The optical cell counter and some theory

In Mie-theory, the scattering coefficient Q (ratio of power of scattered light to power of light striking the projected area of the particle) is a function of the measuring angle θ, the wavelength λ, the projected area A and the refractive index m of the particle (9). Whereas θ and λ can be set by the apparatus, A as a function of the volume V and m are different from cell to cell.

The Technicon H-1 jr.™ calculates V and Hb directly for each red cell from measurements at two measuring angles θ_1 and θ_2. This is possible in red cells (10) because the variation in the refractive index m is reducable to a single factor (haemoglobin concentration) and the cells can be transformed to ideal spheres by a diluent as described below.

Both are impossible in stored platelets. In order to get some information about their scattering characteristics, the scattering function can be divided into three components (9): scattering by diffraction, reflection and refraction.

The total amount of diffracted light depends on the projected area A and its angular distribution on the particle's shape. It forms a forward angle from which one can derive information about the projected area whereas microstructures such as edges and granules increase width of scatter and thus reduce the amount of diffracted light measurable in the forward angle.

The reflective component covers the whole angle equally in both spheres and nonspheres and can thus be considered as „noise".

The refractive component depends on the refractive index m and covers a forward angle. Randomly orientated non-spheres lead to an arbitrary deviation from this angle.

Material and methods

Platelet samples

Platelet rich plasma (PRP) was prepared by centrifugation (10 min, 300 g) of whole blood anticoagulated with either tri-sodium-citrate (2.5 ml 3.8% citrate + 17.5 ml blood) or K^+_2-EDTA (1.3 mg/ml).

Platelet concentrates (PC) were prepared as previously described (11, modifications: first centrifugation 1 000 g for 9 min, second centrifugation 2 900 g for 20 min, anticoagulant: CPDA-1) and stored in Fenwal PL 1240 packs (Baxter Healthcare Ltd.) on an elliptical rotator at 22 °C.

Bleed lines remaining on the PC packs were carefully stripped three times, sealed, and separated to obtain representative samples without opening the packs. Of each sample 475 µl were pipetted into three test tubes, two of them containing 25 µl Na^+_2-EDTA (3% in phosphate buffered saline (PBS, Oxoid Ltd., pH 7.3), end concentration 0.15% w/v), one containing 25 µl PBS without EDTA. The aliquot without EDTA (group 1) and one aliquot with EDTA (group 2) were incubated at 37 °C for one hour, the other one with EDTA (group 3) was left at room temperature (22 °C) for one hour. Platelet morphology, count, and volume were determined on each aliquot.

Design

60 independent platelet units were investigated that way, using the PRP within 2 hours of preparation to achieve optimal morphology as a starting point and PC over a storage period of six days (five to twelve per day) to obtain a relevant spectrum of morphological changes. The number of units was chosen in order to detect even weak correlations with sufficiently high power (power: 0.95, significance level: 0.05; r = 0.45).

In order to find out in principle the capacity of the cell counter to detect morphological changes, a fresh PRP and an old PC (ABO-matched and numerically adjusted with autologous platelet-free plasma to the platelet count of PRP) were mixed in various proportions. The morphology score of PRP and PC was determined and calculated for each mixture. The measurements were carried out after addition of EDTA as above, with one aliquot incubated at 37 °C and another at 22 °C for each sample.

Morphology score

All morphology scores were determined as previously described (2) in duplicates by the same person throughout the experimental period. Assessments were made blind of the identity of each sample. After incubation, the platelets were fixed by the addition of 5% glutaraldehyde in PBS (end concentration 0.19% v/v). Using a phase contrast microscope with a magnification of × 1 000, 100 cells were classified as discs, spheres (lost discoid shape and/or developed up to two small pseudopods), dendrites (more or larger pseudopods) or balloons (lost cell contents, swollen). The percentage of discs (×4) + spheres (×2) + dendrites (×1) gave the morphology score.

Platelet counting

After incubation and before the assessment of platelet count and volume, the aliquots were mixed for at least 10 min on a roller. The optical counter (Technicon H-1 jr.™) diluted the PC automatically after aspiration with a buffered solution, containing Na-dodecyl-phosphate, EDTA, and glutaraldehyde. This diluent makes red cells spherical for an exact determination of MCV, but does not influence the platelet shape (as shown by phase microscopy). The cell suspension then passes through a sheath stream flowcell, where the forward angle light scatter signals are measured cell by cell. Besides counting the cells, the volume of each cell is calculated by its scattering characteristics. For platelets, defined as the particles giving a signal below a distinct threshold in both measuring angles, only the higher angle (5° to 15°) is used for calculation. The mean volume of all counted platelets (mpv) and their distribution width (pdw) are then calculated (pdw = SD/mpv × 100%). For each sample, the differences in mpv and pdw between the EDTA containing aliquots incubated at 37 °C and at room temperature, respectively, were calculated (diffmpv, diffpdw).

Standard statistical methods of analysis were used throughout.

Results

Influence of platelet shape on the volume measurement

Fresh platelets became spheres after using EDTA as anticoagulant. The results of the volume measurement were

compared with those of mainly discoid platelets in citrate anticoagulant. Results are given in tab. 1, showing that the mpv was significantly higher in spherical platelets while the pdw was lover. Platelet volume histograms show a left shift in PRP anticoagulated with citrate compared with EDTA.

On the other hand, there was no significant correlation between mpv or pdw, and morphology in fresh citrate PRP. Pdw and mpv themselves correlated well ($r = -0.88$, $p < 0.0001$, 95 % confidence interval of slope: −5.84 to −3.81).

After mixing the platelets of a fresh PRP and a five days old PC in various proportions, the morphology score correlated very strongly with mpv ($r = 0.97$). The correlation with diffpdw as the difference in pdw due to incubation at 37 °C or 22 °C was weak ($r = 0.70$, fig. 1).

Samples of routine-PC

The platelet counts in PC were within a range of 389 to 1751 × 10^9/l. In analysis of variance there was no significant difference in mpv and pdw between group 1 (without EDTA, incubated at 37 °C; mpv: 5.7 ± 0.8 fl, pdw: 68.4 ± 3.8 %) and group 3 (with EDTA, incubated at 22 °C; mpv: 5.7 ± 0.9 fl, pdw: 67.9 ± 4.2 %). In group 2 (with EDTA and incubation at 37 °C), mpv was significantly higher (6.2 ± 0.8 fl) and pdw lower (64.6 ± 4.2, both $p < 0.0001$).

Mpv decreased during storage ($p < 0.0001$, tab. 2) and pdw increased ($p < 0.01$, fig. 2).

The difference in mpv with incubation at 37 °C and 22 °C (diffmpv) decreased from 0.98 ± 0.29 fl at day 0 to 0.53 ± fl after 6 days ($p < 0.0001$). The difference in pdw (diffpdw) also decreased during storage ($p < 0.0001$, fig. 3).

The morphology score without incubation at 37 °C (group 3: mean score 208.4 ± 51.3) was lower than with incubation ($p < 0.0001$), while the addition of EDTA did

Tab. 1: Percentage of discs and spheres, mpv and pdw in citrate- and EDTA anticoagulated PRP (means ± SD)

	discs (%)	spheres (%)	mpv (fl)	pdw (%)
Citrate-PRP (n = 53)	82.5 ± 9.1	9.8 ±4.5	7.8 ± 0.8	57.0 ± 4.1
EDTA-PRP (n = 12)	4.2 ± 2.8	92.5 ± 4.5	8.4 ± 0.7	47.1 ± 2.7
t-test			$p < 0.05$	$p < 0.0001$

Tab. 2: Changes during the 6-day storage in morphology score (group 1: without EDTA) and mpv (group 2: with EDTA). Means ± SD

	n	morph. sc.	mpv (fl)
fresh	8	356.8 ± 12.7	7.9 ± 0.6*
day 1	12	298.2 ± 41.5	6.5 ± 0.6
day 2	5	292.5 ± 30.4	5.5 ± 0.8
day 3	11	257.4 ± 32.2	6.1 ± 0.6
day 4	8	236.9 ± 17.8	5.9 ± 0.5
day 5	7	190.1 ± 40.6	6.0 ± 0.5
day 6	9	179.1 ± 54.9	5.8 ± 0.8

* n = 4 in fresh PRP

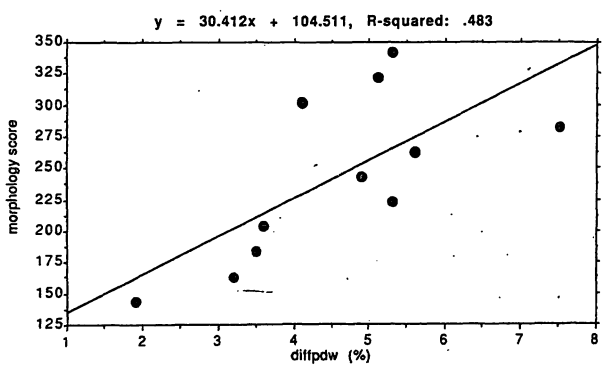

Fig. 1: Morphology score increasing with the difference in pdw with and without incubation at 37 °C, using fresh PRP and 5 days old PC mixed in various proportions

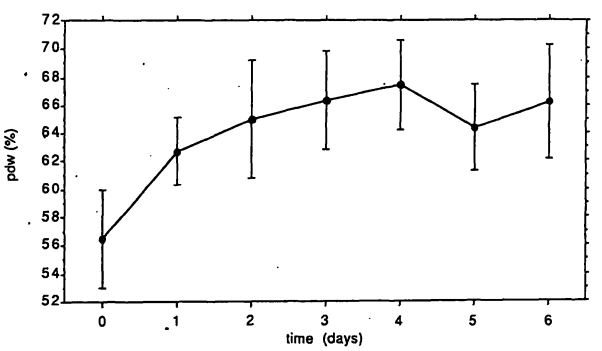

Fig. 2: Increase of pdw (with EDTA and incubation at 37 °C) during storage time. Means and SD

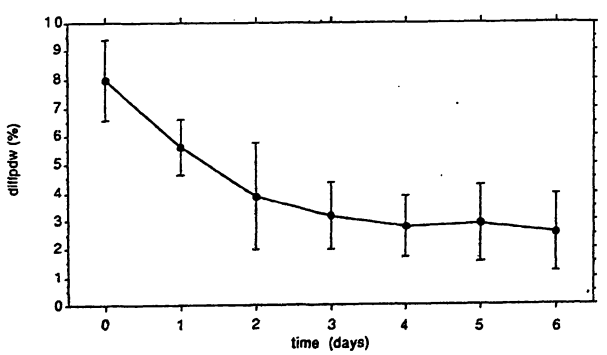

Fig. 3: Decrease of the difference in pdw between incubation at 37 °C and 22 °C during storage. Means and SD

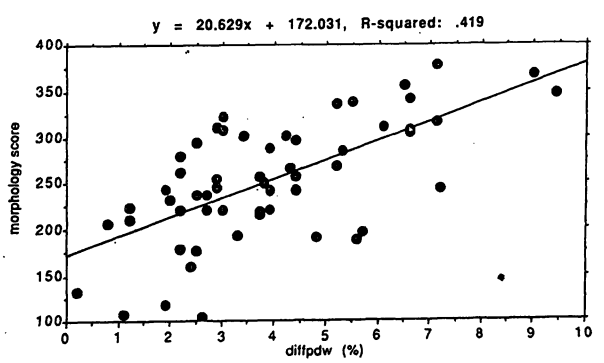

Fig. 4: Morphology score increasing with the difference in pdw between incubation at 37 °C and 22 °C. Std. error of slope: 3.30

not make a difference (group 1: 259.4 ± 67.1, group 2: 257.2 ± 64.2. The score decreased during the storage period ($p < 0.0001$, tab. 2).

The changes in morphology were mainly due to formation of dendrites, while the proportion of spheres maintained stable after a slight increase during the first 24 hours (11.4 ± 3.1 % to 24.6 ± 8.0 %).

The morphology score of group 1 (without EDTA) was chosen as the dependent variable for regression analysis. It correlated with diffpdw ($r = 0.64$, $p < 0.0001$, fig. 4) and with mpv in group 2 ($r = 0.49$, $p < 0.0001$, fig. 5). Multiple linear regression achieved higher correlation with the variables mpv and pdw of group 1, including time as the variable with highest weight ($R_m = 0.87$). 88.3 % of the predictions were within 20 % of the original value.

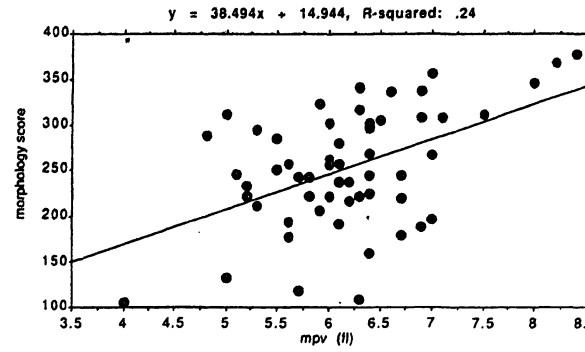

Fig. 5: Morphology score increasing with mpv. Std. error of slope: 9.31

Discussion

The aim of this study was to discover whether an optical single particle counter can give sufficient information about platelet morphology for reliable quality control during the routine counting procedure.

One must emphasise that the term „spheres" as used here for stored citrate anticoagulated platelets means mainly „non-discs" as described above. Platelets in blood anticoagulated by EDTA by itself can be described as „truly spherical" as distinct from the „spheres" with more bizarre forms which develop on storage. The addition of EDTA to platelets previously anticoagulated with citrate did not change their shape (12), but prevented them from redeveloping their preincubation forms during relatively tough treatment on the mixing roller before counting, as shown by the differences between group 1 and group 2.

Anticoagulation with EDTA not only induces platelet sphering but also results in higher mpv and lower pdw with the Technicon H-1 jr.™ (tab. 1). Part of the mpv increase may arise from swelling due to an increase of extracellular K^+ (12), as K^+_2-EDTA was used here. The decrease in pdw strongly supports an explanation by the different shape, as comparison with the regression function of mpv and pdw in fresh citrate anticoagulated PRP shows. This regression function may be artificial due to the pdw computation, for a smaller mpv stands in the approximately log normal volume distribution for a left shift, which automatically increases the pdw calculated from the standard deviation. But the observed pdw of PRP in EDTA is outside the 95 % confidence predictions. Thus, an additional effect must be considered:

The spherical „EDTA-platelets" present a projected area to the laser beam which is dependent only on their actual volume, whereas the projected area of the discoid „citrate platelets" also depends on their orientation about the long axis in the flow, with projection edge-on giving a smaller signal than projection by the whole discoid area (13). This gives a higher „volume distribution" result than the distribution of real volume would give.

The correlations in the experiment where fresh and stored platelets were mixed in different proportions (fig. 1) give further evidence for a dependence of the counter results on platelet morphology. Although the strong correlation between morphology score and mpv may be due to real volume differences of the two mixed preparations, the correlation with diffpdw suggests an additional influence of the platelet shape. Incubation at 37 °C leads to recovery of discoid forms in fresh platelets that may be reversibly altered by in-vitro handling at room temperature. These shape changes result in differences in the pdw results from the cell counter (diffpdw) which tend to decrease with the proportion of platelets in the mixture that are either altered reversibly or not altered at all.

This is confirmed by the statistically significant correlation of diffpdw with the morphology score in a sample of PC collected over a storage period of six days (fig. 4). The correlation of the mean platelet volume (after addition of EDTA and incubation at 37 °C) with the morphology score is weaker (fig. 5). But it still suggests a tendency for mpv as measured by low forward angle light scattering to decrease with a loss of the platelets' morphological integrity during storage, though this is probably further compounded by inter-individual variation.

Results showing similar trends were obtained with experiments using light transmission or scattering in different ways (4, 6, 14), in which the percentage of discs is predicted by differences of scatter/transmission characteristics of platelets randomly orientated and orientated in a laminar flow (by stirring in a aggregometer (14) or pressing through an aperture formed inside the pack (4, 6)). The investigation of different measuring angles by *Bellhouse* et al. (5) suggests that when discs are replaced by non-discs during storage, the reduced proportion of discs leads to less platelet orientation in laminar flow. This in turn leads to a reduced loss of high angle scatter signals (30°), when they are compared with signals generated by randomly orientated platelets. This is shown as a ratio of signal strengths derived from random orientation, and from laminar flow. On the other hand, the low angle scatter (5°) and transmission signals of platelets in laminar motion are reduced with an increasing proportion of non-discs.

Applying this to our experiments, a decrease in the proportion of discs should result in a lower low angle scatter signal expressed as mpv. The projected area of the cell body in „Mie size" becomes smaller as pseudopods are developed and thus scatter less light in the low forward angle (15), whereas the „Rayleigh-Gans size" pseudopods scatter light in high angles. Applying the above stated theory (9), the occurrence of pseudopods might lead to an angular distribution of light scattered by diffraction to higher angles. It might also lead to a higher probability of deviation from the forward angle covered by light that is scattered by refraction. Both result in a lower proportion of light measured in the low forward angle. Further, it is suggested (13, 16) that a loss of dry mass in the

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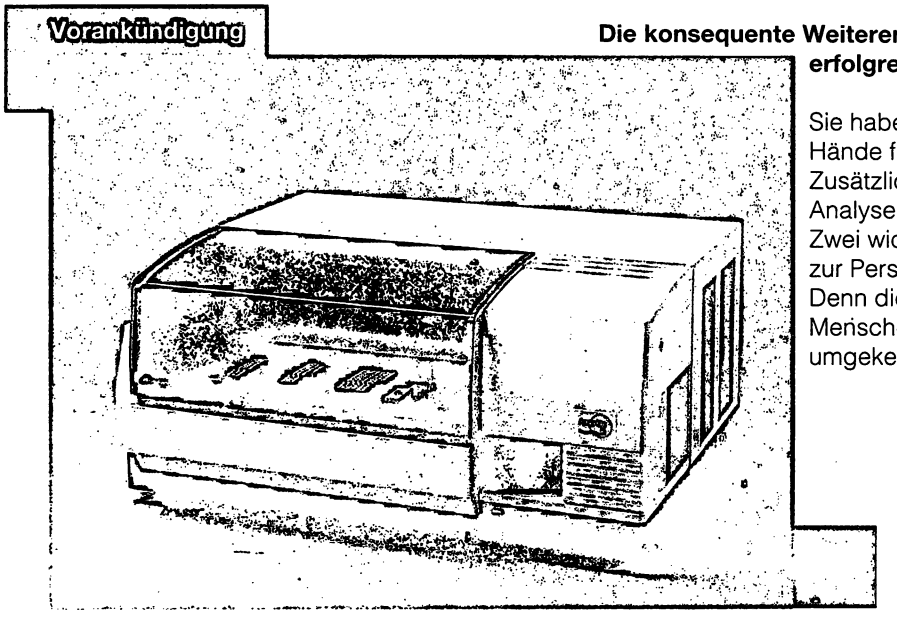

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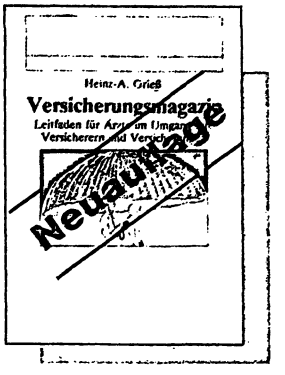

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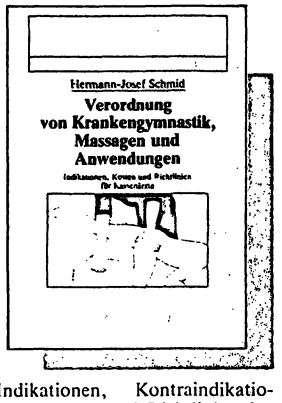

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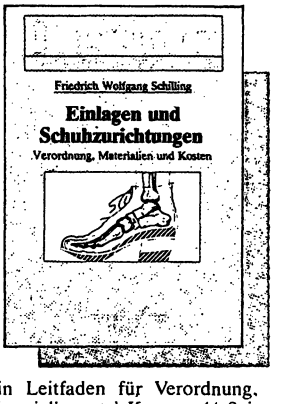

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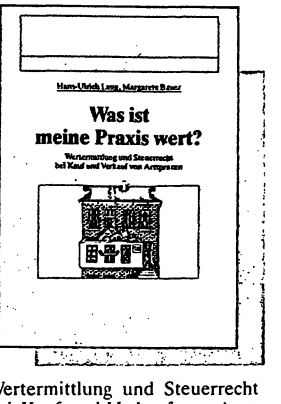

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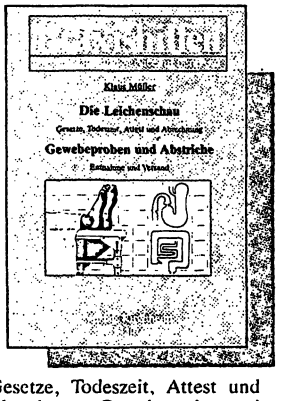

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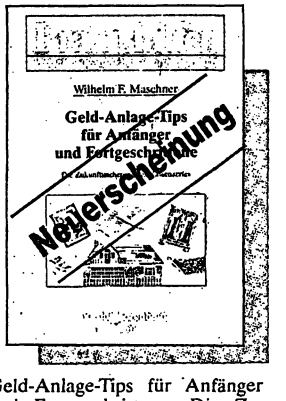

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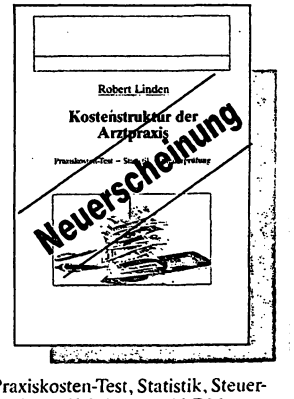

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projected platelet by release during storage and by flow from the cell to its pseudopods, as well as swelling with influx of water (17) decrease the total amount of light scattered by the refractive component due to a decrease in the refractive index (13). In sum, this results in a decrease of mpv during storage as measured by forward angle light scattering, although the real volume, as measured by aperture impedance, increases (13).

The interference of the interindividual variation of the platelet volume might explain at least in part the high variablility in the correlation of mpv and morphology score. By using the better correlating variable diffpdw this factor is avoided. But this variable includes too many other uncertainties. Fig. 4 and 5 show that a reliable prediction of morphology score by the above methods was not achieved. The predictions by multivariate methods including the factor time look more encouraging, but the falsely predicted PC are exactly those which one wishes to detect by effective quality control, i. e. PC of particularly good or bad morphology with respect to their storage age.

The problem of interference of platelet volume and shape in the volume measurements by light scattering might be partly soluble by using two measuring angles, as for volume and haemoglobin determination of red cells. But the angles designed for red cells and used in the Technicon H-1 jr.™ do not help here, because both angles are already covered by the diffraction forward angle of the 2–3 μm diameter platelets. Instead, a high measuring angle could be used in addition to the low forward angle to detect whether a lower low angle signal is accompanied by a higher high angle signal, implying that a platelet without a smooth discoid shape has just passed the chamber. In stored platelets, more reliable volume measurements and additional information about the platelets' morphological condition might be attainable that way.

Morphology score is said to correlate with clinical function (2). Our investigations suggest that optical single platelet counting in its current form cannot give a reliable estimation of platelet morphology in stored concentrates, despite the conclusion that the volume determination is severely affected by morphological changes that occur during storage.

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