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**Introduction of “RTH-II” as a Simple Assay for
Screening PLT Function Specifically and Reliably:
Validation, Standardization and Development of the Procedures of
the Retention Test of Homburg (RTH) in Due of Morphological Control**

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submitted by:

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Born in 1968 in Damascus, Syria

*To my Parents
my Wife,
and my Daughter
with Love and Gratitude*

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I. SUMMARY

1-1. English Summary

Despite platelet (PLT) activation plays a crucial role in the physiology and the pathophysiology of thrombosis and hemostasis, platelet function assays are not well standardized and lack simple and reliable screening possibilities [Breddine 2003, Tsiara 2003, and Reininger 2006]. The Retention Test of Homburg (RTH) was set by the institute of clinical hemostaseology, University of Saarland, in 1993 as an attempt to bridge this gap by introducing a simple and cost effective assay for screening platelet function reliably. The test was based initially on the retention of platelet by exposing citrated platelet rich plasma to a standardized exogenic textured surface (RTH filter: a disc of sintered polyurethane fixed in disposable Eppendorf retention tube: "RTH tube"; pores diameter = 16-22 μ m) under defined conditions of flow forced by centrifugation. The percentage of the difference between platelet count before and after the passage of the filter is defined as the platelet retention index (PLT-RI). High values should be a sign of platelet stickiness, whilst low values are supposed to indicate loss of platelet function.

Since the first developing steps of RTH test, variant procedures were used for performing the assay which have never been standardized [Breddine 2003, Reininger 2006]. The RTH assay delivered unacceptable reproducibility with serious problems in the reliability. The accuracy of RTH has never been studied in healthy individuals and the evidences on its specificity for platelet were inadequate and presented no comprehensive interpretation. Few investigations defined reference ranges for platelet retention index (using citrated platelet rich plasma), but none of them were reliable. The data obtained from a multi-center trial using RTH was at least unacceptable [König 2002]. The serious errors and problems of RTH destroyed the credibility of previous studies and prevented its application in clinical practice. In addition, the sophistications incorporated by the variant procedures e.g., pre-analytic processing of the blood and preparation of variant additives, have damaged the significance of this analysis as a simple and cost effective assay. Some investigations, however, reflected considerable response of platelet retention index to platelet agonists/antagonists promising vital clinical significance of RTH for screening platelet function. This has meanwhile to be interpreted as rather hypothetic and also throws doubts on the reproducibility. Few studies concluded the "athrombogeneity" of the RTH filter system, despite the evidences were not adequate and our study proved that the filter is not as declared completely athrombogenic. The main weakness point of RTH studies was the use of variant and unreliable procedures stepping unacceptably over defining reliable and evidence based standard operation procedure (SOP). Nevertheless, the initially declared athrombogeneity of RTH filter and the relative convenience of the application of RTH in clinical practice represented promising advantages of RTH compared with other retention tests. The objectives of our investigations were therefore: *a.)* Radical validation and improvement of RTH procedures, *b.)* Developing a reliable and simple standardized procedure, *c.)* Setting evidence based interpretation of the specificity for screening platelet function, and *d.)* Defining a reliable reference range in healthy individuals.

The evaluation and the development of the procedures of RTH resulted in excessive modifications and basic improvements on the reliability, the convenience, and the significance of the test implicating the

definition of a new standardized assay on citrated whole blood which was introduced as RTH-II. Stepwise investigations were done by using different Sysmex cell counters and under morphological control for validating and improving the methodology, the reliability and the standardization of RTH. Thus, extensive modifications on RTH besides basic identifications were set and incorporated in RTH-II:

(1) The citrated whole blood was defined as the tested material of choice in clinical practice instead of citrated platelet rich plasma used mainly in RTH. This was essential to avoid the artifact variability attributed to PLT activation by the separation steps as well as to the variability attributed to the diverse interactions of the variant PLT gradients (after preparation by centrifugation) with their medium in the plasma. In addition, the whole blood reflects the physiological status more accurately than plasma and reduces the probable errors related to PLT hyperactivation (by e.g., aggressive mixing). (2) The variable amounts of the tested materials used in RTH were standardized at 500 μ L in RTH-II. (3) The addition of the so in RTH called "reactivating reagent" (Calcium with hirudin) was excluded since it was not justified. Also the resulted platelet reaction was found to be unstandardizable, convertible (from positive to negative) and vary inter-individually. (4) The incubation step (3-10 min for the tested materials in RTH tube) was excluded in RTH-II to avoid possible platelet activation by long contact with the filter and the consequent variability of platelet response related with the variant incubation periods and the inter-individual differences. (5) The measurements before the passage of the filter were applied on the materials in the collection tube (RTH-II) instead of the RTH tube after the incubation step (RTH). The amount of the tested material passing RTH filter was thus standardized at 500 μ L by avoiding the variability related to the variant amounts of the samples aspirated by the cell counters (100-400 μ L). The blood amount passing the filter became subsequently sufficient for performing the measurements on it "in-double" as well as for additional control tests. Putting the collection tube on a rotator assure the homogeneity of the tested material in contrast to the variant rates of blood sedimentations during the stand of RTH tube which leads to unacceptable variability in the measurements. (6) The extremely variable centrifugation conditions used in RTH (79-160 g/ 5-16 min) were standardized at 110 g/ 5 min which allows acceptable platelet distribution in the filter and delivers reliable measurements. Surprisingly, the centrifugation conditions were inadequately specified in some previous RTH studies. (7) The blood collected in the lower part of RTH tube (after centrifugation) was mixed by vortex (3 min in RTH) before performing the measurements. Standardized slight vortex (\approx 3sec) was applied in RTH-II in order to avoid probable unacceptable platelet aggregations, agglutinations, and/or disruption that damage the reliability of the measurements. (8) The measurements on the blood after the passage of the filter should be performed within 2 min after centrifugation to assure the accuracy (preserve the blood homogeneity and minimize the errors attributed to probable occurrence of "platelet particles phenomenon"). (9) The measurements should better be performed "in-double" to assure their reliability. The patterns of the variations between the 1st and the 2nd in-double measurements were also defined as rapid indicators for probable errors (induced by e.g., inadequate or inconvenient mixing). In-double or in-series measurements should be performed by using new tips for each pipetting instead of the same tip in order to avoid unstandardized variation (not specified in RTH related studies). (10) The formula of calculating the large platelet retention index (PLCR-RI) was corrected by using the absolute values of large platelet instead of their ratio (%). The resulted reference range and its correlation with the other retention indices provided

significant meanings/conclusions in contrast to those obtained by using the original formula. Such errors of RTH put also serious questions on the reliability of related previous studies. (11) Nobel identification of additional retention indices (RIs) for leucocytes (WBC-RI), erythrocytes (RBC-RI) and for the platelet size parameters (platelet derived width "PDW-RI" and mean platelet volume "MPV-RI") was set for the first time in any retention test system. Defining these retention indices (including large platelet retention index) besides their correlations with platelet retention index was essential for: a.) defining the errors and the sources of variability of RTH, b) setting and validating necessary modifications, c.) getting comprehensive interpretation for the accuracy, the specificity and the precision of RTH-II, and d.) setting for the first time a key protocol for the interpretation and the quality assurance of RTH-II measurements. (12) Nobel identification of the weight retention index (wRI: the weight of the blood retained in the filter) was set. This index was essential for standardizing and validating the centrifugation conditions as well as the quality assurance of the integrity and the reliability of RTH filter. (13) "Platelet particles" phenomenon was identified as the main source of errors. Platelets activated by shear stress passing through RTH filter are opposed to hyperactivation by inconvenient vortex (RTH) leading to degradable platelet aggregates/agglutinates (platelet particles). These particles are misread by the cell counters as large platelet, erythrocytes and/or leucocytes (whenever the particles are within their size discriminators) and are thus demonstrated by: (a) proportional false decreases in the retention indices of erythrocytes and leucocytes, (b) a false shift to the left for the retention indices of platelet size, and (c) relevant false increase in platelet retention index. Occasional platelet hyper-activation may occur before passing the filter if the blood is inconveniently handled leading to inverse patterns of false variations for the retention indices. The final values of the retention indices vary according to the total of the false variations before and after the passage of the filter. Platelet hyper-activation leads also to rosette forms associated with platelet decrease. The identification of platelet particles phenomenon besides the new retention indices was crucial for defining RTH errors and setting the related modifications. The correlations between platelet retention index and the other retention indices (except large platelet retention index) convert from defined positive patterns to negative ones by the occurrence of platelet particles after passing the filter. Despite RTH-II avoids the occurrence of this phenomenon, it is not completely preventable. Understanding the implications of this phenomenon is therefore mandatory for the right interpretation and quality assurance of RTH-II measurements. (14) The room temperature was defined as a standard condition for performing RTH-II. (15) The time of performing the assay was standardized at <120 min which preserves the reliability of the measurements. Exceeding this limit up to 180 min used in RTH may be associated with marked decrease in the values of platelet retention index.

Diverse approaches were used in our investigations for validating and characterizing the accuracy, the precision and the specificity of RTH-II with the use of multiple morphological control (before and after the passage of RTH filter): A.) In-series precision evaluated in 13 series (n=10) of citrated whole blood and 3 series of Eightcheck control (customary for platelet count check by Sysmex) besides day-by-day precision evaluated by using Eightcheck control. Phase-contrast microscopy was used for control. B.) Exclusion the effect of RTH filter. C.) Performing RTH-II on platelet isolated by gel-filtration compared with citrated whole blood. PLT spreading was used for the morphological control. D.) Validation of a new developed standard for washed and unwashed prefixed platelet under control by phase-contrast micros-

copy. E.) Morphological characterization of the interaction of platelet and blood cells with RTH filter. Semi- and ultra-thin segments of the filter were fixed after the blood passage and then examined by phase-contrast as well as screening and transmission electron microscopy. F.) Establishing reference ranges of the retention indices of platelet and blood cells using citrated whole blood as well as Eightcheck control. The accuracy was controlled by phase-contrast microscopy. G.) Quantity assessment of the filter system besides the blood amounts used and trapped in it. This validation was applied on the materials (citrated whole blood and Eightcheck control) used in the precision and reference range studies.

These approaches proved the accuracy, confirm the acceptable precision of RTH-II and provided evidence based interpretation for the specificity of the assay. The accuracy of platelet retention index was for the first time studied in normal individuals. The extreme falsely elevated levels (mean=2.2 folds) and their increase by time (up to 61%) were substantially corrected. The unacceptably high in-series variation of platelet retention index measurements by RTH was reduced by at least 80% in RTH-II which delivered precise values (standard deviation "SD"=2.9). The reliability of RTH-II measurements is dependent on precise and accurate platelet count besides the biological variability, whilst the errors induced by additional sources of variability in RTH (e.g., platelet particles) increase the variability and false results unacceptably. Using more modern cell counters proved to improve the precision of the measurements remarkably but showed no impact on the accuracy. The day-by-day precision which was evaluated for periods longer than ever before (up to 10 days) using Eightcheck control revealed also acceptable low variation for platelet retention index (SD<2.9).

In 2004, RTH-II could thus be established. For the first time, a reliable reference range for platelet retention index in citrated whole blood with acceptable biovariability (n= 70: mean= 25.2%, SD=6.25) was defined. The impact of different modes of measurements on the accuracy of the results was inspected. In parallel with platelet retention index, the precision and the accuracy of the other retention indices were substantially corrected and reliable reference range for each retention index was for the first time defined. The accuracy of the retention indices of platelet and the other blood cells was proved and characterized by phase contrast and electron microscopy. It was further proved that:

A.) RTH filter is rather athrombogenic in contrast to the other "filter systems" used in previous platelet retention assays (e.g., Hellem 1970).

B.) Under the standardized conditions of RTH-II using citrated whole blood, the retention values of the leukocytes (WBC-RI=4.2%) and erythrocytes (RBC-RI=-3.6%) are related to their sizes which are bigger than the pores of the filter. Whilst platelets which are smaller in size than the pores of the filter interact specifically with it demonstrating basically higher retention values than the blood cells. Platelet retention index reflects directly the response of platelet to activation by shear stress during the passage of the filter. The platelet activation is demonstrated by shape change, extreme adherence and spreading on the filter with remarkable degranulation and excessive formation of wall-bound aggregates that bridge the opposite polyurethane surfaces of the interconnected micro pores, especially in the upper part of the filter. Platelet may occasionally form free aggregates clumping within the lumina of the pores. The platelet response to activation is comparable to the physiological reaction of circulating platelet at a disturbed endothelium and/or subendothelium. The RTH-II assay may therefore reliably screen the

multiple function of platelet in hemostasis and thrombosis. The upper and lower limits of the reference range of platelet retention index in citrated whole blood allow measuring platelet hyper- and hypo-activation which is in contrast to the previous study of Krischek [2000]. The clinical significance of RTH-II was further promoted by several observations e.g., the remarkable negative correlation of platelet retention index with platelet count suggests that the activity of platelet increases in association with the decrease of their count in healthy individuals.

C.) The specificity and reliability of RTH-II was further established by its ability to demonstrate accurately and precisely the inhibited function of platelet in the fixed medium of Eightcheck control (platelet retention index= 2.6%; SD=2.1) compared with the biovariability of platelet activity in citrated whole blood and compatibly with the morphological control. Platelet in Eightcheck control were not activated (spherical) during the passage of the filter and demonstrated no real adhesion on it but only occasional agglutinates with selective contacts to the filter. Whilst RTH studies delivered unexplainably high platelet retention index levels for *fixed* platelet control (>21%; Krischek 2000). The comparability between the retention values of the blood cells in citrated whole blood and that in the fixed medium of Eightcheck control enhances linking the retention of these cells in citrated whole blood mainly to their sizes.

D.) The determinations of the retention indices of platelet size parameters (especially for large platelets) expand the significance of RTH-II assay to the platelet size distribution and show platelet functional heterogeneity. This was evident by e.g., demonstrating the lower activity of large platelet compared with normal sized platelet in normal individuals and their lower response to hyperactivation.

E.) The determinations of the retention indices of leucocytes (WBC-RI) and erythrocytes (RBC-RI) could further extend the significance of RTH-II to the other blood cells which showed some specific signs of activation and interaction with the filter. This may implicate additional hypothetic uses of RTH-II assay such as to find out erythrocyte rigidity and deformability.

The reliability of RTH-II in researches was established by its applicability on the different materials (citrated whole blood, citrated platelet rich plasma, gel-filtered platelet, Eightcheck control, and washed and unwashed prefixed citrated platelet rich plasma) used in our investigations for validating the reliability and specificity of RTH-II with evident compatibility with the multiple morphological control. For example, the RTH-II assay was able to demonstrate the influence of the partial absence of the normal medium of platelets on their predisposition to hyperactivation and stickiness (e.g., forming aggregates and agglutinates) which was basically much less compensated in fixed medium than in citrated whole blood. This was proved by the ca. 2 folds increase of platelet retention index levels in gel-filtered platelets compared with citrated whole blood (57.4% vs. 30.5%) and the basically higher increase of platelet retention index in the washed prefixed platelet standards up to 5 folds compared with the unwashed ones (5.7% vs. 33.5%). The RTH-II played essential role in identifying and characterizing different new phenomena of platelet function (e.g., platelet particles, platelet response to Ca^{2+} with hirudin) besides providing specific beneficial insights for the development of our standards of washed and unwashed prefixed platelet.

The development of the reliability of RTH-II compared with RTH was further associated with substantial improvement in the excellency and the convenience of the test. No preparation for plasma or any

reagent is required and no additives are used in RTH-II. The blood amount required for the test was reduced from >1.5 mL to 0.5 mL. The turnaround time was shortened extensively up to 89% (35-53 min vs. 5.5-6 min). The assay became significantly simpler and less labor intensive. It is performed with greater ease, less equipment (only the standard disposable RTH tube is required with a convenient centrifuge) and higher cost effectiveness. In addition, setting a quality assurance protocol for RTH-II assay for the first time not only enhances the reliability of RTH-II measurements and interpretation but also attains the satisfaction of its use in the clinical laboratory. Our suggestion to automate RTH-II by using conventional electronic cell counter allows on-line reporting, interpretation and quality assurance which enhance further the convenience and cost effectiveness as well as the reliability and quality assurance of RTH-II measurements with greater satisfaction.

Despite previous “adhesion and retention” tests proved to be meaningful, but they could not be established in clinical practice because they are, in contrast to RTH-II, poorly standardized and require experienced personnel and additional equipments (e.g., Hellem-II 1970, O’Brien 1987). Nevertheless, the perspective for setting further improvement in the reliability of RTH-II assay could be seen in view of several observations in our study: e.g.: a.) the negative correlation of the weight of RTH filter with the weight retention index (wRI) and with the platelet retention index, b.) the occasional disruption of platelet after the passage of the RTH filter, and c.) the delicacy of mixing the activated platelet in blood after the passage of the filter that represents the main drawback of RTH-II application in clinical practice since it requires experienced personnel. This might be overcome e.g., by developing more standardized filter system.

In regard of the absence of simple and standardized assay for screening platelet function reliably [Breddine 2003, Tsiara 2003, Tan 2005, and Reininger 2006], the competency of RTH-II assay is evident as a simple and rapid assay for screening PLT function reliably and cost effectively. The essential advantages of RTH-II assay urge for further serious investigations addressing its clinical significance including mainly the response of platelet retention index to pathological variation and the associated implications on platelet size distribution. The significance of the retention indices of leucocytes and erythrocytes in the interpretation and the quality assurance of RTH-II measurements is established, but their clinical significance requires further basic researches. Thus, RTH-II assay can be used as a useful test in researches of platelet function as well as in clinical practice as an ideal adjunctive to refine the tools for the assessment of platelet response and activity in physiology, pathophysiology, and drug monitoring. Several studies on RTH referred to some beneficial uses for screening platelet function. But the unreliability and variability of RTH assay disrupt the reproducibility of data. The recent introduction of RTH-II by our institute as a standardized, reliable and simple assay implicating reliable reference range and evidence-based specificity delivers the right platform for further clinical studies. As first example for this is the recent study of Klinkhardt [2006] on using RTH-II for monitoring “antiplatelet” therapy. The study concluded that RTH-II may be “a simple and easy for monitoring effects on P2Y₁₂-inhibitors on platelet degranulation”. The reliability and the convenience of RTH-II promise major improvement in the medical care delivered for the satisfaction of the patients in the clinical practice as well as in the point of care systems.

1-2. Zusammenfassung

Obwohl die Aktivierung der Thrombozyten („PLT“) eine entscheidende Rolle spielt in der Physiologie und Pathophysiologie der Thrombose und Hämostase, sind Thrombozytenfunktionsteste nicht gut standardisiert. So fehlen einfache und zuverlässige Screening-Tests [Breddine 2003, Tsiara 2003, and Reiningger 2006]. Der Retentionstest Homburg (RTH) wurde entwickelt im Institut für Klinische Hämostaseologie und Transfusionsmedizin des Universitätsklinikums Homburg im Jahr 1993 als ein Versuch die Thrombozytenfunktion orientierend, auf einfache und kosteneffektive Weise sowie zuverlässig zu überprüfen. Der Test basierte initial auf der Retention von Thrombozyten auf standardisierter körperfremder „textured“ Oberfläche (Filter hergestellt aus Polyurethan fixiert in Eppendorf Hütchen: „RTH-Hütchen“) unter definierten Zentrifugationsbedingungen und Verwendung von citrat-antikoaguliertem Plättchen-reichem Plasma. Der Prozentsatz der sich aus der Differenz der Zellzahl der retinierten Thrombozyten zur Plättchenzahl vor Passage des Filtersystems ergibt ist definiert als Thrombozyten-Retentionsindex („PLT-RI“). Hohe Werte sollten als Hinweis für Thrombozyten „Klebrigkeit“ interpretiert werden wohingegen niedrige Werte auf einen Thrombozyten-Funktionsverlust Hinweise geben sollten.

Seit den anfänglichen Entwicklungsschritten zum RTH Test, wurden variable Vorgehensweisen/Verfahren angewandt um den Test durchzuführen. Zu keinem Zeitpunkt konnte eine Standardisierung herbeigeführt werden [Breddine 2003, Reiningger 2006]. Der RTH Test lieferte eine inakzeptable Reproduzierbarkeit und schwerwiegende Probleme bei der Reliabilität. Die Genauigkeit des Testverfahrens wurde zu keinem Zeitpunkt bei Gesunden evaluiert und die Spezifität des Testsystems für Thrombozyten konnte nicht adäquat bewiesen werden, was verständlicherweise Interpretationsschwierigkeiten nach sich zog. Einige Untersuchungen konzentrierten sich auf die Erstellung von Referenzwerten in citrat-antikoaguliertem Plättchenreichem Plasma für Thrombozyten-Retentionsindex; doch waren die Ergebnisse unzuverlässig. So waren auch die Daten, die aus einem Multizenter-Projekt“ gewonnen wurden letztlich inakzeptabel [König 2002]. Die schwerwiegenden Mängel/Probleme beim RTH Test werfen Schatten auf die in früheren Zeiten generierten Daten, respektive durchgeführten Studien, die einer möglichen Einführung in die Praxis entgegenstanden. Zusätzlich stellten die integrierten komplexen Vorgehensweisen, wie z.B. die präanalytische Aufbereitung des Blutes und Präparationen zusätzlicher Reagenzien die Signifikanz der Analyse als simpler und kosteneffektiver Test in Frage. Einige Untersuchungen wurden durchgeführt zur Überprüfung der Einflussnahme von Thrombozyten-Agonisten/ Antagonisten auf den Thrombozyten-Retentionsindex, die im Nachhinein als sehr hypothetisch angesehen werden müssen. Wenige Studien unterstrichen die Athrombogenität des Filtersystems, obwohl keine hinreichende Evidenz hergestellt werden konnte und auch unsere Erkenntnisse hinweisend waren, dass das Filtersystem nicht als völlig athrombogen angesehen werden darf. Trotzdem sollte das Testverfahren aufgrund der anfänglich deklarierten Athrombogenität des Filter-systems und auch relativen Eignung für das Routinelabor – im Vergleich mit anderen Retentionstests – als viel versprechend angesehen werden dürfen. Ziele unserer Untersuchungen waren daher: a.) eine radikale Validierung und Verbesserung des RTH-Verfahrens, b.) Entwicklung und Standardisierung eines einfachen und zuverlässigen Testes, c.) Gewinnung einer Evidenz-basierten Einsicht in die Spezifität des Verfahrens als Thrombozyten-Funktionstest, und d.) Definition verlässlicher Referenzwerte.

Die Auswertung und Entwicklung des Verfahrens zum RTH führten zu zahlreichen Modifikationen und fundamentalen Verbesserungen bzgl. Reliabilität, Eignung und Signifikanz des Testsystems. Diese implizierten die Definition eines neuen, standardisierten Tests („RTH-II“) unter Verwendung von citratantikoaguliertem Vollblut. Mittels unterschiedlicher Sysmex-Zellzählssysteme und unter morphologischer Kontrolle wurden stufenweise Untersuchungen zur methodischen Verbesserung, Reliabilität, und Standardisierbarkeit des Homburger Retentionstestes vorgenommen. Diese führten zu elementaren Entwicklungen von RTH, die in RTH-II Berücksichtigung fanden:

(1) Citratantikoaguliertes Vollblut wurde als Ausgangsmaterial verwendet und für die klinische Routine empfohlen anstelle von citrat-antikoaguliertem Plättchenreichem Plasma (RTH). Dies wurde für essentiell erachtet um die Variabilität der Thrombozyten-Gradienten im Plasma – nach Präparation mittels Zentrifugation – auszuschließen. Zusätzlich reflektiert Vollblut eher einen physiologischen Zustand im Vergleich zu Plasma und reduziert möglicher Fehler die auf die Thrombozyten „Überaktivierung“ (induziert durch z.B. ungeeignete Durchmischungsvorgänge) zurückführbar sind. (2) Die unterschiedlichen Mengen an verwendetem Testmaterial (pro Bestimmung), die im RTH Anwendung fanden wurden standardisiert auf 500 µL im RTH-II. (3) Das Hinzufügen von Kalzium-Hirudin, das im RTH Als so genanntes „reaktivierendes Reagenz“ Verwendung fand, wurde als nicht gerechtfertigt erachtet. Auch war die Thrombozyten-Reaktion hierauf nicht standardisierbar, implausibel (Konversion der „Positiv-Werte“ zu „Negativ-Werten“) variierte intraindividuell. (4) Auf eine Inkubationszeit der verwendeten Materialien im RTH Hütchen (3-10 Min) wurde verzichtet um eine mögliche Thrombozyten-Aktivierung durch lange Kontaktzeiten mit dem Filtersystem und mögliche hierdurch zusätzliche getriggerte inter-individuelle Differenzen zu vermeiden. (5) Die Messungen der Plättchen-Zahl vor der Filterpassage wurden im Sammelröhrchen und nicht im RTH-Hütchen nach dem Inkubationsschritt vorgenommen. Die den RTH-Filter passierenden Menge an Testmaterial wurde standardisiert (500 µL) auch im Hinblick auf die Variabilität der von den unterschiedlichen Zellzähl-systemen aspirierten Proben (100-400 µL). Auch war hinreichen Material für Doppelbestimmungen hiermit gegeben. Die Verwendung eines Rotators sicherte weitestmögliche Homogenität des Untersuchungsmaterials und vermied Fehlbestimmungen, die sich aus einer variablen Sedimentation zellulärer Blutbestandteile in RTH-Hütchen hätten ergeben können. (6) Die extrem variablen Zentrifugationsbedingungen, die im RTH Anwendung fanden (79-160 g/ 5-16 min), wurden standardisiert auf 110 g/ 5 Min was eine akzeptable Thrombozyten Verteilung im Filtersystem sowie zuverlässige Messungen erlaubt. Darüber hinaus wurde in einigen zurückliegenden Untersuchungen mit dem RTH die Zentrifugationsmodalität nicht hinreichend spezifiziert. (7) Im RTH wurde die Plättchen-Zahl nach Filterpassage nach einer Durchmischung (3 Min) mittels „Vortex“ bestimmt. Eine standardisierter Vortex-Methode (<3 Sek.) fand Anwendung im RTH-II im Hinblick auf eine Minimierung von induzierten Thrombozyten-Aggregaten/Agglutinen/Schädigungen, die die Reliabilität der Messungen in Frage stellen würden. (8) Die Plättchen-Zählung nach der Filterpassage sollte binnen 2 Minuten erfolgen um die Genauigkeit mittels weitestmöglicher Homogenität zu gewährleisten und Fehlbestimmungen zu minimieren die auf „Thrombozyten Partikel-Phänomene“ zurückzuführen sind. (9) Die Messungen sollten im Hinblick auf eine verbesserte Reliabilität jeweils als Doppelbestimmung durchgeführt werden. Das Ausmaß der Variation zwischen I. und II. Bestimmung ist auch ein Indikator für evtl. Fehler herangezogen werden (provozierbar z.B. durch inadäquate oder ungeeignete Durchmischung des Materials). Für jede Einzel-

messung der Doppelbestimmungen sowie der Serienmessungen sollten neue Pipettenspitzen verwendet werden auch im Hinblick auf eine mögliche durch die Mehrfach-verwendung derselben Spitzen provozierbare Impräzision (wurde in den RTH Studien ignoriert). (10) Die Formel, die zur Berechnung des große Thrombozyten Retentionsindex („PLCR-RI“) Verwendung fand wurde korrigiert. Anstelle von Prozentzahlen der großen Thrombozyten (%) wurden die entsprechenden absoluten Zahlen angewandt. Die so berechneten Referenzwerte und ihre Korrelation mit den anderen Retentionsindices waren signifikant der alten Berechnungsgrundlage überlegen (Verwendung der „alten Formel“. Diese Fehler stellen auch die Reliabilität vorausgegangener Studien in Frage. (11) Zusätzliche, neue Retentionswerte (RIs) für Leukozyten (WBC-RI), Erythrozyten (RBC-RI) und Thrombozyten-Größe-Parameter („Platelet Derived Width“: „PDW-RI“, und „Mean Platelet Volume“: „MPV-RI“) wurden erstmalig in einem Retentionstest-System definiert. Die Definition dieser Retentionsindices und große Thrombozyten Retentionsindex mit ihrer Korrelationen mit Thrombozyten-Retentionsindex scheint wesentlich für die: a.) Erkennungsmöglichkeit von Fehlern, und Ursachen für eine Variabilität im RTH. b.) Definition/ Validierung notwendiger Modifikationen. c.) Auch wird das Verständnis in die Interpretation von Genauigkeit, Spezifität und Präzision des Testsystems durch die (Mit-) Berücksichtigung dieser Variablen vertieft. d.) Hierauf aufbauend wurde erstmalig ein Schlüsselprotokoll zur Interpretation und Qualitätssicherung der Messungen im RTH-II erstellt. (12) Retentions-Werte für das Gewicht des retinierten Materials („wRI“: Gewicht-Retentionsindex) wurden erstmalig erstellt. Dies schien uns sowohl bedeutsam für die Standardisierung und Validierung der Zentrifugationsbedingungen wie auch die Qualitätssicherung der Integrität und Reliabilität des RTH-Filter Systems. (13) Thrombozyten-Aktivierungs-Mechanismen („Thrombozyten Partikel-Phänomenen“) wurden als Hauptquelle von Messfehlern identifiziert. Scherstress-bedingte Aktivierung von Thrombozyten, wie sie bei der Filterpassage stattfindet wird potenziert durch ungeeignete Vortex-Modalität im RTH und führt zu Aggregaten/Agglutinaten („Thrombozyten Partikel“) und Zelltrümmern. Diese Partikel werden von Zellzähl-Geräten falsch interpretiert als große Thrombozyten, Erythrozyten, und/oder Leukozyten. Die Folgen hiervon sind: (a) ein proportional falscher Abfall der Retentionswerte von Blutzellen, (b) eine falsche „Shift“ nach links (Reduktion) bzgl. der Retentionswerte der Thrombozyten-Größe, (c) ein falscher relevanter Anstieg der Thrombozyten-Retentionsindex Werte. Gelegentlich kann eine Hyperaktivität von Thrombozyten bei der Zellzahlbestimmung vor Filterpassage durch ungeeignetes Handling der Blutprobe zu falschen/ inversen Ergebnissen führen. Eine Thrombozyten-Überaktivierung führt auch zu so genannter Rosetten-bildung, assoziiert mit einem Plättchen-Zahl-Abfall. Die Identifikation von Thrombozyten Partikel-Phänomenen und Erfassung weiterer Retentionswerte (RIs) war entscheidend für die Definition von RTH Fehlern und notwendige Modifikationen. Die Korrelation zwischen Thrombozyten-Retentionsindex und anderen Retentionsindices (mit Ausnahme von große Thrombozyten Retentionsindex) konvertiert von positiven Modells zu negativen durch das Auftreten von Thrombozyten Partikel nach dem Filterpassage. Obwohl RTH-II weitestgehend das Auftreten dieser Phänomene reduziert, so eine Elimination nicht vollständig garantiert werden. Das Verständnis der mit den o.g. Phänomenen verknüpften Implikationen ist deshalb notwendig für richtige Interpretation und Qualitätssicherung der RTH-II Messungen. (14) Die Raumtemperatur wurde als „Standard“ für die Durchführung des RTH-II definiert. (15) Die Zeit für die Durchführung des Testes wurde standardisiert auf <120 Minuten (Zeitfenster nach der Blutentnahme), was einer verbesserten Reliabilität zugute kommt. Ein Überschrei-

ten der Zeitlimits (bis 180 Min) – wie im RTH praktiziert – ist assoziierbar mit deutlich reduzierten Retentionswerten (PLT-RI).

Unter Zuhilfenahme morphologischer Kontrollen (vor/nach Filterpassage) wurden Untersuchungen zur Validierung von Präzision, Spezifität und Genauigkeit durchgeführt: (A) Evaluierung der Präzision in Serie in citratantikoaguliertem Vollblut (13 Serien; n=10) und Eightcheck Control (von Sysmex: 3 Serien) sowie von Tag zu Tag in Eightcheck Control. Phasen-contrastmikroskopische Untersuchungen wurden als Kontrolle hierzu durchgeführt. (B) Ausschluss von filterbezogenen Effekten. (C) Durchführung des RTH-II mittels isolierter (gel-filtrierter) Plättchen im Vergleich mit Citratblut. Thrombozyten-Ausbreitung wurde als Kontrolle benutzt. (D) Validierung eines neu entwickelten Standards von gewaschenen und ungewaschenen fixierten Thrombozyten unter Kontrolle von Phasencontrast-Mikroskopie. (E) Morphologische Charakterisierung der Interaktion von Thrombozyten und Blutzellen mit dem Filtersystem. Ultradünne Segmente des Filters wurden nach der Blutpassage fixiert und anschließend mittels Phasencontrast-mikroskopie und Screening- und Transmissionselektronenmikroskopie untersucht. (F) Einführung von Referenzwerten für die Retentionsindices mittels citratantikoaguliertem Vollblut und Eightcheck Control. Die Genauigkeit wurde kontrolliert mittels Phasencontrast-mikroskopie. (G) Quantitäts-bestimmungen/ Validierung des Filtersystems neben der Bestimmung der Menge des retinierten Materials. Die Auswertung wurde vorgenommen mit citratantikoaguliertem Vollblut und Eightcheck Control. Beide fanden Anwendung in den Präzision- und Referenzwert- Studien.

Diese Untersuchungen bewiesen die Genauigkeit und akzeptable Präzision des RTH-II Testes und lieferten eine Evidenz basierter Interpretation der Spezifität. Die Genauigkeit der Thrombozyten-Retention (PLT-RI) wurde erstmalig bei Gesunden untersucht. Die extrem falsch erhöhten Werte (ca. 2.2 fache) und ihr zeitabhängiger Anstieg (bis zu 61%) wurde korrigiert. Die inakzeptable hohe Variation der Messungen in Serie für Thrombozyten-Retentionsindex wurde reduziert um minimal 80% im RTH-II, welcher präzise Werte lieferte („Standard Abweichung“: SD= 2.9). Die Zuverlässigkeit der RTH-II Messungen ist abhängig von präzisen Plättchen-Zahlen und einer biologischen Variabilität wohingegen die Fehler im RTH (z.B. „Thrombozyten Partikel“) die Variabilität steigern und zu inakzeptablen, falschen Resultaten führen. Die Verwendung von modernen Zellzählgeräten führt zu einer Verbesserung der Präzision im Ggs. zur Genauigkeit. Die Präzision von Tag zu Tag, die erstmalig über einen längeren Zeitraum (10 Tage) mittels Eightcheck Control evaluiert wurde ergab ebenfalls eine akzeptable niedrige Thrombozyten-Retention Variation (SD <2.9; Krichcek 2000).

Im Jahr 2004 konnte RTH-II etabliert werden. Erstmalig wurden zuverlässige Referenzwerte für die Thrombozyten-Retentionsindex in Citratblut mit akzeptabler Biovariabilität definiert (Mittel= 25.2%; SD=6.25). Der Einfluss verschiedener Messarten auf die Genauigkeit des Ergebnisses wurde untersucht. Neben Thrombozyten-Retentionsindex konnte auch die Präzision und Genauigkeit für die anderen Retentionsindices wesentlich korrigiert werden. Erstmalig wurden auch zuverlässige Referenzwerte für die anderen Retentionsindices definiert. Die Genauigkeit von Retentionsindices der Thrombozyten, Leukozyten und Erythrozyten wurden bewiesen und charakterisiert mittels Phasencontrast- und Elektronen-Mikroskopie. Weiterhin konnte bewiesen werden, dass:

A.) Der RTH-Filter relativ athrombogen ist im Ggs. zu den anderen Filter-Systemen, die bei anderen Retentionstest-Systemen bisher zum Einsatz kamen (z.B. Hellem-Test 1970).

B.) Unter standardisierten Blutfluss-Bedingungen im RTH-II, die Retentionswerte von Leukozyten (WBC-RI=4.2%) und Erythrozyten (RBC-RI=3.6%) vorzugsweise abhängig sind von deren Größe, welche im Vergleich zur Filter-Porengröße höher ist. Hingegen werden Thrombozyten, die kleiner sind als die Poren der Filter, spezifisch durch das Filtersystem retiniert. Ihre Retentionswerte liegen deutlich höher, als die der Blutzellen. Thrombozyten-Retentionsindex reflektiert direkt die Antwort der Thrombozyten auf die Aktivierung durch Scherstress – während der Filterpassage. Die Thrombozyten-Aktivierung findet ihren Ausdruck im Formwechsel mit extremer Adhärenz und Ausbreitung auf dem Filter. Darüber hinaus findet eine bemerkenswerte Degranulation mit exzessiver Bildung von Aggregaten statt (speziell im oberen Teil des Filtersystems). Die Aggregate verbinden sich mit gegenüberliegender Polyurethan-Oberfläche (zwischengeschalteten Mikroporen) besonders im oberen Teil des Filters. Darüber hinaus kann es gelegentlich auch zur Bildung freier Thrombozyten-Agregate in den Filter-Lummen kommen. Dieser interaktive Prozess ist vergleichbar mit der an Gefäßwandläsionen (Endothel bzw. Subendothel) stattfindenden Interaktion der zirkulierenden Thrombozyten. Der RTH-II Test ist daher ein Testsystem zu einer zuverlässigen, orientierenden Überprüfung vielfältiger Thrombozyten-Funktionen. Die oberen und unteren Grenzwerte gestatten nun die Messung von Hyper- und Hypo-Aktivität der Thrombozyten was im krassen Gegensatz steht zu den Ergebnissen von Krschek 2000. Unterschiedliche Beobachtungen sprechen für die klinische Signifikanz des RTH-II Testes, z.B. lässt die bemerkenswert negative Korrelation des Thrombozyten-Retentionsindex mit der Plättchen-Zahl vermuten, dass die Thrombozyten Aktivität ansteigt im Verhältnis zur Abnahme ihrer Zahl bei gesunden Individuen.

C.) Die Spezifität und die Zuverlässigkeit des RTH-II Testes wurde weiter etabliert durch die Möglichkeit die gehemmte Funktion der fixierten Thrombozyten in Eightcheck Control (PLT-RI= 2.6%; SD=2.1) verglichen mit der Biovariabilität der Thrombozyten-Aktivität in citratantikoaguliertem Vollblut zuverlässig und mit bewiesener Kompatibilität zu morphologischen Kontrollen zu bestimmen. Thrombozyten in Eightcheck Control während der Filterpassage werden nicht aktiviert und zeigen keine richtige Anhaftung am Filter. Lediglich selektive Kontakte von Thrombozyten Agglutinaten mit dem Filtersystem finden statt. Hingegen beobachtet man in RTH Studien unerklärbar hohe Thrombozyten-Retentionsindex Werte in der Kontrolle fixierte Plättchen (>21%; Krschek 2005). Die Vergleichbarkeit zwischen den Retentionswerten der Blutzellen in citratantikoaguliertem Vollblut und in dem fixierten Eightcheck Control Mediums spricht dafür, dass die Retention dieser Zellen in citratantikoaguliertem Vollblut hauptsächlich im Verhältnis steht zu ihrer Größe.

D.) Die Bestimmungen von den Retentionsindices der Thrombozyten-Größe-Parameter (insbesondere von große Thrombozyten „PLCR-RI“ führten zu weiterer Signifikanz des RTH-II und verweisen auf die zusätzliche Bedeutung der Thrombozyten-Größe und Variabilität. Dies wurde bewiesen durch z.B. eine geringere Thrombozyten-Aktivierung im Falle großer Thrombozyten im Vergleich zu Normalkonfigurierten bei Gesunden wie auch deren geringere Beeinflussbarkeit durch Aktivierung.

E.) Die Bestimmungen von Retentionsindices der Leukozyten (WBC-RI) und Erythrozyten (RBC-RI) könnten ebenfalls die Signifikanz von RTH II auf andere Blutzellen ausweiten, da sie einige spezifische Aktivierungsprozesse und Interaktionen mit dem Filtersystem anzeigen. Dieser Aspekt könnte im Hinblick auf die Möglichkeit einer Überprüfung von Erythrozyten-Rigidität bzw. -Deformabilität bedeutsam werden.

Die Zuverlässigkeit des RTH-II in der Forschung wurde etabliert durch die Anwendbarkeit auf unterschiedlichen Materialien (citratantikoaguliertem Vollblut und Plättchenreichem Plasma, isolierter „gel-filtrierter“ Plättchen, Eightcheck Control, gewaschenes und ungewaschenes fixiertes citratanti-koaguliertem Plättchenreichem Plasma), die in die Validierung des RTH-II verwendet wurden, mit bewiesener Kompatibilität zur multiplen morphologischen Kontrollen. So beispielsweise, gelang es mittels RTH-II Analyse den Einfluss partiellen Entzuges des normalen Thrombozyten-Medium zu zeigen bezüglich ihrer Prädisposition zur Thrombozyten-Hyper- Aktivität und -Klebrigkeit (z.B. Bildung von Aggregaten/Agglutinaten), welche im fixierten Medium (Eightcheck Control) wesentlich weniger kompensiert wird, als in citratantikoaguliertem Vollblut. Dies wurde bewiesen durch den zweifachen Anstieg des Thrombozyten-Retentionsindex Wertes bei isolierter Plättchen versus citratanti-koaguliertem Vollblut (60.6% vs. 32.1%) und dem wesentlich höheren Anstieg des Thrombozyten-Retentionsindex (>5 fache) bei den Standards von fixierten, gewaschenen versus ungewaschenen Thrombozyten (5.7% vs. 30.5%). Der RTH-II dient der Identifizierung und Charakterisierung neuer Thrombozyten-Funktions-Phänomene (z.B. „Thrombozyten Partikel“, Thrombozyten-Antwort auf Kalzium-Hirudin), und liefert spezifische Einsicht in die Entwicklung unseres Standards für gewaschene und ungewaschene Thrombozyten.

Die Verbesserung der Reliabilität im RTH-II – verglichen mit RTH – war auch assoziiert mit einer Verbesserung der „Excellency“ und Eignung des Testes. Keine Vorbereitung von Plasma, oder das Hinzufügen von Reagenzien ist notwendig. Die erforderliche Blutmenge wurde reduziert von >1.5 mL auf 0.5 mL. Die Versuchszeit (insges.) konnte deutlich verkürzt werden – bis zu ca. 90% (35-53 Min vs. 5.5-6 Min). Der Versuch wurde signifikant vereinfacht und ist nun weniger laborintensiv. Er kann einfach durchgeführt werden erfordert keinerlei „technische Voraussetzungen“ (nur RTH-Hütchen mit Zentrifuge), und ist mehr kosteneffektiv. Außerdem wurde durch das erstmalig etablierte Qualitäts-sicherungsprotokoll nicht nur die Verlässlichkeit der RTH-II Messungen und Interpretation verbessert, sondern damit auch die Voraussetzungen für die Implementierung im klinischen Routineeinsatz geschaffen. Unser Vorschlag den RTH-II zu automatisieren, unter Zuhilfenahme konventioneller automatischer Zellzählensysteme, sollte eine Online-Dokumentation, Interpretation und Qualitätssicherung ermöglichen. Dies verstärkt die Eignung und Kosteneffektivität wie auch die Zuverlässigkeit und Qualitätssicherung von RTH-II Messungen.

Obwohl sich bisher angewandte Adhäsions- und Retentionstests als relevant erwiesen haben, konnten sie in der klinischen Praxis nicht angewendet werden, weil sie – im Ggs. zum RTH-II - ungenügend standardisiert waren und zusätzliche technische Erfahrung und Einrichtungen benötigen (z.B. Hellem-II 1970, O'Brien 1987). Trotz der Verbesserungen der Reliabilität des RTH II sind weitere Verbesserungen im Hinblick auf folgende Beobachtungen möglich: a.) die negative Korrelation des Filtergewichts mit den Gewicht-Retentionsindex (wRI) und Thrombozyten-Retentionsindex, b.) die gelegentliche Zerstörung von Thrombozyten nach der Filterpassage, und c.) die Problematik der Durchmischung der durch Filterpassage voraktivierten Thrombozyten mittels geeigneter Vortex-Methode, was ein erfahrenes Laborpersonal benötigt. Solche Verbesserungen mögen nicht zuletzt auch durch ein verbessertes, mehr standardisiertes Filtersystem ermöglicht werden.

Im Hinblick auf das Fehlen eines einfachen, standardisierten Screening-Testes zur Überprüfung der Thrombozyten-Funktion [Breddine 2003, Tsiara 2003, Tan 2005, and Reiningger 2006], ist mit der Etablierung von RTH-II die Möglichkeit eröffnet auf einfache, zuverlässige Art und Weise, kosteneffektiv

und schnell eine Screeninguntersuchung durchzuführen. Die wesentlichen Vorteile von RTH-II ermutigen zu weiteren Untersuchungen mit klinischer Fragestellung wie z.B. der Evaluation von Thrombozyten-Retentionsindex unter pathologischen Bedingungen und die damit verbundene Bedeutung der Thrombozyten-Größenverteilung. Die Bedeutung der Retentionsindices der Leukozyten und Erythrozyten ist wesentlich für die Beurteilung und die Qualitätssicherung der Testergebnisse, ihre klinische Bedeutung erfordert jedoch auch prospektiv eine Konsolidierung. Darüber hinaus kann der RTH-II Test in der Forschung und Klinik hilfreich sein bei der Beurteilung der Thrombozyten-Funktion nicht nur unter physiologischen Bedingungen, sondern auch im Hinblick auf die Differenzierung pathologischer Gegebenheiten, bzw. im Zusammenhang mit Medikamenten-Interaktionen. Einige Studien mit RTH spekulierten mit positiven Effekten wobei die hohe Variabilität der Testergebnisse und auch die fehlende Zuverlässigkeit von RTH die Reproduzierbarkeit der Daten zunichte machten. Die Einführung von RTH-II durch unser Institut liefert eine adäquate Plattform für weitere prospektive Untersuchungen. Als erstes Beispiel hierfür kann die Untersuchung von Klinkhardt (2006) aufgeführt werden, die die Erfassung von Patienten mit Thrombozytenfunktionsdefekten mittels RTH-II ermöglicht. So gesehen könnte mittels RTH-II auch die Erfassung von Thrombozyten-Funktions-Einschränkungen ermöglicht werden. Reliabilität und Eignung des standardisierten RTH-II Testes geben Anlass zu Verbesserungen in der medizinischen Betreuung von Patienten in der klinischen Routine, wie auch als „Point-of-care“ System.

II INTRODUCTION

2-1. History of platelet adhesion, aggregation and retention

As early as 1882, Bizzozero described the platelets (PLT) as the “third morphological blood component” and their role in hemostasis and thrombosis. He could have microscopically insight into the PLT and demonstrated pictures of their thrombotic complications. In 1960, Hellem could demonstrate the adhesion of PLT in vitro by pushing non anticoagulated whole blood through a definite foreign surface of polyvinylchloride column filled with glass beads under well defined flow conditions (flow rate: 5.7 mL/min; column width 3 mm; weight of glass beads 1.3 g). It was hypothesized that PLT adhesion may be interpreted as the first step of thrombus formation induced by the cellular expression of ADP by PLT which get in contact with leisured endothelial vessel walls [Käser-Glanzmann 1962 and Breddin 1968]. This phenomenon was also related to shear stress induced activation of PLT which is more significant in arterial than in venous thrombotic diseases. In contrast to the dynamic approach of Hellem, the PLT adherence on siliconized Bürcker counting chambers was measured in static approach [Breddin 1964]. The modified method of Hellem [Hellem II, 1970] using citrated whole blood (cWB) was, however, easier to practice and less time intensive. Values above 38% were defined as "hyperadhesion of PLT".

In 1962, ADP induced aggregation of PLT could be demonstrated in citrated platelet rich plasma (cPRP) [Born 1962]. This kind of PLT aggregation test (PAT I; Breddin 1965), defining different grades of PLT activation microscopically, was modified later by Breddin in 1976 (PAT III) using cPRP for the performance of a photometrical assay [Breddin 1977]. Spontaneous PLT aggregation (PAT III) was recorded on a universal aggregometer (Braun, Melsungen) using an Eppendorf photometer "1001" by Netheler & Hinz, Hamburg. Angle α (maximal aggregation velocity) >40 degrees was interpreted as “hyperaggregation of PLT” [Breddin 1977, 1986]. Platelet aggregation due to Ca^{++} dependent processes was also related to PLT activation by preanalytical circumstances (e.g., time from withdrawal of blood to the performance of the assay) [Breddin 1974, Bauer 1980]. At least this PLT activation phenomenon did not allow differing between PLT adhesion and PLT aggregation. A vicious circle was hypothesized by which PLT that get into contact with foreign surfaces are activated (or even damaged), express bioactive amines, stimulating further PLT and leading to aggregation and at least thrombus formation [Mc Pherson 1987]. The role of potentially interacting blood components as erythrocytes, leucocytes was postulated [Goldsmith 1971 and 1975], or at least rather unknown [Joist 1980].

In-vitro, PLT aggregation has been studied by monitoring the changes in the optical density of cPRP for PAT III according to Breddin 1976 and 1999. This assay has been routinely used to evaluate hyperactivation of PLT and can be attributed either to arterial or venous vascular diseases [Heidrich 1980, Breddin 1999 (HAPARG study), Schenk 2002, Feinbloom 2005, Phillips 2005, Ginzburg 2006]. The PLT aggregation was also attributed to inflammatory and atherosclerotic processes [Breddin 1974, LaRosa 1998, Ferroni 2003, and Vorchheimer 2006]. An increased PLT aggregation may be interpreted as a thrombophilic risk variable as demonstrated for either patients with arterial diseases [HAPARG-study] as well as for patients with venous thrombotic diseases. Beyond it, PAT III has been proved to be of predictive value for new vascular occlusions in diabetics [Breddin 1985, 1986 and 1986]. Since the blood flow characteristics which might be more or less laminar in arterial vessels are quite different from venous

ones, the PLT activation process have to be interpreted differently. Due to higher shear stress in stenotic arterial vessels or even due to vasospasms the PLT are stimulated leading to aggregation which can be demonstrated in vitro without the additional apply of agonists e.g. ADP, or collagen [Konstantopoulos 1995]. After the development of PAT I [Breddin 1965], several approaches were developed to measure PLT aggregation other than PAT III e.g., the Wu & Hoak test [Wu 1974, Velaskar 1982] using flow cytometric analysis which was of limited value since it was not standardized [Schmitz 1998]. Platelet induced thrombin generation is of interest to measure drug effects [Schenk, 1996, 1999 and 2001, Van 2005].

A series of "filter tests", modified from the dynamic approach of Hellem 1960 using mainly cPRP, were developed to measure the adhesion (retention) of PLT after the passage of unphysiological filter systems composed of glass beads, glass wool, plastic surfaces, or extra cellular matrix coated surfaces either with or without shear forces e.g., Salzman [1963], Hellem-II [1970], O'Brien [1987]. The difference in PLT count before and after the passage of the filter system reflected the retention of PLT which was attributed primarily to PLT adhesion. The microscopic investigation of the filter used by Salzman [1963], however, showed extensive PLT aggregates on the glass beads. It was concluded that the PLT retention by such filters is a measure of PLT aggregation and adhesion [Breddin 2003, Kaneko 2003]. The latest filter test was the retention test of Homburg (RTH) developed in Homburg 1993 [Krischek 2000]. None of these quantitative or semi-quantitative retention tests was applied in the routine clinical laboratory while they could not be standardized [Scharer 1985, Breddin 2003, Tsiara 2003, Tan 2005, and Reiningger 2006].

2-2. Platelets and their crucial role in hemostasis and thrombosis

The data accumulated by the different PLT function assays and clinical studies proved that PLT are the most important group of "blood cells" in the context of hemostasis [Margaret 2005, Furie 2006]. These relatively small discoid cells (0.5-3.0 μ m) are synthesized in bone marrow and represent a highly specialized and matured differentiated form the megakaryocytes [Alan 1994, Frederick 2001]. The PLT cytoskeleton is rich in partly polymerized actin filaments (0.55 mmol/L) which form microtubules close to the plasma membrane. Parts of the actin filament connect directly to the PLT membrane protein. A second structure is formed by the planar spectrin-based membrane skeleton. The PLT cytoskeleton contributes significantly to the shape changes which are observed after PLT activation and helps the PLT to spread over an injured area. Metabolically, the PLT are active and rich of granules that contain variant ingredients (e.g., calcium ions, PLT activators, vasoconstrictor, and proteins involved in coagulation or its regulation) but have lost their protein biosynthesis capability. Energy is generated by glucose degradation and oxidative phosphorylation and stored in the form of ATP (adenosine tri-phosphate). In resting state PLT are not thrombogenic, but after stimulation they become a potent and active player in hemostasis. Activation of PLT plays a crucial role in the pathology and the pathophysiology of thrombosis and hemostasis. The series of events triggered after PLT activation depends on the applied stimulus. Resting PLT transform rapidly from a discoid to spherical shape upon activation via the contractile system of their cytoskeleton. Weak stimulation leads to shape change and mild activation result in PLT

spreading, adherence and aggregation. More intense stimulation trigger the liberation of arachidonate and secretion of the contents of PLT granules; part of them are translocated to the plasma membrane and serve as cell adhesion receptor e.g., P-selectin. Compared with the plasmatic coagulation factors, the importance of PLT has been increasing in the last decade especially in the primary hemostasis [Jurk 1995]. For immediate blood coagulation after an injury, the initial reactions are mediated by an interaction of the vessel wall and PLT. The timing of primary hemostasis in normal individuals is as follows: vasoconstriction (immediately), PLT adhesion (seconds), PLT aggregations (minutes) [White 1994, Morgenstern 1997, Miller 2001, Breddin 2003, Levi 2005, Jurk 2005 and Furie 2006]. In addition PLT are also tightly linked to the immune system; activated PLT secrete several cytokines, growth factors and many other proteins or low molecular substances [Wagner 2003, Levi 2005, and Weber 2005]. Also other blood cells mediate numerous function in hemostasis from the very simple effect of thickening or dilution of the blood and pressing the PLT against the vessel wall by the erythrocytes to the release of multiple cytokines and other hormones from the different leukocytes and their exposure to the procoagulant surfaces under certain conditions [Jurk 2005, Afshar 2006 and Furie 2006].

The interaction of PLT in the sequence of events leading to coagulation activation could be summarized as follows: (1) PLT activated by contact with injured tissues secrete substances such as (ADP), serotonin and especially thromboxane which activate other PLT and thus increase the local PLT density and activation leading to the constriction of the vessel wall and consequently to reduced diffusion. (2) Activated PLT secrete also Ca^{2+} and several coagulation and fibrinolysis proteins increasing the local concentration of the reactant. (3) Simultaneously, activated PLT become more sticky (e.g., more binding of vWF, fibrinogen, collagen of the vessel wall). This leads to an increase of local concentration, reduced diffusion by formation of PLT plug and adherence to vessel wall. (4) Activated PLT transport negatively charged phospholipids to the surface which bind collagen factors leading to local increasing in the concentration. The kinetic conditions are improved and allow thrombin formation by plasmatic coagulation proteins. (5) The formation of thrombin results in: a.) triggering additional activation of PLT, b.) catalysis of fibrin crosslinking and inhibition of fibrinolysis, and c.) activation of endothelial cells which leads to the amplifications of all previous events. (6) The formation of an insoluble fibrin network by fibrin cross-linking reduces the blood diffusion, protect against inhibitors from blood and stabilize the PLT plug. (7) The activated leucocytes invade later the affected area, release cytokines with multiple functions, express tissue factor activity and expose procoagulant surface. (8) The fibrinolytic inhibitors prevent an early dissolution of the clot. Inhibitors of the activated coagulation factors prevent a systemic activation of coagulation throughout the vasculature. Thus the clot is stabilized during the healing of the injured tissue and thrombus formation is limited [Van 2001, Huntington 2005, Jurk 2005, Levi 2005, Home 2005, and Furie 2006].

2-3. Availability of simple platelet function assays in clinical practice

Despite the plenty of PLT function assays, there has been always a striking need for a simple, reliable and cost effective assay for assessing PLT function and activity in clinical practice [Breddin 2003, Reiningger 2006]. The old and world-wide used simple test "bleeding time" e.g., according to Ivy or Duke

is poorly standardized, temperature dependant, unreliable and lacks specificity and sensitivity. Actually this method is not standardizable. Simple approaches such as measuring the mean PLT volume (MPV) or PLT release products need further evaluation. The new automated procedure of platelet function analyzer (PFA-100) designed to detect vWF deficiency and PLT adhesion and aggregation defects is rather convenient but it provides limited measuring range in samples from patients with strongly defective primary hemostasis and it is dependant on normal count of platelets and erythrocytes. The Ultegra-RPFA is a promising method, but the claim of a correlation between the test results and the clinical outcomes needs further evaluation. The cone and plate analyzer (CPA; Diamed Impact) has the advantage of being a fully automated rapid system for the point of care testing. Extensive studies are needed to determine the usefulness of this test for evaluation of patient's diathesis. The methods measuring spontaneous or agonist-induced PLT aggregation deliver reliable results, but they require specialized laboratories, experienced personnel, and big amount of the blood (ca. 10 mL) and can not serve as a simple approach for screening PLT function cost effectively. Even these reliable methods are not consistently predictive for vascular risk. A major advance in the assessment of PLT activation was the introduction of flow cytometric assays for the receptors involved in adhesion and aggregation. Flow cytometric assays can detect early PLT activation but they are time consuming, need specialized and high cost equipment and reagents as well as experienced personnel. These drawbacks have been serious limitations for the wide application in clinical practice. None of the mentioned analyses can provide a practical index of platelet activity that can reliably detect or predict probable vascular risk. Actually none of the presently available platelet function tests is well standardized. So there is much space for the improvement of simple, reliable, and cost effective PLT function assay [Breddin 2003, Rand 2003, Tsiara 2003, Jurk 2005, Haubelt 2005, Levi 2005, Tan 2005, and Reininger 2006].

2-4. Objectives and methodology of the study

The Retention Test of Homburg (RTH) was established by the institute of clinical hemostaseology, Univ. of Saarland, in 1993 as an attempt to introduce a simple and cost effective assay for screening PLT function reliably. RTH test is based on the retention of platelets resulting from exposing them (cPRP was mainly used) to a standardized exogenic surface (the polypropylene filter of Eppendorf retention tube) under defined conditions of flow forced by centrifugation. The percentage of the difference between PLT count before and after the passage of the filter is defined as the retention index (RI) of PLT (PLT-RI). High PLT-RI should be a sign of PLT hyperactivity/stickiness, whilst low PLT-RI is supposed to indicate PLT hypoactivity/stickiness [Wenzel 2000]. Since then, variant procedures were used [Wieding 2001, Schenk 2002, Wieding 2004], and the assay has never been standardized [Breddin 2003]. Furthermore, the reproducibility of RTH assay has been unacceptable and opposing serious problems and the accuracy was never studied in healthy individuals. The evidences on the specificity of this assay for PLT were inadequate and presented no comprehensive interpretation. Few investigations addressed reference ranges for PLT-RI by using cPRP [Kricheck 2000], but none was reliable. The data obtained from a multicentre trial using RTH were at least unacceptable [König 2002] and the assay could never be introduced for the clinical practice [Reininger 2006]. In addition, the need for processing the blood to

prepare cPRP and for preparing the variant additives used in these procedures deteriorated the significance of this analysis as a simple and cost effective assay.

On the other hand, the RTH assay had major advantages on earlier filter tests e.g., [Salzman 1963, Hellem-II 1970 and O'Brien 1987] attributed to: a.) the "athrombogeneity" of its filter system compared with other materials (collagen, glass beads etc.), as described by the morphological investigations despite the evidences were not adequate [Schenk 2002, Koscielny 2002]. b.) the relative convenience for the routine clinical laboratory by using disposable retention tube. The competent principle of RTH and the promising results of the studies demonstrating proportional correlation between PLT-RI and specific PLT agonists and inhibitors [Wieding 2001 and 2002, Schenk 2001] urged the need for radical validation of RTH procedures, developing a reliable and simple standardized operation procedure (SOP), addressing evidence based interpretation of the specificity for screening PLT function, and defining a reliable reference range in healthy individuals. These objectives represented actually the aim of our study and could be acceptably achieved. In order to avoid bias by reporting with the earlier unreliable procedures of RTH, the newly developed reliable SOP of RTH incorporating our basic improvements and modifications was introduced as "RTH-II". The rationale for using a methodologically simple test was to screen PLT hyperactivation or defects in healthy subjects and in point of care systems. Beyond it, diagnosing individual PLT disorder is supposed to be detected by specific PLT function assays.

A stepwise approach was followed for the validation and the development of RTH procedures. Each step was evaluated and modified and new procedures or identifications were addressed and incorporated in RTH assay in order to: a.) primarily exclude or at least minimize the sources of variability and errors and assure the reliability and the specificity of the measurements, and b.) secondarily improve the convenience of the RTH as a simple and cost effective assay. Multiple approaches were applied for the investigations defining the sources of variability and errors and validating the due modifications as well as the resulting newly developed SOP which was based on using cWB. Specific investigations were designed to validate and get more comprehensive insights into the precision, the accuracy and especially the specificity of RTH. In addition to cWB, our investigations used cPRP, PLT isolated by gel-filtration, Eightcheck control and our developed PLT standards. Multiple conventional morphological approaches (PLT spreading, phase-contrast microscopy and screening and transmission electron microscopy) were performed on the tested materials before and after the passage of RTH filter as well as on the filter fixed sections in order to control the reliability of RTH measurements and to provide further insights into the implications of our modifications as well as the significance and the interpretation of RTH assay. Developing a reliable SOP of RTH was a pre-requisite for addressing reliable reference ranges in healthy individuals which was performed also under morphologic control.

III SUBJECTS AND METHODS

3-1. Subjects of the study (healthy blood donors)

Unless otherwise is mentioned, the investigations included in our study were performed on apparently healthy blood donors (BD). The blood donors were recruited from blood donor unit in the Institute of Clinical Hemostaseology and Transfusion Medicine, Saarland University Clinics in Homburg/Saar. Blood samples were obtained before completing the blood bank safety tests, but the results of a sample were included in the study only after passing the safety tests. The blood donors were non-selectively recruited in regard of gender or age. Blood donors who took Aspirin-like drugs within the last two weeks before blood withdrawal were excluded from the study.

3-2. Equipment and Apparatuses

3-2-1. Standardized Eppendorf retention tubes (RTH tubes) The standardized Eppendorf retention tube (RTH tube) is designed especially for performing RTH assay. This tube (1.5 mL) consists of an upper and a lower part. A newly developed filter system is fixed in the bottom of the upper part, as shown in Fig.3-2-1. The RTH filter is a disc (height 2.3 mm; diameter: 6.9 mm) with standardized textured surface composed of sintered polyurethane (PU) with inter-connected micro pores of 16 to 22 μm in diameter. RTH tubes (including RTH filter) are disposed for one time measurement and they are produced and supplied by Eppendorf Co. [Wenzel 2000].

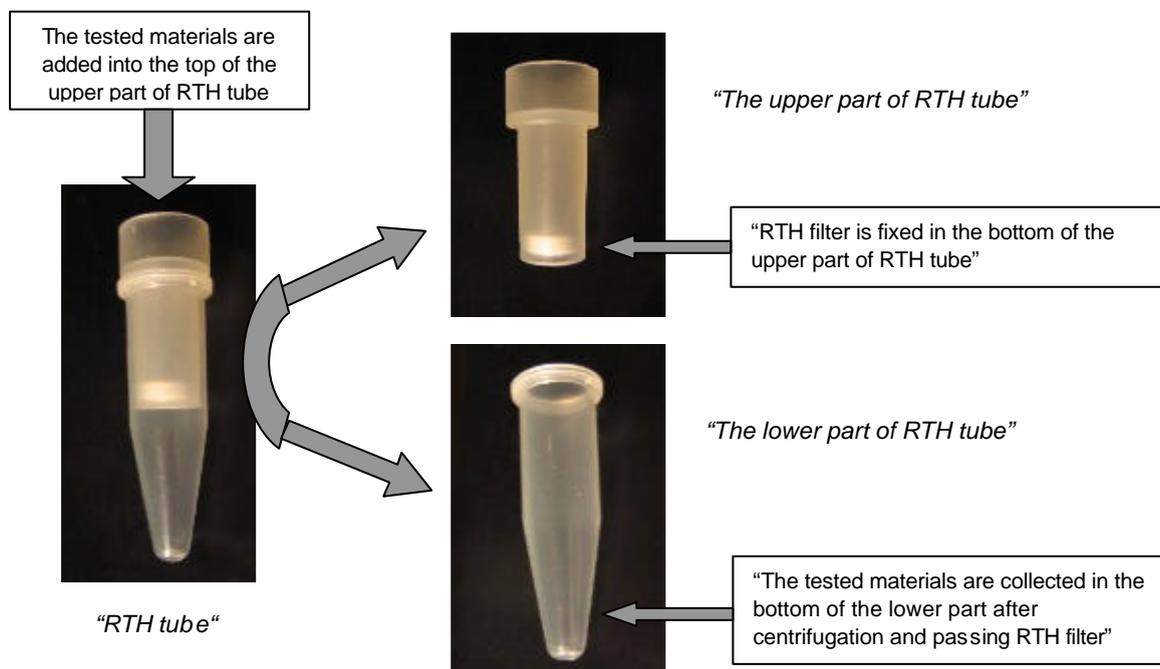


Fig. 3-2-1: Eppendorf retention tube "RTH Tube".

3-2-2. Eppendorf tubes Eppendorf tubes (Epp tubes; 1.5 mL) were used for some specific experiments. Epp tubes are similar to RTH tubes (p.3-2-1) in size, but consist of only one part that has no filter inside.

3-2-3. Convenient centrifuges A centrifuge with a standardized phase of acceleration, centrifugation and deceleration that can provide constant temperature (8-37°C) was required for centrifuging of RTH tubes. The centrifuge should provide the centrifugal forces 90-110 g (p.3-4-3-2-2-5 and p.5-1-1-2). We used mainly the centrifuge "Eppendorf 5417C" which may provide 110 g at 1000 rpm. Other centrifuges supplied with plates adaptable to the RTH tubes were occasionally used (e.g., in our experiments on gel-filtration in the Dept. of Experimental Surgery; p.4-2-2-1). A centrifuge was also used for preparing cPRP (110 g/ 15 min; p.3-4-2) and for preparing the PLT standard (p.4-2-5-1). The rotational speeds of the centrifuge that comply with the specified centrifugal forces (g) were calculated by considering the radius (*r*) of the centrifuge head and using the following equation:

$$RCF \text{ (relative centrifugal force)} = 1.118 \times 10^{-5} \times r \times (\text{rpm})^2$$

in which *RCF* is the relative centrifugal force in units of "g" (i.e., multiples of the gravitational force); 1.118×10^{-5} is a constant; "*r*" is the radius (cm) between the axis of rotation and the center of the centrifuge tube; and "*rpm*" is the speed in revolutions per minute [Bauer 1980].

3-2-4. Reliable electronic PLT counter Multi-channel whole blood cell counters incorporating PLT (PLT) counting¹ capabilities were required for the measurements of RTH and the complete blood cell count (CBC)². Our investigations were done in the Institute of Clinical Hemostaseology where we used the available models of cell counters: Sysmex M2000, Sysmex KX21, Sysmex SF3000, and Sysmex K1000. The cell counters were controlled daily by the low and the normal level of Eightcheck control (p.3-3-2). The cell counter "Coulter ACT" was used for performing the investigations on isolated PLT in the Dept. of Experimental Surgery.

3-2-5. Vortex instrument Heidolph Reax 2000, supplied with different vortex speeds that range from grade 1 to 9, was used to mix the tested samples by vortex.

3-2-6. Mechanical rotator Blood samples (p.3-4-1) were put on a mechanical rotator to assure homogeneity before performing the analyses.

3-2-7. Chromatography column for PLT isolation The chromatography column for PLT isolation by gel-filtration using Sepharose 2B (p.3-4-4-1) was composed of 3 parts: A.) 50 mL perfusor syringe (for single use); supplied by BBraun. B.) Cell strainer; nylon mesh (pore size 40 μm), sterile/gamma irradiated, by Becton Dickinson. C.) 3 ways stopcock for infusion, supplied by BBraun.

3-2-8. Light, phase-contrast, and electronic microscopes For the morphological control (p.3-5).

3-2-9. Athrombogenic plastic slides and covers These slides were essential for performing PLT spreading (p.3-4-5-2-4). They were also used along with plastic covers for the phase-contrast microscopy (p.3-5-2). The use of glassware was avoided since they may activate the PLT by contact [Vogler 1995]. The plastic athrombogenic slides (76 x 26 mm, 0.75 mm thick) and covers (22 x 22 mm) are manufactured by Heinz Herenz and Plano W Planet respectively.

3-2-10. pH meter "PHM 64 research" pH meter, by Radiometer Copenhagen, was used for adjusting the pH of the reactivating reagent (p.3-3-3), the buffers used in PLT spreading (p.3-3-6), and in the preparation of PLT standard (p.3-3-14).

¹ Platelet counting includes: platelet count (PLT) and platelet size distributions PDW (platelet arithmetic width), MPV (mean platelet volume) and PLCR (platelet large cell ratio).

² Complete blood cell count (CBC) includes: platelet counting, leukocytes and main normal cell types, and erythrocytes and their indices.

3-3. Reagents

3-3-1. Citrated plastic tubes (syringes) Green Sarstedt monovettes (5 and 10 mL containing 0.5 and 1 mL of 3.8% Na-citrate respectively) were used for preparing isotonic citrated whole blood (cWB; p.3-4-1) and/or citrated PLT rich plasma (cPRP; p.3-4-2).

3-3-2. Eightcheck control The normal and the low level of the Eightcheck control (4.0 mL tubes contain stabilized blood cells; by Sysmex) were used to control PLT count measurements by electronic cell counters (p.3-2-4). The normal level of the Eightcheck control (EC) was also used as a "PLT standard" to evaluate/validate the retention phenomena demonstrated by RTH assay (p.4-2-3-1, 4-1 and 6-1). EC tubes were stored at 2-8°C and allowed to equilibrate at room temperature before use.

3-3-3. Reagents of RTH assay (calcium chloride and r-Hirudin) Calcium chloride (CaCl₂; 5 mL vial) and r-hirudin (500 ATU/vial) are included in the kit of RTH assay, provided by Eppendorf-Pentapharm, for preparing the reactivation reagent used in this assay, as described in p.3-4-3-2-1.

3-3-4. Distilled water (D.W.) Sterile and pyrogenfree distilled water, produced by Delta-Pharma for the external use. The D.W. was employed for rinsing and dilution purposes.

3-3-5. Sepharose 2B gel Sepharose 2B is a bead-formed Agarose-based gel-filtration matrix manufactured by Pharmacia Biotech. The content of agarose in Sepharose 2B is 2%. The broad fractionation range of Sepharose make it suitable for characterizing or cleaning-up samples containing components of diverse molecular weight; such as for the isolation of PLT from cWB [Grignani 1998]. The gel (Sepharose) was stored at 4 to 8°C in the presence of a bacteriostatic agent (20% ethanol).

3-3-6. Platelet buffer (PLT buffer) This buffer was prepared for the isolation of PLT by Sepharose 2B (p.3-4-5). The formulas of the stock and the use solutions are described in Tab.3-3-6, and the qualifications of the components of these solutions are as follows:

Table 3-3-6: Formulas of the stock and the use solutions of the PLT buffer.

Stock solution (10x) ^a		Use Solution (1x) ^b	
NaCl	42.4 g	stock solution	100 mL
HEPES	11.9 g	Glucose	1.8 g
Na ₂ HPO ₄	0.35 g	BSA (Bovine Sigma Albumin)	3.0 g
KCl	8.3 mL of 3 M	D.W.	S.Q.1000 mL
MgCl ₂	10 mL of 1 M		
D.W.	S.Q. 500 mL		
pH 7.4			

^aTo be sterile filtered after preparation and then kept in refrigerator (2-8°C). If any turbidity or precipitation developed, the buffer should not be used. ^bTo be kept at -20°C in aliquots of 50 mL polypropylene glycol tubes.

A.) Stock solution (10x):

- HEPES: N-[2-hydroxyethyl] piperazin-N'-[2-ethane-sulfonic] sodium hydrogenphosphate, by Sigma (order code H0891) for molecular biology (99+%). EC No. 230-907-9; pKa =7.5 at 25°C, useful pH range 6.8-8.2 at room temperature.
- NaCl, Na₂HPO₄, KCl and MgCl₂ for analysis, by Sigma and MERCK. The physiologic NaCl solution for washing as well as the KCl (3M) and MgCl₂ (1M) solutions have to be prepared according to the required amount of PLT buffer. Further detailed descriptions are available in Ab.3-3-6.

B.) Use Solution (1x):

- Glucose: D(+)-Glucose monohydrate for biochemistry and microbiology use, by MERCK.
- BSA: Bovine Sigma Albumin. Cell cultured tested and desiccated for laboratory use, EC No. 232-936-2; nitrogen content 15.43%. Stored at 2-8°C. Order code A-9418.

- 3-3-7. Isotonic sodium chloride** 100 mL vial (for IV infusion) by Delta-Pharm. Sterile and pyrogenfree solution; theoretical osmolality 309 m.osm/L, pH=5-7.
- 3-3-8. Sodium citrate solution** 10 mL Amp, 3.13% solution by Braun used for lowering the viscosity of the blood reactions. Sterile without preserving material.
- 3-3-9. Formaldehyde solution 20%** One liter (i.e., 1.04 kg) stock solution for analysis stabilized with 10% methanol (toxic); produced by MERCK.
- 3-3-10. Potassium permanganate solution** Titrisol Amp; 3.161 g KMnO_4 / 100 mL; produced by MERCK; and stored in dark place.
- 3-3-11. Giemsa stain** Azur-Eosin-Methylene blue solution for microscopy (contains Methanol); produced by Diagnostika MERCK and stored at 15-25 °C.
- 3-3-12. Glutardialdehyde 25%** For electron microscopy; by ROTH. Stored at 4°C.
- 3-3-13. Sodium acid research grade (NaN_3 : min. 99%)** Reagents for sulfides and thiocyanates (Mr = 65.0) produced by SERVA, Heidelberg/NY). Stored at 4°C.
- 3-3-14. Buffers used for preparing PLT standard** Two buffers were used: A.) Phosphate buffer saline (PBS; pH=7.4-7.6) and B.) Phosphate buffer (0.1 M; pH=7.4). These buffers are stable for weeks at 4°C. The formulas of these buffers were modified several times throughout the series of experiments performed in order to develop a convenient protocol for the preparation of our own unstimulated PLT standard. The final formula of each buffer is addressed in the results of developing the PLT standard (p.4-2-5-1-2). The appearance of turbidity or precipitation in either buffer indicates its decay and the buffer should be discarded in such a case.

3-4. Designs of the standard operation procedures (SOP)

3-4-1. Blood sampling and collection: Preparation of citrated whole blood (cWB)

Blood was withdrawn from the cubital vein into a 5 or 10 mL syringe (green Sarstedt monovette; 1 part isotonic citrate : 9 parts whole blood; p.3-3-1). The puncture was carefully performed with a 1.1-gauge (1.1x40 mm) of a sterile disposable needle. Blood and anticoagulant were thoroughly and gently mixed to obtain the cWB ready for further tests. If the tube has been standing, this requires at least 2 min on mechanical rotator (p.3-2-6) to assure homogeneity and precise results. Special interest was paid to fill the tube exactly up to the measure line. To prevent tissue thromboplastin from reaching the blood sample by vein puncture, the two-syringe method was applied [Bauer 1980]. The first syringe was discarded (or used for tests other than our study) after aspiration of a minimum of 3 mL blood. The needle was not removed and a second plastic syringe was attached and the specimen was obtained by gentle traction.

3-4-2. Preparation of citrated platelet rich plasma (cPRP)

The cWB in the collection tube (5 or 10 mL Sarstedt monovettes; p.3-3-1) was centrifuged at 110 g for 15 min without brake at room temperature. The resulted cPRP was transferred carefully by micro-pipette (using new disposable plastic tips) into a plastic collection tube to get ready for further analyses. The aspiration of the cPRP was performed with a specific care not to disturb the lower layers of leukocytes and erythrocytes. The cPRP was pipetted gently on the inner wall of the collection tube.

3-4-3. Retention Test of Homburg (RTH)

3-4-3-1. Principle of the test

PLT are exposed to a standardized exogenic surface (the filter of Eppendorf retention tube) under defined conditions of flow forced by centrifugation. The percentage of the difference between PLT count before and after the passage of the filter is defined as the retention index (RI) of PLT (PLT-RI). The test is performed mainly by using cPRP and possibly by cWB. Decreased PLT-RI indicates a reduced PLT stickiness and function. In contrast, high levels of PLT-RI indicate increased PLT stickiness that is associated with an increased PLT aggregation as seen in e.g., vascular diseases.

3-4-3-2. First SOP of RTH assay “SOP-1”

The procedure of RTH (SOP-1), which was the subject of our validation and standardization, was based mainly on the newest guideline of RTH assay at the time we start our study [Wenzel 2000].

3-4-3-2-1. Preparation of the reactivating reagent 5 mL vial CaCl_2 is added into the vial of r-hirudin (500 ATU) which is dissolved within 3-5 min. Both reagents, CaCl_2 and r-hirudin, are provided with the kit of RTH (p.3-3-3). The resulting solution is stable for at least 3 months, if refrigerated.

3-4-3-2-2. Performance of the assay

3-4-3-2-2-1. 450 μL cPRP or cWB (p.3-4-1 & 2) is pipetted in the upper part of the RTH tube (p.3-2-1).

3-4-3-2-2-2. *Blank:* 50 μL of the reactivating reagent (r-Hirudin with CaCl_2 ; p.3-3-3) is added.

3-4-3-2-2-3. *Pre-incubation:* The RTH tube is then left at room temperature for 3-10 min.

3-4-3-2-2-4. *The measurements before the passage of RTH filter (b.F measurements):* A part of the tested substance (cWB or cPRP) is aspirated from the upper part of RTH tube by an electronic cell counter (p.3-2-4) to measure PLT count and PLCR before centrifugation.

3-4-3-2-2-5. *Centrifugation:* RTH tube is centrifuged. Variant conditions could be applied (79 to 160 g for 5-16 min; Wienefoet 2003); the most common “g” values are 80-120 g [Wieding 2004].

3-4-3-2-2-6. *The measurements after the passage of RTH filter (a.F measurements):* The upper part of RTH tube is removed and the cWB is mixed thoroughly by vortex for 3 min. PLT count and PLCR ratio are then measured by a cell counter. Whenever the Eppendorf filter is removed while using cPRP, it should not be sampled from the layer containing erythrocyte; instead the cPRP should be withdrawn by pipette from top layer.

Note 1: Due to the critical role of the PLT counting (b.F and a.F) in RTH assay, it is preferable to be performed *in-double* and the mean is considered for the next calculations.

Note 2: The RTH test should be performed within 30-180 min after blood withdrawal.

This SOP (SOP-1) was substantially modified according to the conclusions of our evaluation to it and to our successive investigations on RTH (p.4-1 & 2). New SOPs of RTH (SOP-2 and SOP-3) were consequently addressed in our study; as described in sections p.4-1-1-7 and p.4-1-2-5 of the results.

3-4-3-3. Calculation of the retention indices (RIs) of the total platelets (PLT-RI) and the large platelets (PLCR-RI)

The retention index (RI) of the platelet (PLT-RI) as well as of the large platelet (PLCR-RI) was calculated by measuring PLT count and PLCR ratio respectively before (b.F) and after (a.F) the passage of the filter:

$$\text{RI (\%)} = \frac{(\text{b.F measurement} - \text{a.F measurement})}{\text{b.F measurement}} \times 100$$

3-4-4. Platelet isolation by gel-filtration using Sepharose 2B

3-4-4-1. Making the chromatography column

The plunger of a 50 mL syringe (p.3-2-7-A) is taken off and a nylon mesh (Cell strainer; p.3-2-7-B) is replaced and fixed in the bottom of the cylinder of the syringe by a suitable adhesive. The borders of the mesh should first be cut to suit the inner diameter of the cylinder of the syringe. A sufficient quantity of the adhesive is applied to the borders of the lower side of the mesh. The nylon mesh is then put down the cylinder by using the plunger. The needle of the syringe should be thrown away and replaced by a 3 ways stopcock (p.3-2-7-C).

3-4-4-2. Preparation of the " Sepharose 2B gel"

Put 100 mL of the Sepharose 2B (p.3-3-5) in a plastic filter (Sarstedt; 22 μ m) and use water vacuum to remove alcohol from the Sepharose. Wash with 800-900 mL NaCl solution (100 mL 1.45 M NaCl and 900 mL distilled water). After washing, bring the Sepharose into solution with NaCl and pour in the prepared column. Fill the column to 50-60 mL and keep moist (turn running off). While spinning, equilibrate the Sepharose in the column with PLT buffer (PLT buffer; p.3-3-6. This step is to get rid of the saline in the Sepharose; use as much PLT buffer as the column is filled with Sepharose. Usually this is 50-60 mL of the PLT buffer that has to run through the column. Just before adding the PLT buffer, the column must be dry on the top for a few seconds. The column is then ready for use, and it can be covered by a parafilm and kept in the refrigerator (2-8°C). The Sepharose in the column should be kept moist in the whole following steps using PLT buffer (p.3-3-6).

3-4-4-3. SOP of the platelet isolation

- 3-4-4-3-1. The Chromatography column must be vertically fixed on a suitable stand and a 50 mL glass container is put under the open of the 3 ways stopcock. Then, let the column to equilibrate to the room temperature before using it.
- 3-4-4-3-2. Allow sufficient numbers of the aliquots of PLT buffer to dissolve in a water-bath chamber, and then put them on a suitable porter. Open one of the PLT buffer tubes and put in it a disposable plastic pipette to ready it for use.
- 3-4-4-3-3. Put the PLT collection tubes, ready for use, on another suitable porter (three 5 mL plastic tubes for each 10 mL blood sample).
- 3-4-4-3-4. In parallel, by the use of 10 mL cWB drawn from one blood donor, cPRP is prepared, collected in a plastic tube and allowed to rest before use (SOP; p.3-4-2). Add the cPRP carefully on the inner-wall of the column. The top of the column must be dry for seconds just before the cPRP is added.

Note: All the materials that are supposed to be added to the column (PLT buffer, cPRP, NaCl) should be applied carefully on its inner-wall.

- 3-4-4-3-5. Let the upper surface of the Sepharose dry for seconds, then wash the column with PLT buffer.
- 3-4-4-3-6. Collecting the isolated PLT (iPLT). When the plasma (the yellow ring) is at the level of 30-40 mL, the outcoming PLT buffer drops become gradually cloudy bearing the iPLT. Collect as much iPLT as cPRP put on the column, or a little bit more. The outcoming is collected into 3 tubes (T1, T2 & T3; ca 2 mL in each), so the second one is supposed to bear

the most cloudy drops and consequently the highest concentration of iPLT. When the outcoming drops become again unclouded stop collecting PLT buffer. Let the iPLT rest in the 37°C water-bath for 30 min. In the course of collecting PLT, keep the Sepharose in the column moist using PLT buffer.

3-4-4-3-7. The column can either be thrown away, or kept in refrigerator if it is still in a good condition. In the second case, the following steps should be performed:

- The column is washed with 50 mL PLT buffer.
- The stopcock is closed, and then filling the column with PLT buffer is completed.
- The top of the column is closed tightly with a parafilm.
- The column is stood carefully in the refrigerator (2-8°C).

3-4-4-3-8. The 50 mL glass container is washed with normal water, and let it dry. The 50 mL plastic (polypropylene) tubes are washed with normal water and then D.W. The tubes are then let dry (only if they are still in a good condition, otherwise they should be thrown away).

3-4-5. Platelet spreading (morphological test by light microscopy)

This test was adapted from the method introduced by Marx [1960] and modified by Breddin [1968].

3-4-5-1. Preparation of the use solutions

3-4-5-1-1. *Isotonic sodium citrate solution* Sodium citrate is diluted with isotonic NaCl (1:9); 10 mL are subtracted from a sealed vial of 100 mL isotonic NaCl (p.3-3-7) and substituted with the content of 10 mL sodium citrate ampoule 3.13 % (p.3-3-8).

3-4-5-1-2. *Formaldehyde 10%* 42.5 mL of Formaldehyde 20% stock solution (p.3-3-9) is diluted with 50 mL D.W. (p.3-3-4).

3-4-5-1-3. *Potassium permanganate 0.1 N.* KMnO_4 Amp (3.161 g/ 100 mL; p.3-3-10) is mixed with 1000 mL D.W. to get 0.1 N solution as described as follows: (1) Insert the ampoule in the neck of the volumetric flask and push the pointed glass rod (Cat No. 9998) through the upper membrane. (2) Push the lower membrane. 3) Withdraw and rinse the glass rod. Rotate the ampoule and rinse it with D.W. Fill the volumetric flask to the mark 20°C with D.W. and mix thoroughly.

3-4-5-1-4. *Giemsa stain solution.* The stock solution (p.3-3-11) is diluted with D.W. before use (1:2).

Note. The resulted solutions are stable for one week at room temperature. Only fresh solutions were used for each batch of PLT spreading preparations to avoid contamination and changes in pH.

3-4-5-2. SOP of the platelet spreading

3-4-5-2-1. PLT count is performed on the collected cWB (SOP; p.3-4-1).

3-4-5-2-2. Preparing cPRP (SOP; p.3-4-2). Gel-filtered PLT could be also examined as well as cPRP, and in this case there is no need to perform the next dilution step (p.3-4-5-2-3).

3-4-5-2-3. The dilution step. The PLT count of the cPRP is adjusted to 30×10^6 with isotonic Na-citrate solution (p.3-4-5-1-1).

3-4-5-2-4. About 24 mL of the resulted platelet suspension is poured on a specific athrombogenic plastic slide (p.3-2-9) and incubated at room temperature for 30 min allowing the platelets to adhere and spread on the slide.

3-4-5-2-5. The slide is rinsed carefully with the isotonic Na-citrate solution (p.3-4-5-1-1).

- 3-4-5-2-6. Fixation step. The slide is stood in the 10% formaldehyde solution (p.3-4-5-1-2) for 7 min.
- 3-4-5-2-7. The slide is rinsed with isotonic Na-citrate solution (p.3-4-5-1-1).
- 3-4-5-2-8. Oxidation step. The slide is stood in the 0.1N potassium permanganate solution (p.3-4-5-1-3) for 5 min.
- 3-4-5-2-9. The slide is rinsed with isotonic Na-citrate solution.
- 3-4-5-2-10. Staining is accomplished by standing the slide in Giemsa's solution (p.3-4-5-1-4) for 60 min.
- 3-4-5-2-11. The slide is rinsed gently with D.W. and let the slide dry.
- 3-4-5-2-12. Examine the preparation by light microscope at the magnification of x100 and x 400.

3-5. Morphological control/validation

Conventional morphologic approaches were performed on the tested materials before and after the passage of RTH filter as well as on the filter after centrifugation in order to provide further insights into the significance and implications of RTH assay and to inspect/assure the reliability of RTH measurements. This was quite essential for the basic developments in the initial SOP of RTH as well as for the validation the developed SOP (SOP-3) and introducing RTH-II. Three approaches were used:

3-5-1. Platelet spreading

PLT spreading was applied in the investigations performing RTH by using PLT isolated by gel-filtration (p.3-4-4) compared with that in cWB. This test was performed on the tested material before and after the passage of RTH filter in order to assess the adhesion, spreading, and spontaneous aggregation or agglutination of PLT from a morphological point of view. The preparations of PLT spreading (p.3-4-5) were examined by light microscopy. According to Scharrer [1985], five types of PLT can be distinguished in healthy individuals: (1) *Giant forms* with a surface of more than 200 μm . (2) *Big forms* with minimum one diameter more than 10 μm . (3) *Small forms* with an average diameter less than 10 μm . (4) *Intermediate forms (round, etc.)* which are not completely spread and have little hyalomere between pseudopodia. (5) *Spider forms* which are not spread PLT with one or more short and relative thick pseudopodia. The forms 1-3 are defined as spread PLT forms while the forms 4 and 5 are not spread.

In our microscopic examination, hundred PLT were counted in each PLT spreading preparation. The findings were recorded on an application (Ab.3-5-1) that was designed to include the complete descriptions of the examined preparations as follows:

- A.) The technical quality of the preparation (thickness, coloring, dirties if any, etc.) was differentiated. The thickness of the preparations reflect firstly the PLT count of the tested sample and secondly the adhesion of PLT.
- B.) The relative presence in percentages (%) of the spread (giant, big, and small) and non-spread (intermediate and spiders) PLT as well as the disrupted and abnormal forms.
- C.) The grade of PLT aggregates (Aggr) and/or agglutinates (Aggl) were assessed semi quantitatively and differentiated according to their range of size (Tab.4-2-2-3).
- D.) Summary describes whether the examined preparation demonstrated a normal picture, more or less spreading depression, or increased big spread forms (left shifted).

3-5-2. Phase-contrast microscopy

The phase-contrast microscopy was used as a reference method [Joist 1980] for the direct examination (with no additions or coloring) of PLT morphology in cWB and variety of the samples used in our investigations on RTH i.e., cPRP, washed and unwashed prefixed PLT, and Eightcheck control. The focus in this examination was on the following:

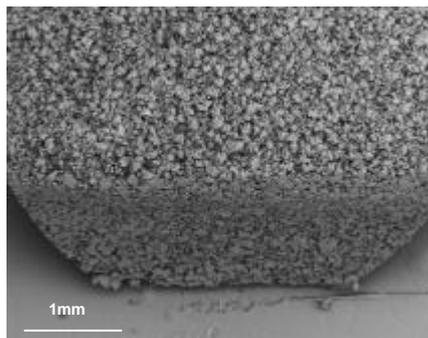
- A.) The activation state of the PLT: Resting PLT present intact discocytes. Partly activated PLT demonstrate discocytes with small pseudopodia (echinodiscocytes) that should not exceed 10% in normal cases. The presence of PLT with long pseudopodia or activated forms is a sign of fully activated PLT that should not exist in normal individuals.
- B.) The suspension state of PLT: Normal resting PLT should be separately suspended in the fluid phases of cWB, cPRP and washed PLT forming no aggregates (Aggr) or agglutinates (Aggl).
- C.) The presence of abnormal findings in the blood cells, if any. A special care was paid on the concentration of WBC and RBC in the investigations of preparing the PLT standard (p.4-2-5).
- D.) Semi-quantitative control on the accuracy of the PLT and blood cells measurements. Specific care for “quantitative” count was paid for controlling the measurements of the reference ranges of RIs.

3-5-3. Transmission and scanning electron and phase-contrast microscopy of RTH filter

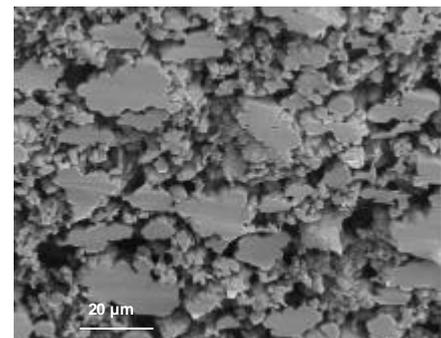
The transmission (TEM) and scanning (SEM) electron microscopy were used besides phase-contrast microscopy for characterizing and specifying the PLT and the blood cells interactions with PU surfaces of RTH filter by the use of cWB from healthy blood donors (p.3-4-1) and Eightcheck control (EC). The procedure of the examination was adapted from Geyer [Geyer 1973] as summarized as follows:

Immediately after the centrifugation of the RTH tubes (110 g/ 5 min; acc to SOP-3 unless otherwise is mentioned), the filter was removed out and fixed in 5% glutaraldehyde in 0.1M cacodylat buffer solution for 30 min at 4°C. The fixing solution composed of 2 mL glutaraldehyde (25% for electron microscopy; p.3-3-12) in 8 mL 0.1M cacodylate buffer (pH=7.4). The RTH filter was thereafter split in four segments using a razor blade on a water-repellent Teflon surface (Fig.3-5-3) taking into consideration its top and bottom faces. The segments were fixed for further 30 min at 4°C within the fixing solution and then were incubated for washing in 10 mL of the washing-buffer (0.1M cacodylate buffer containing 7.55% sucrose) over night at 4°C. After postfixation with osmium tetroxide solution the segments were dehydrated with acetone and embedded in Araldite. After embedding, ultra-thin sections were prepared

Fig. 3-5-3:
RTH filter
preparation
for microscopic
examinations.



Segment of RTH filter



Ultra-thin section of RTH filter

and stained with uranylacetate and lead citrate. The resulting preparations were examined by TEM and SEM. For the phase-contrast microscopy, semi-thin sections (0.5 μm) were prepared and stained by methylene blue polychrome.

3-6. Statistical methods

The statistical analyses of the results were performed by the use of the statistical program "SPSS" (Statistical Package for Social Sciences; version 11) and by Excel program of Microsoft (XP version). The levels below 0.05 were considered for the significance of the " p " (probability) values. The Kolmogorov Smirnov (KS) statistic with Lilliefors significance correction was used for testing the normality of distribution. The Shapiro-Wilk statistic was calculated if the sample size was low. The significance of the differences between the mean values and the variances of two independent samples were inspected by "Independent Sample t test" and by "Levene's test" respectively. For the comparison of more than two samples, "One-Way Anova" test was used for testing the significance of the differences of means and "Levene" for the homogeneity of variances. "Post Hoc Multiple comparisons" was performed whenever necessary by the use of LSD and Tamhane's tests. The significance of the differences between two related samples was inspected by applying "Paired Sample ttest". For non-parametric samples, the significance of the differences were computed using the Mann-Whitney U test for independent samples and by "Wilcoxon" or "Sign" tests for two related samples. The significance of the correlation (2-tailed) between the values of two variables was investigated by "Pearson" and "Spearman's rho" tests for parametric and nonparametric samples respectively. The correlation coefficient (r) was calculated in parallel with " p " value in both tests. The employed statistical tests are described by in details by Wagner [Wagner 2004]. The methodology for establishing the reference values for the tested parameters was adapted from Galen [1980] and Tetrault [2001]. The normal Q-Q Plot test was used for further inspection of the distribution of the RIs in normal individuals.

3-7. Strategy of writing

The thesis was constructed in respective order. For reference purposes, we use the abbreviation "p" to refer to the respective section or chapter e.g., p.4-2-3-1: refers to the first section of chapter 4-2-3. RTH measurements before (b.F) and after (a.F) the passage of the filter (p.3-4-3-2-2) were performed mainly in-double and, unless otherwise is mentioned, the mean values were used for further evaluations and/or comparisons (e.g., descriptive statistics). Due to the huge output of the investigations of our study, the results presented in this thesis are mainly *descriptive statistics* and/or *summaries* of the *key* results. The detailed results are recorded in abandons (Ab). The Ab. of certain data is referred to by the respective number of the corresponding data e.g., Tab.Ab.4-3-2-3 refers to the detailed results of the data described in Tab.4-3-2-3. The references were referred to by the name of the first author along with the year of publication. The bibliography chapter (p.7) presents the references in alphabetical order.

IV RESULTS

4-1. Identifications and investigations to improve

RTH procedures

4-1-1. Identifications and investigations addressing to the second SOP of RTH assay (SOP-2)

By reviewing the first standard operation procedures (SOP-1; p.3-4-3-2) of RTH after the initial application of RTH assay according to this SOP, some analytical problems and difficulties became evident. A stepwise approach was followed therefore to evaluate and improve the procedure of RTH. Specific identifications and modifications were consequently incorporated in the methodology of the SOP of RTH, as described in the following:

4-1-1-1. Fixing/Identifying the conditions of the centrifugation of RTH tubes

According to the original SOP of RTH (SOP-1; p.3-4-3-2-2-5), no specific standard condition was defined for the centrifugation of RTH tube. Diverse conditions were adapted ranging initially from 79 to 160 g for 5-16 min [Wienefoet 2003]; the most common centrifugal forces (gravitational forces) used in previous RTH studies ranged between 80-120 g [Wieding 2004]. We defined, however, the speed 110 g / 5 min as a standard condition for our further investigations of RTH assay because of:

- A.) Reviewing our preliminary experiments as well as some previous analyses of RTH [Koscienly 2002] showed that the values of PLT-RI measured by using the speed 110 g were meaningful (95% reference ranges=13-35%) and allowed acceptable margin for significant pathological variations. Lower “g” values (e.g., 90 g) revealed to deliver too high PLT-RI values (mean=73%; Schenk 2002) implicating no adequate margin for pathological thrombophilic variations, and vice versa by higher “g” forces.
- B.) Using centrifugation period longer than 5 min would be of no advantage and prolong the turnaround time of the test.

The initial fixation of this standard condition was, however, further evaluated/validated in the course of our investigations (using this condition) for the improvement, the standardization and the validation of RTH assay which proved its reliability, as explained in p.5-1-1-2.

4-1-1-2. Investigation of the effect of the reactivating reagent (CaCl₂ with r-Hirudin) on the retention of PLT and on PLT size distribution in cPRP

A. Aim and methodology of the study

The SOP-1 of RTH included an additional use of 50 µL of the reactivating reagent (5 mL CaCl₂ with 500 ATU of r-Hirudin; p.3-3-3) to the tested materials (cWB, cPRP) before the centrifugation step (p.3-4-3-2-2-5). The necessity of this addition was on question in since the retention index (RI) is a relative value and it does not require, therefore, the addition of CaCl₂ as a blank. In addition, we believed that this addition may induce further variability. As an attempt to improve and simplify RTH procedures, we investigated the effect of the reactivating reagent on the PLT-RI and on PLT size distribution in cPRP. Four samples

of cPRP were prepared from 4 blood donors (p.3-1) and 450 μL was transferred from each sample into a series of 10 RTH tubes. Different concentrations of the buffered reactivating reagent were applied on each series, as described in Tab.4-1-1-2-B, and RTH was performed on each RTH tube. The concentration of the undiluted reagent was detected in the Dept. of Clinical Chemistry and found to be equal to 19.0 mmol CaCl_2/L . Na Citrate was added to the undiluted reagent to deliver the required dilutions. The r-hirudin was diluted with only Na-citrate in order to provide a reagent free from CaCl_2 .

B. Results of investigating the effect of the reactivating reagent on the retention of PLT and on PLT size distribution in cPRP

The key results are summarized in Tab.4-1-1-2-B. No significant differences were found in the measurements before the passage of the filter.

Table 4-1-1-2-B: Effect^a of adding different concentrations of the reactivating reagent (CaCl_2 with r-Hirudin) on the retention of PLT-RI and PLCR-RI in cPRP.

CaCl ₂ mmol/L	Sample 1				Sample 2				Sample 3				Sample 4			
	PLT ^b x10 ³ / μL	PLT-RI %	PLCR _b %	PLCR-RI %	PLT ^b x10 ³ / μL	PLT-RI %	PLCR _b %	PLCR-RI %	PLT ^b x10 ³ / μL	PLT-RI %	PLCR _b %	PLCR-RI %	PLT ^b x10 ³ / μL	PLT-RI %	PLCR _b %	PLCR-RI %
19.0	343	27.1	15.0	49.5	230	23.9	16.5	35.0	192	38.5	17.6	37.8	249	12.0	24.7	8.8
19.0	334	41.9	17.2	71.3	235	13.2	16.4	22.2	190	40.5	17.2	39.1	254	16.5	25.6	14.9
9.5	371	42.9	9.8	43.4	237	24.9	15.8	30.6	186	46.2	16.5	44.9	247	26.3	25.4	22.3
9.5	359	NA	10.4	NA	245	34.3	15.6	42.7	193	45.1	18.7	43.3	246	28.9	24.2	26.5
4.75	350	32.9	9.9	39.0	233	22.7	15.7	27.2	189	44.4	18.2	44.1	250	30.4	23.1	27.1
4.75	339	NA	9.6	NA	236	39.8	15.8	49.7	184	45.1	16.1	40.0	255	34.1	24.6	32.2
2.38	337	27.9	10.5	41.6	223	33.2	14.8	38.6	184	33.7	17.5	32.2	248	29.8	24.3	27.5
2.38	369	25.5	9.7	31.6	236	36.0	17.1	47.6	190	30.0	16.4	27.4	252	31.0	24.9	27.3
0.0	369	22.2	10.6	23.7	242	24.4	16.6	33.0	189	26.5	17.8	24.4	249	20.5	25.8	17.1
0.0	337	17.5	10.7	19.8	241	NA	16.5	NA	192	21.9	18.3	20.6	253	23.3	23.5	19.1
Mean	350.8	29.7	11.3	40.0	235.8	28.0	16.1	36.3	188.9	37.2	17.4	35.4	250.3	25.3	24.6	22.3
SD	14.9	9.0	2.6	16.2	6.3	8.3	0.7	9.2	3.2	8.7	0.9	8.7	3.1	7.1	0.9	7.2
CV (%)	4.3	30.2	22.9	40.4	2.7	29.7	4.1	25.4	1.7	23.5	5.0	24.7	1.2	28.0	3.6	32.3

^aThe detailed results are described in Tab.4-1-1-2. ^bThe measurements before the passage of the filter (i.e., in the collection tube; p.3-4-2). PLT= platelet count; PLT-RI= the retention index of platelet; PLCR= platelet large cell ratio; PLCR-RI= the retention index of PLCR.

Within each series of samples 1-4, the a.F (after the passage of the filter) measurements of PLT and consequently PLT-RI varied significantly by applying different concentrations of Ca^{+2} ($p < 0.05$), as described in Tab.4-1-1-2-B. Although no statistically significant correlation between the variation of these retention indices and Ca^{+2} concentration was objected ($p = 0.188$; Spearman's rho test), these results showed a dose-dependent effect of Ca^{+2} on PLT-RI, as demonstrated in Fig.4-1-1-2-B-1. This effect varied markedly among samples 1 to 4. PLT-RI increased proportionally with the concentration of Ca^{+2} up to a cut-point ranging about 5-12 mmol/L. Higher levels of Ca^{+2} led to marked decreases in PLT-RI which varied inter-individually (Fig.4-1-1-2-B-1). This effect was less pronounced in PLCR-RI, as illustrated in Fig.4-1-1-2-B-2.

It was decided therefore to perform RTH without the addition of the reactivating reagent (p.5-1-1-3). The amount of the tested material that should be pipetted into RTH tube increased consequently to 500 μL instead of 450 μL . The a.F measurements of MPV (mean CV=1.8%) and PDW (mean CV=5.7%) within the series of samples 1-4 were rather homogeneous.

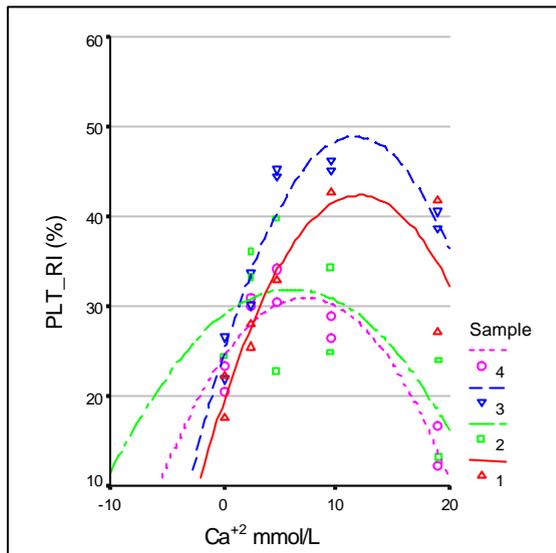


Fig. 4-1-1-2-B-1: Inter-individual variation in the response of PLT-RI to CaCl_2 in the reactivating reagent added to cPRP.

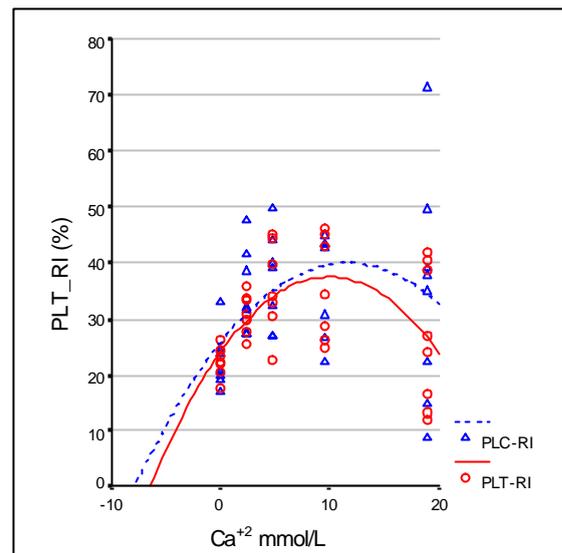


Fig. 4-1-1-2-B-2: Effect of the reactivating reagent on PLT-RI and PLCR-RI in cPRP.

4-1-1-3. Measurements before the passage of RTH filter comparing RTH tubes with the original collection tubes

In SOP-1, the measurements performed before the passage of RTH filter (b.F measurements; p.3-4-3-2-2-4) were applied on the 450 μL tested material added into RTH tubes. The measurements were performed after the resting period and before the centrifugation step. This methodology had led to several problems and errors:

A.) The normal run of the available cell counter (Sysmex M2000) required about 400 μL for each measurement leaving less than 100 μL of the tested material to pass RTH filter. The use of the micro run of that counter (pre-dilution of the tested material) consumed less amounts of the material (10 μL) but required additional reagents for the pre-dilution which might increase the probability of the variability of these measurements as well as the time and costs. The other available cell counters (p.3-2-4) consumed 100 to 150 μL per measurement. Thus, performing the b.F measurements on the material added to RTH tube made it impossible to perform these measurements in-double (as recommended by the first SOP; p.3-4-3-2) or at least unacceptably minimize the amount of the rest material that should be centrifuged and then measured in-double (a.F measurements). In addition, the morphological assays on the tested material before and after the passage of RTH filter, performed in our studies for control and validation purposes, required further amounts.

B.) The variable amounts used by the different cell counters for b.F measurements resulted in variances in the rest amounts of the tested materials that should pass RTH filter and thus might lead to more variations in the determinations of RTH.

Consequently, we suggested performing the b.F measurements on the tested materials in the original collection tube after transferring 500 μL (instead of 450 μL since the addition of 50 μL reactivating reagent was excluded; p.4-1-1-2) of the materials to RTH tubes. Further advantages of this step are explained in another chapter (discussion; p. 5-1-1-4).

4-1-1-4. Exclusion of the incubation period of cWB in RTH tube before centrifugation

According to SOP-1 (p.3-4-3-2-2-3), after the addition of the tested material in the RTH tube, it was rested for up to 10 min in order to calm down the disturbed PLT before performing the b.F measurements on it. Due to our previous modification (p.4-1-1-3), the b.F measurements were no longer performed on the material added in RTH tubes and no need was found therefore to rest RTH tubes. Further obligations for this step are described in p. 5-1-1-5.

4-1-1-5. Quantity assessment of the tips of the micropipette used in RTH

A. Aim and methodology of the study

Aspirating and pipetting samples tested by RTH should be performed by the use of new tips for each RTH test. Repeating RTH on the same sample may suggest using the same tip to distribute this sample into the different RTH tubes for more convenience and saving tips. An experiment was designed to inspect the reliability of employing the same tip for successive pipetting from the same sample by using cWB from healthy blood donors (p.3-1). This was critical to address the right methodology of performing RTH in-double as well as studying the precision of RTH and similar investigations where successive pipetting from the same sample is applied. The cWB of each blood donor was distributed into 4 Epp tubes (p.3-2-2), 500 μ L in each, by using 3 different modes of pipetting as described as follows: (A) using the same tip for the first two tubes and a new tip for the other ones. (B) using the same tip for the first three tubes and a new tip for the 4th one. (C) using a new tip for each pipetting.

B. Results of the quantity assessment of the tips of the micropipette used in RTH

The statistics of these results are described in Tab.4-1-1-5-B. The mean weight of the cWB pipetted by employing pre-used tips (504.1 mg) was significantly higher ($p < 0.0005$; paired sample t test) than that pipetted by using new tips (528.1 mg). The mean increase in the cWB amount delivered by the pre-used tips compared with new ones was 4.1%. Interestingly, the variation of the pre-used tips was 2 times higher than that of the new ones (SD=1.7 vs. 3.4). Only new tips were therefore used for all the measurement in our investigations including the RTH ones.

Table 4-1-1-5-B: Descriptive Statistics^a of the quantity assessment of the tips of the micropipette used in RTH.

Descriptive Statistics (n=36)	WB Weight ^b		Increase ^c (%)
	New Tip (mg)	Used Tip (mg)	
Range	6.4	11.6	81.3
Minimum	501.0	521.3	4.1
Maximum	507.4	532.9	5.0
Mean	504.1	528.1	4.8
SD	1.7	3.4	101.4
CV (%)	0.3	0.6	92.2

^a The detailed results are described in Tab.Ab.4-1-1-4.

^b WB Weight= the weight of the 500 μ L cWB pipetted into Eppendorf tubes by using new or used tips.

^c Increase= the percentage of the increase in the cWB amount pipetted by using used tips compared with that pipetted by using a new tip.

4-1-1-6. Correction of the identification of the retention index of large platelet (PLCR-RI)

The original formula of PLCR-RI was based on the percentages of large PLT (p.3-4-3-3)³, whilst the corrected was calculated by using their absolute count in order to improve its significance, as described in p. 5-1-1-7 and p.5-4-3-3-A. The new identification of PLCR-RI was as follows⁴:

$$\text{PLCR-RI (\%)} = \frac{[(\text{b.F-PLCR} \times \text{b.F-PLT}) - (\text{a.F-PLCR} \times \text{a.F-PLT})]}{\text{b.F-PLCR} \times \text{b.F-PLT}} \times 100$$

4-1-1-7. Addressing the second SOP of RTH (SOP-2)

Due to the improvements implicated by the modifications suggested in this chapter (p.4-1-1-1 to 6), the SOP-1 of RTH described in p.3-4-3-2 was improved to the "SOP-2" which took into consideration the following alteration:

- 4-1-1-7-1. The amount of the tested material to be added into RTH tube should be 500 µL instead of 450 µL in the SOP-1(p.3-4-3-2-2-1) and by the use of a new tip for each measurement.
 - 4-1-1-7-2. No addition of the reactivating reagent to the tested materials is required (p.3-4-3-2-2-2).
 - 4-1-1-7-3. The b.F measurements (before passing RTH filter; p.3-4-3-2-2-4) should be performed on the tested material in the original collecting tube just before transferring 500 µL of this material to RTH tube. The collecting tube should be rested (on rotator) before performing the b.F measurements to assure homogeneity and PLT calmness.
 - 4-1-1-7-4. Exclusion of the pre-incubation of RTH tubes before centrifugation (p.3-4-3-2-2-3).
 - 4-1-1-7-5. The centrifugation of the materials in RTH tubes (p.3-4-3-2-2-5) is defined at 110 g/ 5 min.
 - 4-1-1-7-6. The next steps of performing RTH i.e., applying the a.F measurements on RTH tube after mixing the centrifuged cWB thoroughly by vortex for 3 min are performed as described in the SOP-1 (p.3-4-3-2-2-5 and 6).
 - 4-1-1-7-7. The corrected formula of PLCR-RI (p.4-1-1-6) should be considered in RTH determinations.
- These points combined represented the second SOP of RTH (SOP-2) performed on cWB.

³ The initial formula: $\text{PLCR-RI (\%)} = ((\text{b.F-PLCR}) - \text{a.F-PLCR}) / (\text{b.F-PLCR}) \times 100$

b.F-PLCR= PLCR ratio measured before the passage of RTH filter; a.F-PLCR=PLCR ratio measured after passing RTH filter.

⁴ b.F-PLT= PLT count measured before the passage of RTH filter; a.F-PLT= PLT count measured after passing RTH filter.

4-1-2. Identifications and investigations addressing to the third SOP of RTH assay (SOP-3)

4-1-2-1. Improving protocol: Aim and methodology of the study

After addressing the second SOP of RTH (SOP-2; p.4-1-1-7), it was essential to investigate the precision and the reliability of RTH as a "simple" assay applied on cWB for screening the function of PLT. An improving protocol was designed for this purpose. Our investigations represented one part of an inter-laboratory study held in different university centers in Europe [König 2002]⁵. Blood was withdrawn from healthy blood donors (BD; p.3-1), collected in 10 mL citrated tubes (p.3-3-1) and then distributed into a series of 10 RTH tubes (p.3-2-1); 0.5 mL in each. RTH was performed according to SOP-2 (p.4-1-1-7). The b.F measurements (before the passage of the filter) were performed on the cWB in the collection tubes. The series of 10 RTH tubes were centrifuged simultaneously and the a.F measurements (after the passage of the filter) were performed on the centrifuged cWB collected in the lower parts of RTH tubes after mixing the blood by vortex for 3 min. The complete blood cell count (CBC) was determined in each measurement by the use of an automatic cell counter; the values of PLT count and PLCR ratio were registered and the corresponding retention indices (PLT-RI and PLCR-RI) were accordingly calculated. Both b.F and a.F measurements were performed in-double and these two repetitions were referred to as "*horizontal measurements*". A third repetition was performed whenever possible to have more insight, but only the values of the 1st and the 2nd repetitions were used to compute the mean of the horizontal measurements and correlated determinations. The measurements performed on the series of 10 RTH tubes of each BD were referred to as "*in-series measurements*", and the corresponding values were consequently referred to "in-series ones". The 10 in-series a.F measurements of each cWB sample were not simultaneously performed but successively with increasing time intervals since the measurements by the cell counters required minimum time (ca. 3 min) and were occasionally interrupted by the routine work. Nevertheless, this was helpful to study the influence of time on the retention of PLT by RTH filter within the limit of 180 min after blood withdrawal under which the whole a.F measurements had to be performed. The improving protocol was performed in 4 stages; the stages 1 to 3 are described in the section studying the influence of time on PLT-RI (p.4-1-2-2-1), and the stage 4 is described in the section handling the influence of mixing cWB by vortex on PLT-RI (p.4-1-2-3-1).

4-1-2-2. Initial results and the influence of time on the retention of platelet

4-1-2-2-1. Methodology of the successive stages investigating the influence of time

In the 1st stage of the study, the improving protocol (p.4-1-2-1) was performed on 5 BDs within 180 min after blood withdrawal. The initial results of this stage pronounced highly elevated levels of PLT-RI that increased apparently with time. The influence of time was therefore inspected in the next 2 stages. In the 2nd stage, cWB was collected from one BD and distributed into 2 collecting tubes (samples 6a and 6b) that underwent the same improving protocol but with different low and high time intervals (<80 min and >120 min after blood withdrawal respectively; Tab.4-1-2-2-2). This was an attempt to check whether

⁵ Berlin (Grigorove), Frankfurt (Klinkhardt), Giessen (Mucha/Lauer), Hamburg (Gutensohn), Ludwigshafen (Hellstern), Wien (Dostal).

increasing time intervals required for performing the successive a.F measurements on the 10 RTH tubes within each series were responsible for the in-series increase of PLT-RI in the 1st stage. Another confirmatory inspection on the influence of time was done in *the 3rd stage* of the study in order to avoid a probable exceptional individual response in the 2nd stage (samples 6A, 6B) that was performed on one BD. The improving protocol was performed on another 2 BDs (samples 7 & 8) but with a special care to finish the measurements within 60 min, as described in Tab.4-1-2-2-2.

4-1-2-2-2. Initial results of the improving protocol and investigation of the influence of time on the retention of platelets

The main key findings are described in Tab.4-1-2-2-2. In *the 1st stage*, the values of PLT-RI were remarkably elevated (mean=45.7-81.7%) with high variation (SD=12.0-14.9), as shown in Fig.4-1-2-2-2-1. The mean of PLT-RI within each series increased significantly by time ($r= 0.68$; $p<0.0005$: Fig.4-1-2-2-2-2); where the last in-series measurements (RTH tube No. 10) were extremely higher (33.2-45.7%) than that of the first ones (RTH tubes No 1), as described in Tab.4-1-2-2-2. On the other hand, the second a.F measurements implicated another significant increase in PLT-RI compared with the first ones (horizontal increase up to 40%; Fig.4-1-2-2-2-3). In *the 2nd stage*, the paired t-test revealed no significant difference between the mean PLT-RI values of samples 6a and 6b (77.9% vs. 85.9%; $p>0.3$: Fig.4-1-2-2-2-1) which were comparably high with the means of PLT-RI in the 1st stage (82.1% vs. 60.3%: Tab.4-1-2-2-2). The variation of sample 6a (SD=20.7) sustained the high level found in the 1st stage (mean SD= 15.3), as shown in Fig.4-1-2-2-2-1. Surprisingly, the variation of PLT-RI in sample 6b, that was tested with high time intervals, delivered much lower values than that of sample 6a which was tested with lower time intervals (SD= 4.3 vs. 20.7: $p<0.05$; Fig.4-1-2-2-2-1). Both samples 6a and 6b revealed positive correlation between PLT-RI and time ($r=0.80$ vs. 0.36) which was significant only in sample 6a ($p<0.01$: Fig.4-1-2-2-2-a). The horizontal increase of PLT-RI was slightly higher in sample 6a (10.5%) than in 6b (8.1%), as demonstrated in Fig.4-1-2-2-3.

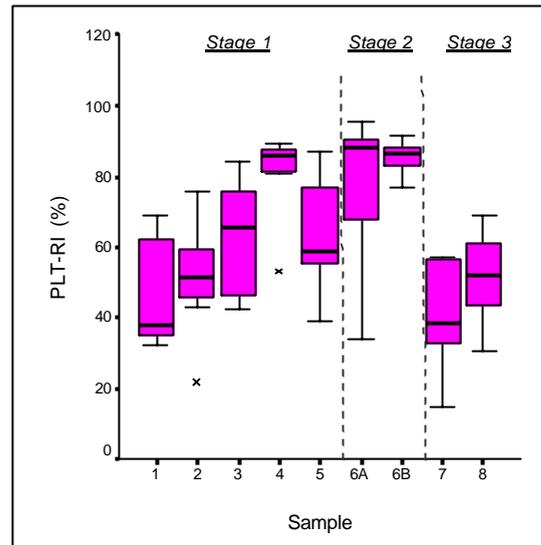


Fig. 4-1-2-2-2-1: Distribution of the in-series measurements (n=10) of PLT-RI in stages 1-3.

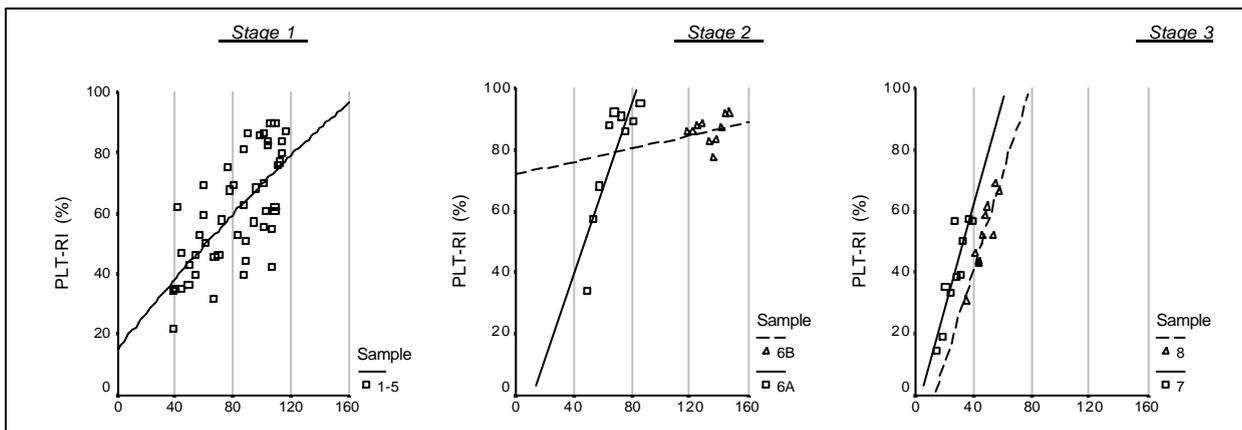


Fig. 4-1-2-2-2-2: PLT-RI against time intervals of the in-series measurements (n=10) in stages 1-3.

Table 4-1-2-2-2: Main key results* of RTH measurements in the different stages performing the improving protocol (SOP-2: stages 1-3 and SOP-3: stage 4).**

Stage ^a No.	Sample ^b No.	Time ^c min	b.F PLT Count		b.F Measurements					PLT-RI				PLCR-RI			PDW-RI		MPV-RI		WBC-RI			RBC-RI %	HGB-RI %	HCT-RI %
			Mean x10 ³ /μL	CV %	PLCR %	PDW fL	MPV fL	WBC x10 ³ /μL	RBC x10 ⁶ /μL	Mean %	SD	s.Incr %	h.Incr %	Mean %	SD	RI-Diff %	Mean %	SD	Mean %	SD	Mean %	SD	s.Decr %			
1	1	38-77	253	0.0	15.9	9.3	9.1	11.1	4.2	45.8	14.7	33.2	6.8	25.9	12.7	19.9	-15.3	13.1	-6.3	11.5	-5.0	4.0	4.5	-4.7	-5.0	-3.6
	2	39-80	141	0.0	15.1	9.9	8.2	5.0	4.3	52.3	14.9	47.5	8.7	18.7	18.6	33.6	-27.6	11.7	-19.5	6.4	-8.2	8.5	10.0	-6.2	-5.5	-5.1
	3	54-115	407	0.3	6.8	8.2	7.0	7.2	3.8	64.1	15.4	37.7	-0.2	15.4	34.6	48.6	-5.7	5.5	-18.3	4.2	-35.6	17.9	37.8	-7.4	-7.4	-5.8
	4	83-112	261	0.5	15.6	10.1	8.4	3.9	3.9	81.7	12.0	36.4	13.3	65.3	24.1	26.2	-38.7	22.8	-14.5	6.1	-48.6	19.9	48.7	-7.2	-6.0	-4.4
	5	87-117	193	1.8	18.5	11.1	8.9	7.0	3.6	62.3	14.5	47.5	14.5	34.2	18.5	31.5	-28.7	11.8	-19.7	8.2	-7.9	10.4	26.4	-5.2	-6.5	-3.9
2	6a	50-86	147	0.5	33.8	14.6	11.2	6.6	4.1	77.9	20.7	61.4	10.5	68.4	26.2	22.2	-28.4	10.5	-7.0	2.8	-31.3	14.4	25.0	-5.8	-4.9	-4.7
	6b	118-147	147	0.5	33.8	14.6	11.2	6.6	4.1	85.9	4.4	6.1	8.1	81.0	6.4	21.1	-35.2	16.6	-7.7	6.3	-31.4	7.2	-1.5	-7.3	-5.9	-6.0
3	7	15-39	207	3.1	12.5	9.2	7.9	6.7	4.2	40.0	15.4	41.6	8.1	11.0	16.6	29.0	-4.7	9.6	-12.5	8.3	-4.4	2.0	6.0	-7.1	-4.0	-7.1
	8	35-58	140	3.0	28.9	14.0	10.2	9.0	4.2	52.1	11.7	35.4	23.2	48.9	9.0	3.3	-6.9	6.3	-4.0	5.5	-3.1	1.5	2.8	-4.8	-4.6	-4.0
4	9	29-118	210	1.0	14.3	10.0	8.1	4.3	4.3	25.6	6.2	-7.6	2.6	23.3	6.3	2.1	-1.7	2.5	-1.1	0.7	3.0	1.6	1.2	-5.3	-4.0	-5.1
	10	59-98	149	0.5	21.1	11.0	9.3	3.4	3.9	29.9	5.1	2.7	1.5	30.5	7.3	-0.6	-3.2	3.2	0.9	2.4	7.2	2.0	0.0	-2.5	-1.1	-2.4
	11	31-74	159	2.7	15.3	10.3	8.3	6.4	4.9	28.2	3.0	-3.1	-6.0	26.0	3.8	2.2	1.8	4.5	-1.9	1.5	7.7	2.6	-5.5	-3.3	-2.5	-2.6
Stages 1-3			210	1.1	20.1	11.2	9.1	7.0	4.0	62.5	13.8	38.5	10.3	41.0	18.5	26.2	-21.2	12.0	-12.2	6.6	-19.5	9.5	17.7	-6.2	-5.5	-5.0
Stage 4			172	1.4	16.9	10.4	8.5	4.7	4.4	27.9	4.7	-2.7	-0.6	26.6	5.8	1.2	-1.0	3.4	-0.7	1.5	5.9	2.0	-1.4	-3.7	-2.5	-3.3
Sig.*** "p value" of the corrections by using SOP-3 (stage 4) vs. SOP-2 (Stages 1-3)									0.000	0.000	0.000	0.000	0.002	0.002	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.012	0.010	0.000	0.000	0.000

Note: Unless otherwise is mentioned, the values of the different retention indices (RIs) are the in-series means of the first in-double measurements.

* This table was intensively summarized from the complete 72-page table (Tab.Ab.4-1-2-1), where the detailed results of the successive horizontal and in-series measurements before and after the passage of RTH filter as well as the resulting RIs and their horizontal and in-series variations are described.

**Compared with SOP-2, the main modifications adapted in SOP-3 were: A.) The blood after the centrifugation of RTH tubes should be mixed by applying the new standardized slight vortex (=3 sec; p.4-1-2-4-4) instead of 3 min in SOP-2 (p.4-1-1-7) and SOP-1 (p.3-4-3-2). The a.F measurements should be performed within 1-2 min afterwards. B.) Performing RTH assay within 120 min after blood withdrawal. Further details are explained in p.4-1-2-3-1 and p.4-1-2-5.

*** The significance of the differences between SOP-3 and SOP-2 measurements (p = the probability value) was computed by using t-test for equality of means and Levene's test for equality of variances (the value $p=0.000$ is equivalent to $p<0.0005$).

^a The different stages performing the improving protocol of this study (p.4-1-2-1).

^b Each sample was obtained from one healthy blood donor (p.3-1) except for samples 6a and 6b which were taken from the same blood donor for comparison purposes (p.4-1-2-2-1).

^c The time intervals of performing the a.F measurements (on the blood after passing RTH filter) on the first and the last RTH tube of the 10-RTH tube series for each sample (the delay of performing RTH after blood withdrawal was also related with the routine run of the laboratory "Institute of Clinical Hemostaseology and Transfusion Medicine).

b.F PLT Count= PLT count measured in cWB before the passage of RTH filter where the mean and the CV of the in-double measurements are given.

PDW-RI, MPV-RI, WBC-RI, RBC-RI, HGB-RI and HCT-RI= the retention indices of PDW, MPV, WBC, RBC, HGB and HCT respectively which we identified in p.4-1-2-3-1-1.

SD = the standard deviation;

s.Incr = the series increase i.e., the increase in PLT-RI observed in the last in-series a.F measurement (RTH tube #10) compared with that of the first one (RTH tube #1).

h.Incr = the horizontal increase i.e., the in-series means of the increases of PLT-RI in the second in-double measurements compared with the first ones.

RI-Diff = the decrease of the values of PLCR-RI in comparison with PLT-RI.

s.Decr = the decrease in WBC-RI observed in the last in series measurement (RTH tube #10) compared with that of the first one (RTH tube #1).

The mean of PLT-RI (46.1%) in the 3rd stage showed a remarkable decrease compared with that in the previous stages (mean=67.1%). But the variation (mean SD= 13.6) and the in-series (38.5%) as well as the horizontal (15.6%) increase of PLT-RI were relatively high and comparable to that encountered in the earlier stages (Fig.4-1-2-2-2-1 & 3). Actually, the increasing time intervals kept on influencing PLT-RI positively in this stage ($r>0.8$; $p<0.005$), as demonstrated in Fig.4-1-2-2-2-2.

Our investigation revealed strong positive correlation between PLCR-RI and PLT-RI within the stages 1-3 ($r=0.78$; $p<0.0005$), as illustrated in Fig.4-1-2-2-2-4. In parallel with PLT-RI, PLCR-RI revealed high values in stages 1-3 but they were less elevated than PLT-RI and demonstrated higher in-series variations (Fig.4-1-2-2-2-4 and Fig.4-1-2-3-2-8) and similar horizontal ones (Tab.Ab.4-1-2-1).

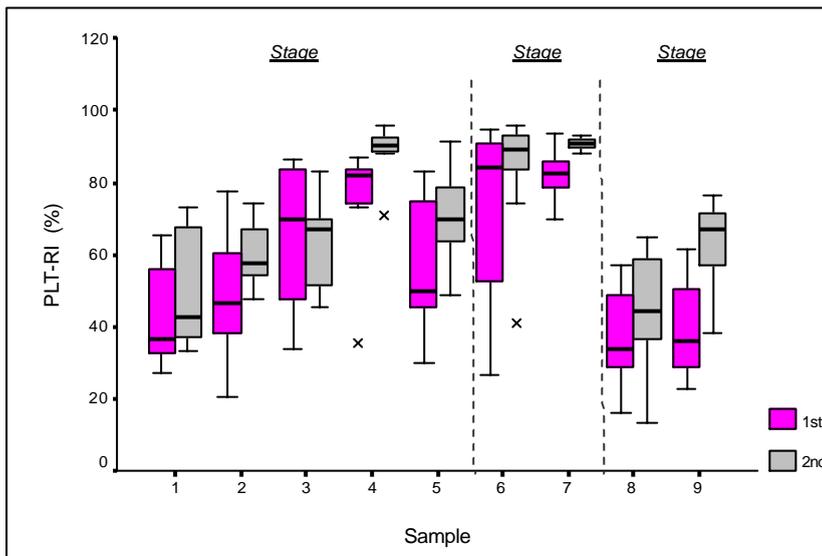


Fig. 4-1-2-2-2-3: Distribution of the first (1st) and the second (2nd) in-double measurements of PLT-RI within the different series (n=10) of samples in stages 1-3.

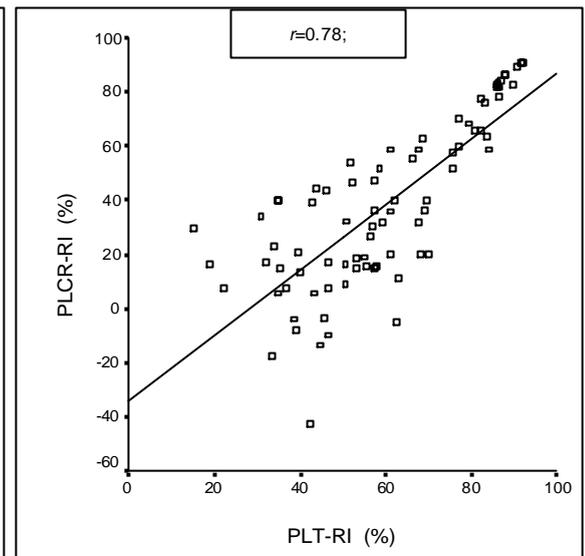


Fig. 4-1-2-2-2-4: PLCR-RI against PLT-RI in stages 1-3.

4-1-2-3. Influence of mixing cWB on PLT-RI and interacting retention indices (RIs)

4-1-2-3-1. Background, aim, identifications and methodology

A. Enlarging the improving protocol: Identification of new retention indices for the parameters of complete blood counting (CBC)

The application of different time intervals in the improving protocol in stages 1-3 (p.4-1-2-2-2) failed to decrease the elevated PLT-RI values and the corresponding high variation. In addition, *three main observations had initiated our investigation* to the influence of mixing the cWB by vortex on PLT-RI:

- The b.F measurements of PLT delivered low variations (mean CV=1.1%; Tab.4-1-2-2-2). Whilst the significant variations were restricted in the a.F measurements; where RTH tubes were vortexed thoroughly (ca 3 min) according to the SOP-1 of RTH assay (p.3-4-3-2-2-6).
- The horizontal increase in PLT-RI of the same RTH tube (i.e., in the second compared with the first in-double a.F measurements) could not be attributed to the differences of RTH filters.
- The elevated levels of PLT-RI and their in-series as well as horizontal increases (p.4-1-2-2-2) were signs for probable PLT aggregation due to hyperactivation by shear stress (centrifugation).

As an attempt to have better understanding to the reasons of PLT-RI variability and the probable interacting factors as well as to have more comprehensive insight into the specificity of RTH, we determined to

enlarge the improving protocol and identify additional retention indices (RIs). In parallel to PLT and PLCR measurements (p.4-1-2-1), the whole parameters detected by CBC (complete blood counting i.e., PLT size distributions, leukocytes and their main normal types, erythrocytes, and RBC indices) were measured before and after centrifugation in order to identify a corresponding retention index (RI) for each i.e., PDW-RI, MPV-RI, WBC-RI, NEUT-RI, LYMP-RI, MXD-RI, RBC-RI, HGB-RI, HCT-RI, MCV-RI, MCHC-RI and RDW-RI. The formula of these new (RI) was identified as follows:

$$\text{RI (retention index \%)} = \frac{(\text{b.F measurement} - \text{a.F measurement})}{\text{b.F measurement}} \times 100$$

B. Methodology of investigating the influence of mixing cWB on platelet and RTH assay

Two approaches were used for this purpose:

(a) A morphological pilot study of “blood mixing effect” was designed to investigate the influence of different modes of mixing cWB (mainly vortex) on PLT count and PLT clumping as well as on PLT-RI and interacting RIs (p.4-1-2-4). A “*new standardized vortex method*” was addressed to minimize PLT clumping and the a.F variations (after centrifugation and passing RTH filter).

(b) New RIs were identified in the enlarged improving protocol (p.4-1-2-3-1-A) for stages 1 to 3 (samples 1-8). The b.F and a.F measurements required for calculating RIs were obtained from the corresponding CBC (complete blood count). The enlarged protocol (i.e.. including the new identified RIs; p.4-1-2-3-1-A) was performed on 3 BDs (stage 4: samples 911) taking into consideration the new standardized vortex method addressed by the study of “blood mixing effect” (p.4-1-2-4-4) and finishing the RTH assay within 120 min after blood withdrawal. The series of the last sample (No.11) consisted of 13 RTH tubes instead of 10 to have even further confirmation about the results. The horizontal and the in-series variations for the b.F and a.F measurements as well as for the RIs were calculated thereafter for the whole 4 stages. A comparison between the findings of the enlarged protocol with and without applying the standardized vortex (stage 4 vs. stages 1-3) as well as a comparison among the results of applying different time intervals in stage 4 may then give further insight into the influence of mixing cWB by vortex after the passage of RTH filter. We aimed to inspect the probable improvement by applying the new standardized vortex method in (p.4-1-2-4-4) stage 4. The results of “approach B” are presented in the next section (p.4-1-2-3-2), whereas the results of approach A are described in section p.4-1-2-4.

4-1-2-3-2. Results of the enlarged improving protocol after applying the new standardized vortex method compared with that before (stages 4 vs. 1-3)

The detailed results for the a.F and b.F measurements, RIs and variations are reported in a 72-page table (Tab.Ab.4-1-2-1). The main key findings (summarized in Tab.4-1-2-2-2) may be described as follows:

A.) PLT-RI corrections: No significant difference was recognized between the means as well the variances of PLT-RI in samples 911 (stage 4) despite different time intervals were applied in each (29-118 min), as shown in Fig.4-1-2-3-2-1. The mean values of PLT-RI in these samples (9-11) were strongly lower (55.6%; $p < 0.0005$) than that found in samples 1-8 (stages 1-3: mean= 27.7% vs. 62.5%), as illustrated in Fig.4-1-2-3-2-1. The significant increase by time found in stages 1-3 was substantially eliminated in stage 4 (Fig.4-1-2-3-2-2). On the contrary to the sharp in-series increases of PLT-RI values in stages 1-3 (mean=38.5%) and their significant correlation with time ($r=0.717$; $p < 0.0005$), these

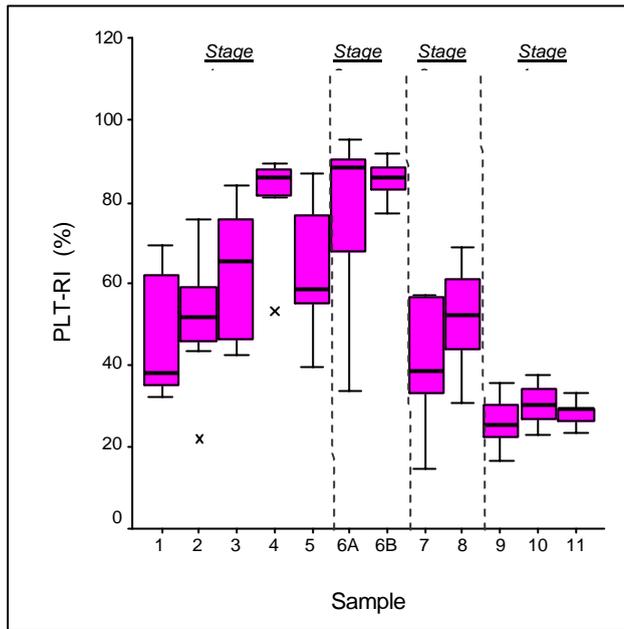


Fig. 4-1-2-3-2-1: Distribution of the in-series measurements (n=10) of PLT-RI in stage 4 compared with that in stages 1-3.

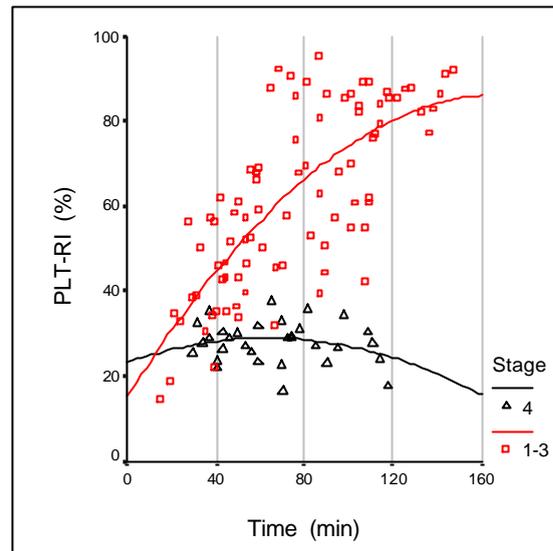


Fig. 4-1-2-3-2-2: PLT-RI against time intervals of the in-series measurements of stage 4 compared with that of stages 1-3.

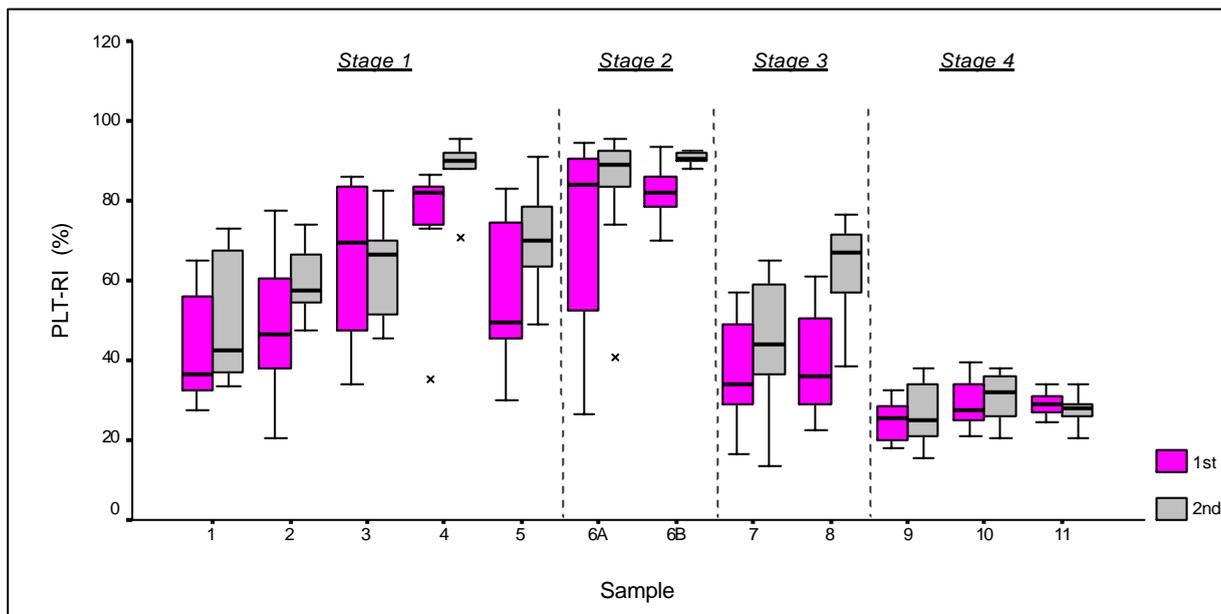


Fig. 4-1-2-3-2-3: Distribution of the first (1st) and second (2nd) in-double measurements of PLT-RI within the different series (n=10) of samples in stages 1-4.

increases were minimized to very low levels in stage 4 (range= -7.6% to 2.7%), and the average correlation switched from positive to negative ranges ($r=-0.046$). Levene's test spelled out significant difference in the variation of PLT-RI between stages 1-3 and 4 (mean SD= 13.8 vs. 4.7; $p<0.0005$; Fig.4-1-2-3-2-1). Also, the high horizontal increase of PLT-RI observed in stages 1-3 (mean=10.3%) declined substantially in stage 4 ($p<0.0005$) reaching negative level (mean= -2.7%) which implicated actual decrease (Tab.4-1-2-2-2), as demonstrated in Fig.4-1-2-3-2-3.

B.) WBC-RI and RBC-RI correction: The improvements in the measurements of PLT-RI in stage 4 were associated with essential coincidences in the RIs of WBC and RBC. The relationship between WBC-RI and PLT-RI converted from strongly negative in stages 1-3 ($r= -0.82$; $p<0.0005$) to insignificant positive in stage 4 ($r=0.30$), as illustrated in Fig.4-1-2-3-2-4. The extremely negative values

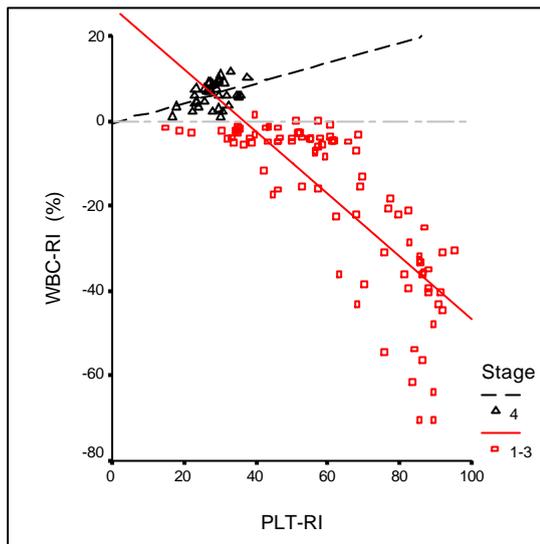


Fig. 4-1-2-3-2-4: Conversion of the correlation between WBC-RI and PLT-RI from strongly negative in stages 1-3 to positive in stage 4.

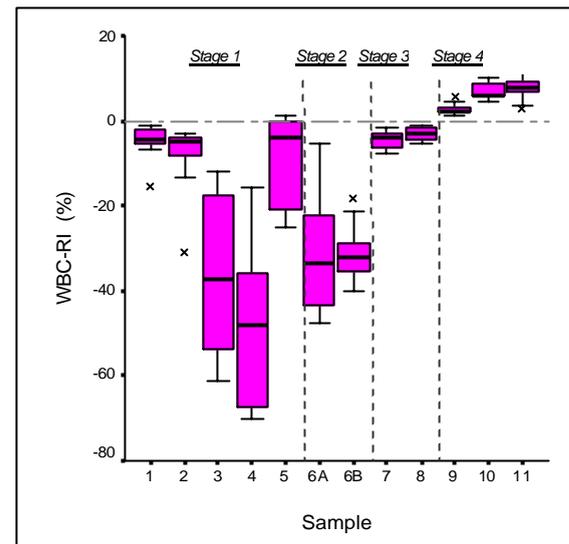


Fig. 4-1-2-3-2-5: Distribution of the in-series measurements (n=10) of WBC-RI in stages 1-4.

of WBC-RI in stages 1-3 rose up consequently to low positive values in stage 4 (mean= -19.5% vs. 5.9%; $p < 0.0005$) along with significantly lower variation (mean SD=9.5 vs. 2.0; $p = 0.012$), as shown in Fig.4-1-2-3-2-5. A similar pattern of converted correlation was pronounced between RBC-RI and PLT-RI where it switched from a slight negative and significant correlation in stages 1-3 ($r = -0.33$; $p = 0.002$) to a positive one in stage 4 ($r = 0.49$; $p = 0.004$), as illustrated in Fig.4-1-2-3-2-6). This conversion was associated with a significant rise in the RBC-RI values in stage 4 compared with that in stages 1-3 (-3.7% vs. -6.2%; $p < 0.0005$). The relative increase of RBC-RI values in stage 4 was remarkably lower than that of WBC-RI (40.4% vs. 130.5%), as demonstrated in Fig.4-1-2-3-2-4 & 6.

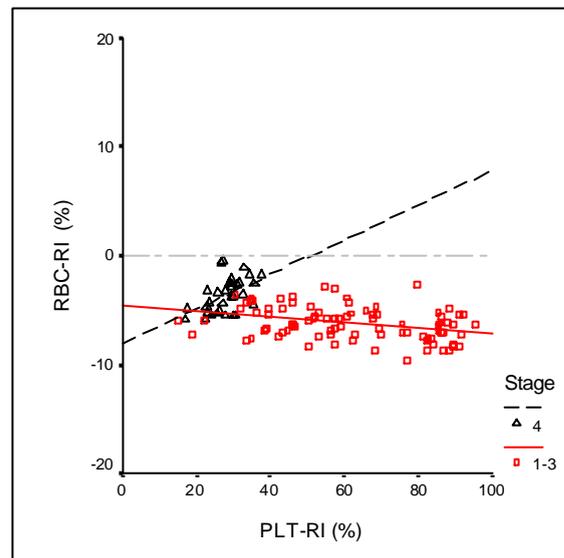


Fig. 4-1-2-3-2-6: Conversion of correlation between RBC-RI and PLT-RI from negative in stages 1-3 to positive in stage 4.

C.) PLCR-RI correction: The values of PLCR-RI in stage 4 held the strong positive correlation with PLT-RI found in the stages 1-3 ($r = 0.75$; $p < 0.0005$). But the big decrease of the values of PLCR-RI compared with PLT-RI values (RI-Diff; Tab.4-1-2-2-2) founded in stages 1-3 was minimized sharply in stage 4 (21.5% vs. 1.3%; $p < 0.0005$), as illustrated in Fig.4-1-2-3-2-7. Consequently and in parallel with PLT-RI, the high variations of PLCR-RI in stages 1-3 decreased significantly ($p = 0.002$) in stage 4 (mean SD=18.5 vs. 5.8). Also, the mean of the high in-series increase of PLCR-RI measurements in stages 1-3 (32.4%) was eliminated in stage 4 and converted to insignificant decrease (-3.4%), as shown in Fig.4-1-2-3-2-8.

D.) PDW-RI and MPV-RI correction: Both PDW-RI and MPV-RI were extremely negative in stages 1-3 and rose up significantly ($p < 0.0005$) to low negative levels in stage 4 (mean PDW-RI= -21.1% vs. -1.0 %; mean MPV-RI=-12.2 vs. 0.7%). The high variation of both RIs in stages 1-3

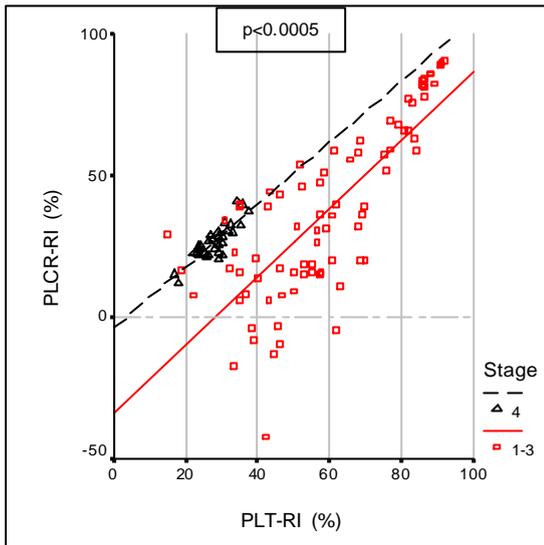


Fig. 4-1-2-3-2-7: PLCR-RI against PLT-RI in stage 4 with line of significant fit comparable to that in stages 1-3.

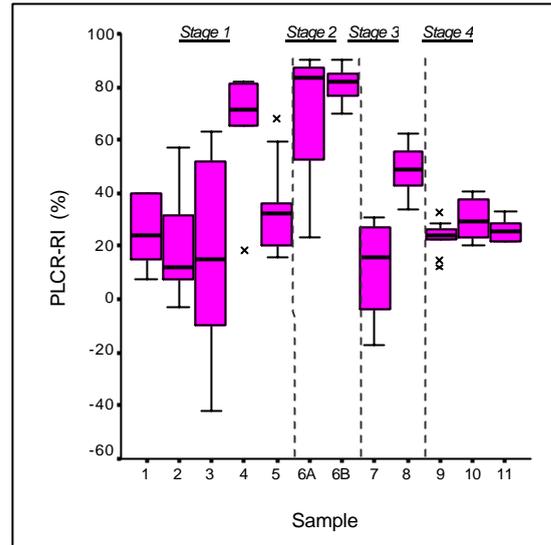


Fig. 4-1-2-3-2-8: Distribution of the in-series measurements (n=10) of PLCR-RI in stages 1-4.

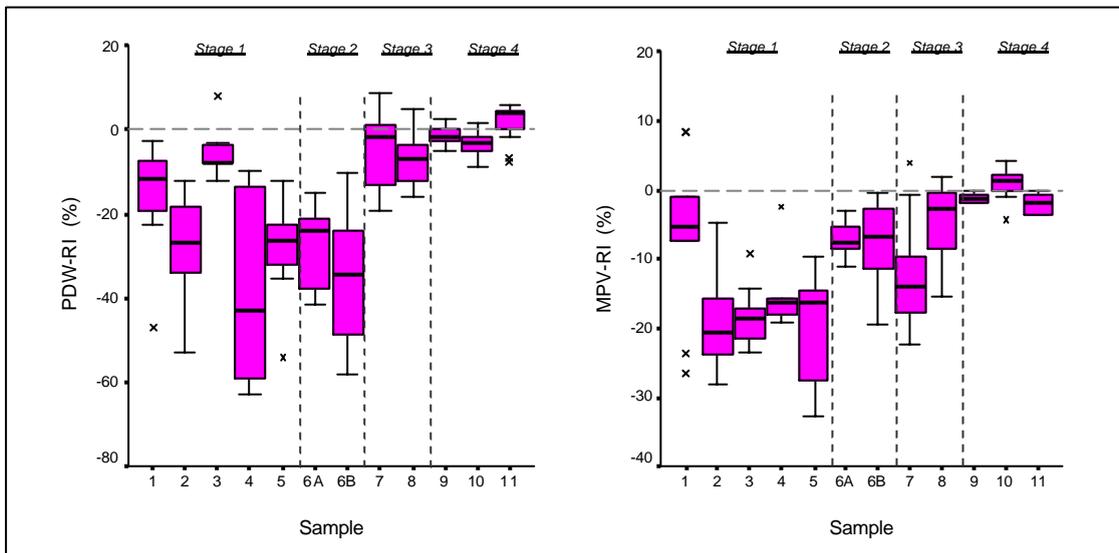


Fig. 4-1-2-3-2-9: Distribution of the in-series measurements (n=10) of PDW-RI and MPV-RI in stages 1-4.

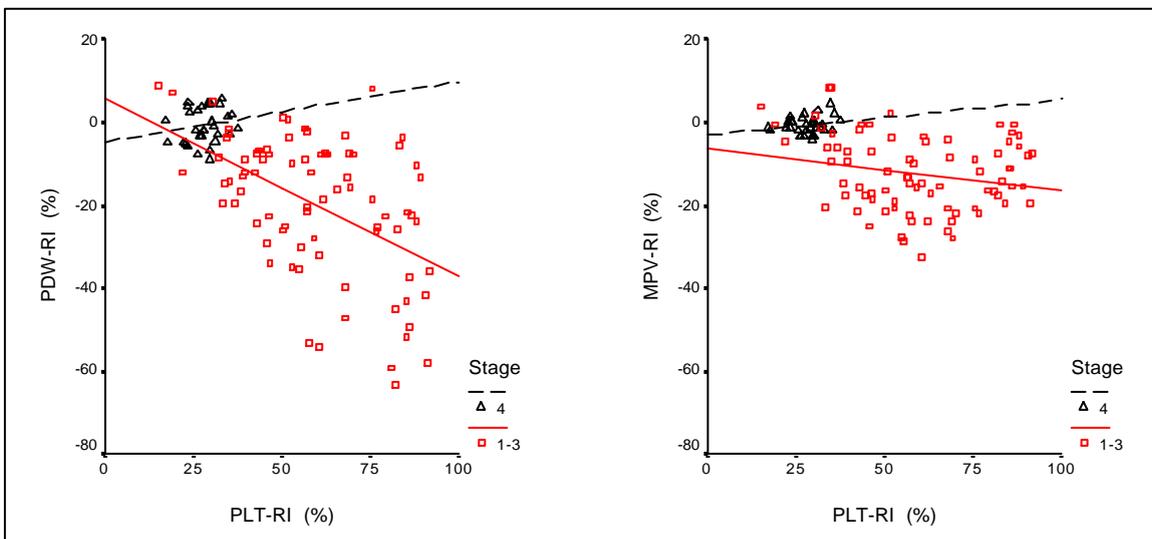


Fig. 4-1-2-3-2-10: Conversion of the relationship of PDW-RI and MPV-RI with PLT-RI from negative in stages 1-3 to positive in stage 4.

decreased significantly in stage 4 (mean SD = 12.0 vs. 3.4 and 6.6 vs. 1.5 respectively; $p < 0.001$), as shown in Fig.4-1-2-3-2-9. Of specific interest was the conversion of correlation between PLT-RI and each of PDW-RI and MPV-RI from an inverse relationship in stages 1-3 ($r = -0.44$ and -0.16) to a positive one in stage 4 ($r = 0.12$ and 0.03), as demonstrated in Fig.4-1-2-3-2-10.

E.) RIs of the main normal types of leukocytes i.e., LYM-RI, NEU-RI and MXD-RI: These RIs (identified in section p.4-1-2-3-1-A) had intimate and proportional relationships with WBC-RI throughout the whole stages (1-4) of the improving protocol ($p < 0.001$). Consequently these indices demonstrated the same pattern of correlation with PLT-RI as WBC-RI did (Fig.4-1-2-3-2-11). No sign of a significant correlation between the variations of WBC-RI and the RI of any specific type of leukocytes was observed.

F.) RIs of the the erythrocytes indices i.e., HGB-RI, HCT-RI, MCV-RI, MCHC-RI and RDW-RI:

The retention indices of the different RBC indices (identified in section p.4-1-2-3-1-A) demonstrated diverse correlations with RBC-RI. Both HGB-RI and HCT-RI were significantly and positively correlated with RBC-RI ($p < 0.0005$), but this correlation was stronger in stage 4 than in stages 1-3 (HGB-RI: $r = 0.87$ vs. 0.43 ; HCT-RI $r = 0.96$ vs. 0.46). There was insignificant low positive correlation between RDW-RI and RBC-RI, but it was more remarkable in stage 4 than in stages 1-3 ($r = 0.43$ vs. 0.17). An inverse pattern of correlation with RBC-RI was delivered by MCV-RI, MCH-RI and MCHC-RI which demonstrated more remarkable negative effects with RBC-RI in stages 1-3 ($r = -0.26$ to -0.58) than in stage 4 ($r = -0.07$ to 0.28), as illustrated in Fig.4-1-2-3-2-12.

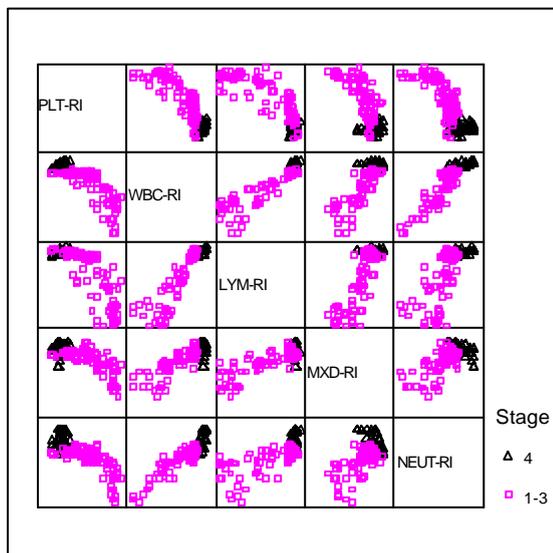


Fig. 4-1-2-3-2-12: Scatter matrix of the mutual correlations between the RIs of the main types of leukocytes, WBC-RI and PLT-RI.

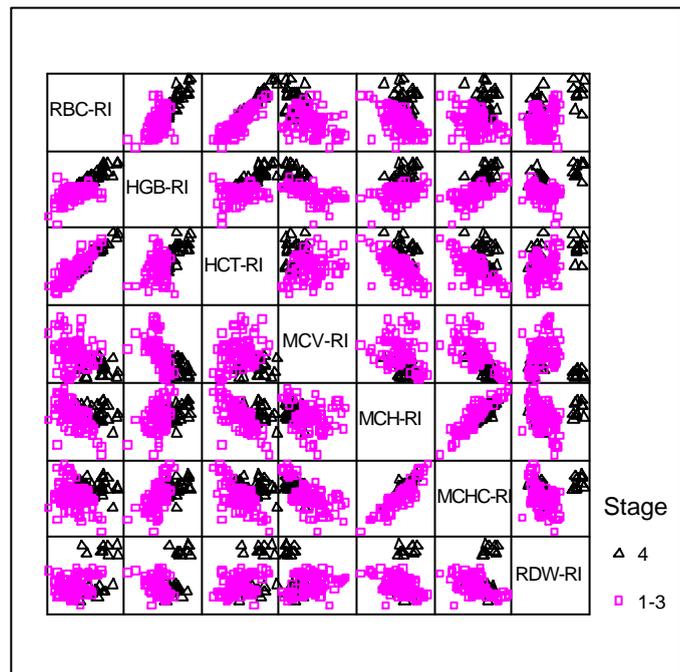


Fig. 4-1-2-3-2-11: Scatter matrix of the mutual correlations between the RIs of the main indices of erythrocytes and RBC-RI.

4-1-2-4. Morphological pilot study to the influence of mixing cWB on PLT count, PLT-RI and the interacting RIs: Addressing a standardized mixing method

4-1-2-4-1. Objectives and methodology

This study was designed as an attempt to interpret the unacceptable high means and variability of PLT-RI delivered in stages 1-3 of the improving protocol (p.4-1-2-2-2). The influence of different modes of mixing the blood on PLT clumping and PLT count as well as on the measurements of PLT-RI and the probable interacting retention indices (RIs) were investigated under morphological control by phase-contrast microscopy. We focused our interest on the vortex (V) as a mean of mixing the blood since it was recommended by the first SOP of RTH (p.3-4-3-2). Different modes of vortex were applied within each experiment where S.V, M.V, H.V, >H.V referred to slight, moderate, high, and further high (for about 1 min) vortex modes respectively. The study composed of series of small experiments and was performed on cWB from “healthy” blood donors (BD; p.3-1). We intended to reflect in this chapter *only the key experiments* where *two approaches* were considered on the influence of mixing the blood on PLT:

A. Experiments on resting cWB (First Approach)

Two experiments were performed on normal resting blood (undisturbed by vortex or centrifugation); the tested tubes were rested on rotator for 10 min before starting the experiments.

(a) First experiment: Blood drawn from one blood donor was distributed into 4 Epp tubes (Eppendorf tubes; p.3-2-2); 0.5 mL in each. Different vortex modes were applied on each Epp tube, and thereafter CBC (complete blood count; including PLT count) was performed on blood (Tab.4-1-2-4-2-a).

(b) Second experiment: 0.5 mL of the cWB drawn from one blood donor and collected in citrated SM tube (Sarstedt monovett 5 mL; p.3-2-1) was transferred into one Epp tube (p.3-2-2). Variant modes of vortex were successively applied on each of SM and Epp tube. A comparison between the influence of vortex on the blood in the big tube (SM) and that in the small one (Epp) was possible by performing CBC after the application of each vortex mode, as described in Tab.4-1-2-4-2-b.

B. Experiments on cWB after centrifugation and passing RTH filter (Second Approach)

After finding out the significant inverse correlation between the rate of vortexing the resting blood and the consequent PLT count in the first approach of experiments (p.4-1-2-4-1-A), we stepped ahead to examine this phenomenon on the blood in RTH tubes after centrifugation and passing RTH filter which might have serious impact on RTH assay reliability. The SOP-2 of RTH (p.4-1-1-7) was applied taking into further consideration the new RIs for the other blood cells identified in the enlarged practical protocol (WBC-RI, RBC-RI; p.4-1-2-3-1-A). The b.F measurements were performed on the SM tubes. *After the centrifugation of RTH tubes*, variant methods of mixing were applied on each tube before performing the a.F measurements. Three key experiments are mentioned here (p.4-1-2-4-3):

a.) First experiment: Blood was drawn from one blood donor and collected into 2 SM tubes. The blood from the first and the second SM tube was transferred then to 3 RTH tubes (“Series 1”) and to 4 RTH tubes (“Series 2”) respectively (0.5 mL each). Different modes of vortex were applied on “Series 1” after centrifugation. The results demonstrated the initial superiority of the “*slight vortex*” spelled by the earlier experiments on resting blood (p.4-1-2-4-3). Slight vortex was therefore applied after centrifugation in “Series 2” in comparison with mixing by a plastic pipette (successive aspiration and pipetting) for further validation, as described in Tab.4-1-2-4-3-a.

b.) Second experiment: To confirm the influence of vortex and standardize the “slight vortex” mode for mixing the a.F blood in RTH assay, a *second experiment* was done. Blood was drawn from one blood donor and distributed into 6 RTH tubes. After centrifugation, the blood was mixed by applying increasing vortex rates in comparison with mixing by pipette, as shown in Tab.4-1-2-4-3-b.

c.) Third experiment: Due to the evident influence of the inconvenient mix and/or vortex on the a.F measurements of RTH and the initial superiority of “slight vortex” revealed by the earlier experiments (p.4-1-2-4-3 & 4), further investigations were done to standardize the slight vortex mode in order to minimize possible variations and spurious results. Mixing by pipette and micropipette was used to compare data (Tab.Ab.4-1-2-4-3-d.). Among these investigations, we described here (Tab.4-1-2-4-3-c) one key example on the cases where PLT count was not significantly influenced by increased vortex rates and the slight vortex mode at least confirmed its superiority.

The findings of CBC and RTH assay in both approaches (p.4-1-2-4-1-A & B) were controlled by phase-contrast microscopy (p.3-5-2). The detailed results of the complete experiments of this pilot study (CBC and RTH measurements and their variations) are described in Ab.4-1-2-4. Only key examples representing the main successive steps of this study are summarized in the next sections (p.4-1-2-4-2 & 3) concluded by the description of the standardized “slight vortex” in section p.4-1-2-4-4.

4-1-2-4-2. Results of the key experiments studying the influence of vortex on resting cWB

a.) In *the first experiment* (Tab.4-1-2-4-2-a), the higher rate of vortex delivered significant decrease in PLT (81.1%) associated with a marked increase in WBC (29.9%), as demonstrated in Fig.4-1-2-4-2-a & b. Interestingly, the microscopic control showed no increase in WBC and revealed significant increases in PLT aggregates (Aggr: up to 70%) proportional to the decreases in PLT count. Moreover, significant amounts of these Aggr formed WBC like particles (Tab.4-1-2-4-2-a) that were positively correlated with the increased count of WBC.

Tables 4-1-2-4-2-a & b: Summary^a of the key results of the main two experiments studying the influence of vortex on resting cWB.

Table 4-1-2-4-2-a: First experiment.

Mixing Mode	PLT (x10 ³ /μL)	PLCR (%)	WBC (x10 ³ /μL)	RBC (x10 ⁶ /μL)	Microscopic Findings
A.F	122	38.9	6.7	4.15	Intact PLT, r. s.Aggr
M.V	102	41.0	7.1	4.16	Intact PLT, Aggr 10%: s+, m+++, b+. Ros++, Rol++
H.V	67	43.3	7.4	4.18	Intact PLT, Aggr 35%: s++, m+, b+++ . Ros++, Rol++
>H.V	23	31.0	8.7	4.02	Intact PLT, Aggr 70%: s+, m+++, b++. Ros+, Rol++

Table 4-1-2-4-2-b: Second experiment.

Test Tube	Mixing Mode	PLT (x10 ³ /μL)	PLCR (%)	WBC (x10 ³ /μL)	RBC (x10 ⁶ /μL)	Microscopic Findings
SM (5 mL)	A.F	175	19.3	3.8	3.76	Intact PLT, r. s.Aggr
	H.V	136	20.6	4.2	3.68	Intact PLT, Aggr 15%: s+++ , m+, b++. Ros+, Rol+
	Rest ^b	137	20.0	4.1	3.68	Intact PLT, Aggr 15%: s+++ , m+, b++. Ros+, Rol+
	M.V	111	25.2	4.2	3.72	Intact PLT, Aggr 25%: s+++ , m+, b++. Ros++, Rol+
	H.V	112	22.1	4.5	3.71	Intact PLT, Aggr 25%: s+++ , m+, b++. Ros++, Rol+
Epp (1.5 mL)	A.F	185	20.8	3.8	3.68	Intact PLT, r. s.Aggr
	H.V	106	21.3	5.2	3.65	Intact PLT, Aggr 35%: s++ , m+++ , b++. Ros+, Rol+
	>H.V ^c	18	NA	4.8	3.73	Intact PLT, Aggr 75%: s++ , m+, b+++ . Ros++, Rol+

^aThe detailed results of the 1st and the 2nd experiments are described in Tab.Ab.4-1-2-4-2-a & b respectively. ^bRest for 10 min. ^cEpp tube was rested for 60 min before applying further H.V. SM= Sarstedt monovett 5 mL (p.3-3-1); Epp= Eppendorf tube (p.3-2-2); A.F= after filling with a pipette; M.V, H.V = mixing the resting blood by relatively moderate and high vortex modes respectively; >H.V= further high vortex (for about 1 min); Intact PLT = PLT had intact discocytes with less than 10% echinodiscocytes and no spherical or fully activated forms; r=rare; Aggr % = the percentage of PLT aggregates; s, m, b = small (2-4 PLT), medium (5-17 PLT), and big (>18 PLT) particles of PLT aggregations; +, ++, +++ = the relative presence; Ros= rosettes forms (conjugates of PLT adhered around WBC); Rol= rouleaux (coin-rolls) forms of erythrocytes.

b.) Similarly to the first experiment, the increasing rates of vortex applied on the Epp tubes of the 2nd experiment delivered significantly elevated PLT count (90.3%) along with WBC increase (26.3%) associated with increased PLT aggregation (Aggr: 75%) found microscopically. But the increase in WBC declined slightly by applying further high vortex despite there was a permanent decrease in PLT count, as illustrated in Fig.4-1-2-4-2-b. This controversy could be microscopically explained by the size of the different Aggr (Tab.4-1-2-4-2-b). The coincidence of PLT decrease and WBC increase by applying increased vortex rates was less pronounced in the SM tube than in the Epp one; the PLT decrease was relatively lower (36.0% vs. 90.3%) and so was the increase in WBC (18.4% vs. 26.3%).

Fig. 4-1-2-4-2-a: PLT and WBC counts measured after filling (A.F) the Epp tube with blood and after applying moderate (M.V), high (H.V) and further high (>H.V) vortex modes.

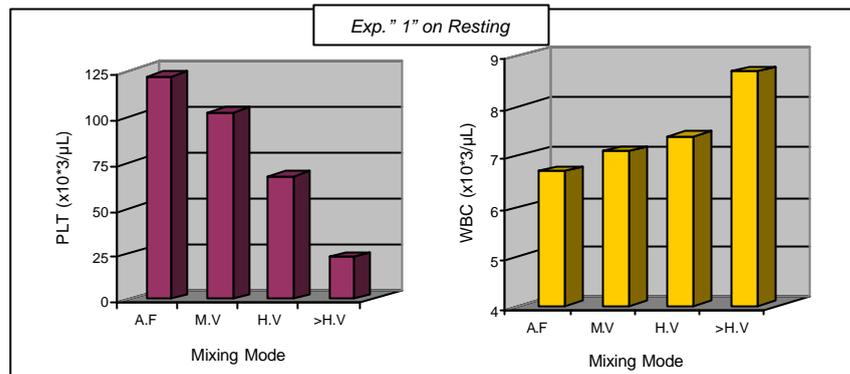
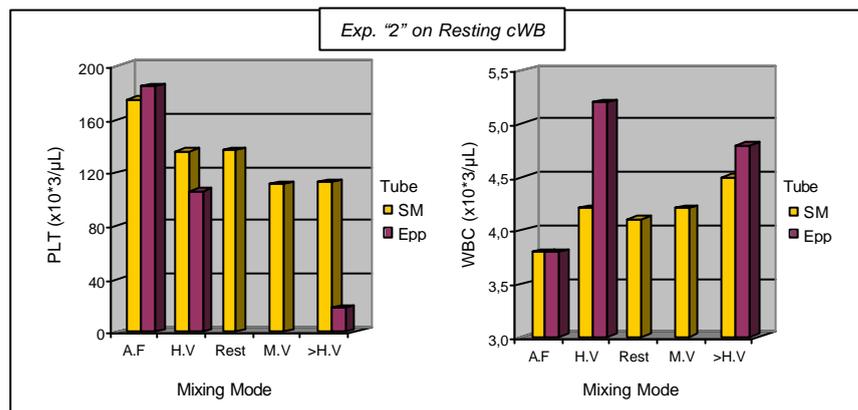


Fig. 4-1-2-4-2-b: PLT and WBC counts measured after filling (A.F) the tube with blood and after applying moderate (M.V), high (H.V) and further high (>H.V) vortex modes and a rest of 60 min (Rest).



Microscopically, the increases of Aggr in the blood in SM tubes observed after the suc-

cessive modes of vortex were proportional to the relatively low decreases in PLT count; the percentages of Aggr were found to be lower than those observed in the smaller tube i.e., Epp tube (25% vs. 75%). In both experiments, the decreases in PLT-RI were associated with relevant decreases in PLCR-RI and the microscopic control showed that PLT preserved their integrity after the different modes of vortex, as described in Tab.4-1-2-4-2-a & b. The satellitosis phenomenon (rosette forms; p.3-5-2) was encountered in all cases of the moderate and high vortex modes and so were the rouleaux forms of RBC. The rosette forms rose up markedly with the increase of PLT aggregations, as described in Tab.4-1-2-4-2-a & b.

4-1-2-4-3. Results of the key experiments performed on cWB after centrifugation and passing RTH filter

a.) The increased vortex rates applied on RTH tubes in the series "1" of the 1st experiment revealed evident decreases in the corresponding a.F measurements of PLT count (51%; Tab.4-1-2-4-3-a). This led to relevant increases in PLT-RI (40.1%), as illustrated in Fig.4-1-2-4-3-a1. The increases in PLT-RI were closely correlated with the incidences of Aggr (up to 35%) and rosette forms observed microscopically as well as with the significant decrease in WBC-RI (17.3%). The decrease of WBC-RI was attributed to the increase in a.F count of WBC (17.8%) which was proportional to the increase of WBC-like Aggr observed

microscopically (m.Aggr; Tab.4-1-2-4-3-a). Compared with WBC-RI, RBC-RI values decreased less significantly (1.9%). Nevertheless, the microscopic control showed no increase in the a.F counts of WBC or RBC. PLCR-RI increased significantly in parallel with PLT-RI (Fig.4-1-2-4-3-a1).

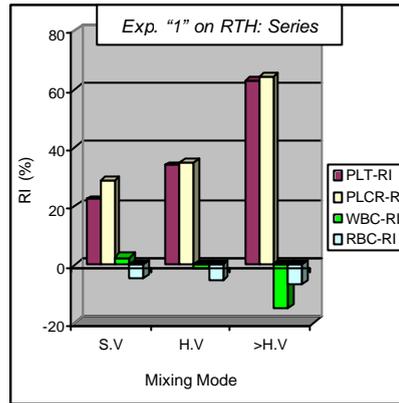


Fig. 4-1-2-4-3-a1: RIs measured at increased vortex modes in the 1st experiment on RTH.

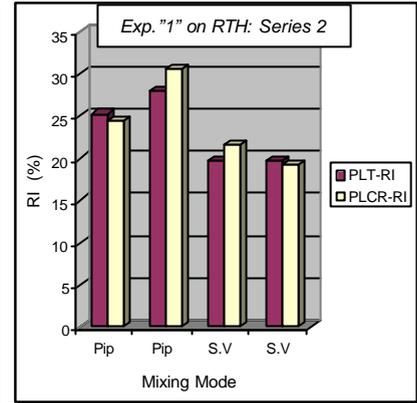


Fig. 4-1-2-4-3-a2: Comparison between the RIs delivered by mixing with pipette (Pip) and slight vortex (SV) in the 1st experiment on RTH.

In the series "2" of the 1st experiment, mixing by pipette led to slightly elevated PLT-RI values

(mean=26.6%) and higher variation (SD= 1.9) compared with the use of slight vortex mode (mean 19.8%. SD= 0.0; Fig.4-1-2-4-3-a2). Similar findings were spelled out by PLCR-RI values after mixing by pipette and by slight vortex (mean=27.6% vs. 20.4%; SD=4.3 vs. 1.7). Microscopically, PLT preserved their integrity and disposed few Aggr and rosette forms after mixing by pipette, while these forms were rare by using slight vortex mode (Tab.4-1-2-4-3-a).

b.) In the 2nd experiment, applying increased vortex rates delivered also proportional increases in PLT-RI levels (up to 48.6%; Fig.4-1-2-4-3-b) which were intimately related to the increases in Aggr (up to 50%) and in lower extent to the presence of rosette forms (Tab.4-1-2-4-3-b). PLT-RI increases were significantly associated with the marked decreases in WBC-RI (25.6%). The decreases in WBC-RI were evidently relevant to the increases in WBC like Aggr since no remarkable increase in WBC was found microscopically (Tab. and Fig. 4-1-2-4-3-b). The decreases in RBC-RI were less pronounced compared with those of WBC-RI (2.2% vs. 25.6%). The microscopic control revealed marked increases in RBC size-like Aggr by the application of higher vortex rates but no significant variation in the counts of RBC was seen. On the other hand, mixing by pipette led to low variation in PLT-RI comparable to that by using slight vortex mode (SD=0.8) but RBC-RI values were found to be positive and significantly higher by the use of pipette (2.9% to 9.1% vs. -1.9% to -2.2%; Tab. & Fig.4-1-2-4-3-b). This increases in RBC-RI was attributed to lower counts of RBC in a.F measurements (marked microscopically) due to inadequate mixing by pipette and it reflected the advantage of using slight vortex on pipette. PLCR-RI followed the same patterns of the variation spelled by PLT-RI, as demonstrated in Fig.4-1-2-4-3-b.

c.) Further experiments were performed to standardize the slight vortex. The results are presented in Tab.4-1-2-4-3-c & d, whilst the standardized vortex method is described in the next section (p.4-1-2-5). Among these experiments, we mention here in the 3rd experiment, the results of a key example (Tab.4-1-2-4-3-c) where increased vortex rates did not lead to significantly higher levels of PLT-RI compared with that spelled by the slight vortex (17.6% vs. 17.3%). Beyond it, there were even higher variations in PLT-RI as well as errors in the other RIs of RTH (PLCR-RI and RBC-RI; Fig.4-1-2-4-3-c). Mixing by slight vortex was superior to the use of pipette since the latter demonstrated higher variation in PLT-RI (SD=7.2 vs. 1.0) as well as in the other RIs (Tab.4-1-2-4-3-c), as shown in Fig.4-1-2-4-3-c. Microscopically, PLT discocytes were intact demonstrating rare Aggr and rosette forms. No configurations of RBC

rouleaux were objected. No remarkable variation in WBC or RBC counts was observed microscopically in all RTH tubes except for the 2nd RTH tube which was mixed by pipette and showed few Aggr (<10%), as described in Tab.4-1-2-4-3-c.

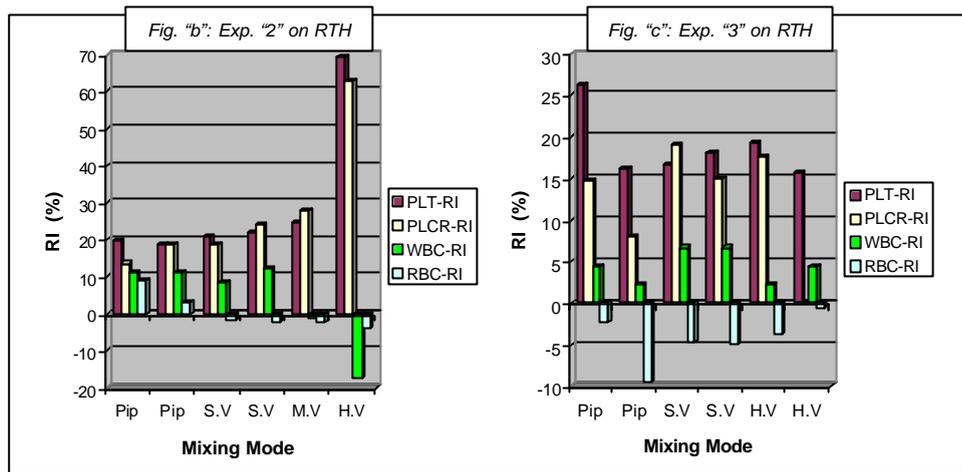


Fig. 4-1-2-4-3-b & c: RIs delivered by the use of pipette (Pip) in comparison with increasing vortex rates in the 2nd and the 3rd experiments on RTH.

Tables 4-1-2-4-3-a to c: Key* results of the main three experiments studying the influence of vortex and mixing the blood after centrifugation and passing RTH filter on PLT-RI and the main interacting retention indices (RIs).

Table 4-1-2-4-3-a: First experiment.

Tube No.	Measurement	time min	PLT x10 ³ /μL	PLCR %	WBC x10 ³ /μL	RBC x10 ⁶ /μL	PLT-RI %	PLCR-RI %	WBC-RI %	RBC-RI %	Microscopic Findings
SM1 "1 st Series"	b.F	12	182	32.3	7.5	3.74					Intact, r. s.Aggr
	a.F #1. S.V	22	141	29.6	7.3	3.91	22.5	29.0	2.7	-4.5	Intact, r. s-m.Aggr. r.Rol
	a.F #2. H.V	31	120	31.7	7.6	3.94	34.1	35.3	-1.3	-5.3	Intact, Aggr 10%: s+++; m+, b++. Ros+, Rol+
	a.F #3. >hv	36	68	30.7	8.6	3.98	62.6	64.5	-14.7	-6.4	Intact, Aggr 35%: s+, m+++; b++. Ros+++; Rol+
SM2 "2 nd Series"	b.F	10	182	30.0	6.9	3.74					Intact, r. s.Aggr
	a.F #1. Pip	24	136	30.3	6.9	3.85	25.3	24.5	0.0	-2.9	Intact, Aggr 5%: s+, m+++; b+. r.Ros, r.Rol
	a.F #2. Pip	27	131	28.9	6.3	3.98	28.0	30.7	8.7	-6.4	Intact, Aggr 10%: s+++; m+, b++. r.Ros, r.Rol
	a.F #3. S.V	28	146	29.3	6.7	3.95	19.8	21.7	2.9	-5.6	Intact, r. s-m.Aggr, r.Ros. r.Rol
a.F #4. S.V	31	146	30.2	6.5	3.86	19.8	19.2	5.8	-3.2	Intact, r. s-m.Aggr, r.Ros. r.Rol	

Table 4-1-2-4-3-b: Second experiment.

Measurement	time min	PLT x10 ³ /μL	PLCR %	WBC x10 ³ /μL	RBC x10 ⁶ /μL	PLT-RI %	PLCR-RI %	WBC-RI %	RBC-RI %	Microscopic Findings
b.F	10	179	21.3	8.2	4.18					Intact, r. s.Aggr
a.F #1. Pip	23	144	22.9	7.3	3.80	19.6	13.5	11.0	9.1	Intact, r. s-m.Aggr, r.Rol
a.F #2. Pip	24	146	21.3	7.3	4.06	18.4	18.4	11.0	2.9	Intact, r. s-m.Aggr, r.Rol
a.F #3. S.V	27	142	21.9	7.5	4.26	20.7	18.4	8.5	-1.9	Intact, r. s-m.Aggr, r.Rol
a.F #4. S.V	36	140	20.7	7.2	4.27	21.8	24.0	12.2	-2.2	Intact, r. s-m.Aggr, r.Rol
a.F #5 M.V	29	135	20.3	8.3	4.28	24.6	28.1	-1.2	-2.4	Intact, Aggr <5%: s+, m+++; r.Ros, r.Rol
a.F #6. H.V	35	55	25.6	9.6	4.35	69.3	63.1	-17.1	-4.1	Intact, Aggr50%: s+, m+++; b++. Ros+, Rol+

Table 4-1-2-4-3-c: Third experiment.

Measurement	time min	PLT x10 ³ /μL	PLCR %	WBC x10 ³ /μL	RBC x10 ⁶ /μL	PLT-RI %	PLCR-RI %	WBC-RI %	RBC-RI %	Microscopic Findings
b.F	11	217	27.5	4.5	3.47					Intact, r. s.Aggr
a.F #1 Pip	31	160	31.8	4.3	3.55	26.3	14.7	4.4	-2.3	Intact, Aggr <10%: s+, m+, b+. r.Ros
a.F #2 Pip	33	182	30.2	4.4	3.80	16.1	7.9	2.2	-9.5	Intact, r. s-m.Aggr, r.Ros
a.F #3 S.V	34	181	26.7	4.2	3.63	16.6	19.0	6.7	-4.6	Intact, r.s-m.Aggr, r.Ros
a.F #4 S.V	36	178	28.5	4.2	3.64	18.0	15.0	6.7	-4.9	Intact, r.s-m.Aggr, r.Ros
a.F #5 H.V	38	175	28.1	4.4	3.6	19.4	17.6	2.2	-3.7	Intact, r.s-m.Aggr, r.Ros
a.F #6 H.V	38	183	32.6	4.3	3.49	15.7	0.0	4.4	-0.6	Intact, r.s-m.Aggr, r.Ros

* The detailed results of the 1st, the 2nd, and 3rd experiments are described in Tab.Ab.4-1-2-4-3-a, b & c respectively. SM= Sarstedt monovett 5 mL (p.3-3-1). b.F= the measurements performed on cWB before passing RTH Filter i.e., on the blood collected in SM tubes (p.3-4-1); a.F #1, a.F #2, etc = the measurements performed on the blood after passing RTH filter i.e., in the lower part of RTH tube (p.3-2-1); S.V, M.V, H.V, >H.V= mixing the a.F blood by relatively slight, moderate, high, and further high (for about 1 min) vortex modes respectively; Pip= mixing the a.F blood by successive aspiration and pipetting by a plastic pipette; Intact = PLT had intact discocytes with less than 10% echinodiscocytes and no spherical or fully activated forms; r=rare; Aggr % = the percentage of PLT aggregates; s, m, b= small (2-4 PLT), medium (5-17 PLT), and big (>18 PLT) particles of PLT aggregations; +, ++, +++ = the relative presence; Ros= rosettes forms (conjugates of PLT adhered around WBC); Rol= rouleaux (coin-rolls) forms of erythrocytes.

4-1-2-4.4. Standardized method for the vortex of RTH tubes after centrifugation

The results of the morphological pilot study of “blood mixing effect” (p.4-1-2-4-2 & 3) proved the superiority as well as the convenience of the slight vortex mode for mixing the a.F blood (after centrifugation and passing RTH filter) in order to minimize PLT clumping and the variations in the a.F measurements. The resulting standardized slight vortex could be consequently described as follows:

“Vortex should be performed carefully using slight vortex (vortex speed= 3.3-3.7; p.3-1-5) 3 times before the 1st measurement and 2 times for further measurements; <2 seconds for each one-time vortex (=3 sec per measurement). The rate and the time of vortex for the further a.F measurements depend on the degree of sedimentation of the cWB pipetted in Eppendorf tubes. However, the aF measurements on the blood should be performed within 1-2 min after vortex to avoid further sedimentation. Frequent vortex and rest may lead to unacceptable results. The blood should be mixed thoroughly by vortex without forming foams or air bubbles. The Eppendorf tube has to be placed in the center of the plate of the vortex apparatus and hanged from the top of the Eppendorf tube. “Over-vortex” may lead to unacceptable PLT hyperactivation and PLT aggregation that is demonstrated in falsely high PLT-RI levels with unacceptable high variation (p.4-1-2-4-2 & 3). Inadequate mixing may lead to falsely high levels of PLT-RI associated with falsely high levels of HGB-RI (p.4-1-2-4-3-b).”

4-1-2-5. Addressing the third SOP of RTH assay (SOP-3)

The application of the standardized vortex method led to an essential improvement (p.4-1-2-3-2). We addressed therefore the third SOP (SOP-3) of RTH which included the same procedures as mentioned in SOP-2 (p.4-1-1-7) besides considering the following aspects: **A.)** Tested samples should be carefully handled especially after passing RTH filter (a.F). The a.F blood should be mixed by the new standardized vortex (=3 sec; p.4-1-2-4-4) instead of 3 min as in SOP-1 (p.3-4-3-2). The a.F measurements should be performed within 1-2 min after mixing to avoid errors attributed to blood sedimentation (p.5-1-2-7-C-b). **B.)** RTH should be performed within 120 min after blood withdrawal to avoid spurious reduced results (Fig.4-1-2-3-2-2). **C.)** The new identified RIs that revealed significant implication on the interpretation of RTH (i.e., MPV-RI, PDW-RI, WBC-RI, RBC-RI, HGB-RI and HCT-RI; p.5-1-2-3, 4, 6) were termed “secondary” RIs and could be calculated in parallel with the “main” RIs (PLT-RI and PLCR-RI) as far as required. **D.)** The reliable results after applying the standardized vortex (p.4-1-2-3-2) support firmly the reliability of our centrifugation condition (110 g/ 5 min). Thus, the SOP-3 of RTH may be summarized as follows:

4-1-2-5-1. 500 µL of cWB (or the tested material) is pipetted carefully into the inner surface of the upper part of the RTH tube (p.3-1-2).

4-1-2-5-2. *The b.F measurements:* CBC is performed on the blood in the collection tube (p.3-4-1).

4-1-2-5-3. *Centrifugation:* The RTH tube is centrifuged at 110 g/ 5 min.

4-1-2-5-4. *The a.F measurements:* The upper part of RTH tube is removed and the cWB (in the lower part of RTH tube; p.3-2-1) is mixed thoroughly by slight vortex (standardized slight vortex; p.4-1-2-4-4). CBC is measured shortly afterwards (1-2 min) by a cell counter.

Note 1: RTH should be performed within 120 min after the withdrawal of the blood.

Note 2: It is preferable to perform the measurements *in-double* and the mean is considered for further calculations of the main RIs as well as the secondary RIs.

4-2. Validation and quality control of the third SOP (SOP-3) of RTH

4-2-1. Inspection of the specificity of RTH filter by measuring RIs with and without RTH filter

4-2-1-1. Aim and methodology of the study

This study was designed to inspect whether the acceptable precision of RTH resulted by applying the third SOP of RTH (implicating the new standardized vortex; p.4-1-2-3-2) was attributed only to precise procedures or to a further specific effect of RTH filter on PLT in cWB. We suggested that by passing RTH filter, PLT are retained *specifically* leading to a significant decrease in PLT count. The blood withdrawn from a given healthy blood donor was collected in a citrated tube (p.3-3-1). The citrated blood (cWB) was distributed into one RTH tube (p.3-2-1) and into one Epp tube (without RTH filter; p.3-2-2); 500 µL in each. Both RTH and Epp tubes were centrifuged according to SOP-3 of RTH (p.4-1-2-5) and the a.F measurements were performed in-double on both tubes after centrifugation. The RIs (retention indices) of RTH for the cWB in both RTH and Epp tubes were calculated by the comparison with the b.F measurements performed on the collection citrated tube. Sysmex SF 3000 was used for performing CBC (complete blood count) in this study. A comparison of the resulting RIs i.e., with and without using RTH filter, was held to inspect our suggestion about the specificity of RTH filter.

4-2-1-2. Results of performing RTH with and without RTH filter

Table 4-2-1-2: Descriptive statistics^a of the results of performing RTH with and without RTH filter.

Parameter	RTH Filter ^b	(n)	Minimum (%)	Maximum (%)	Mean (%)	SD	KS ^c P	t-Test ^d p
PLT-RI (%)	with	19	16.9	31.6	23.8	4.3	0.200 ^e	
	no	19	-7.3	10.1	3.2	3.9	0.200 ^e	<0.0005
PLCR-RI (%)	with	19	3.4	29.7	18.4	8.9	0.047	
	no	19	-25.7	23.3	-1.2	11.2	0.073	<0.0005
PDW-RI (%)	with	19	-27.0	4.2	-7.6	7.9	0.089	
	no	19	-17.6	8.1	-4.1	5.8	0.200 ^e	0.063
MPV-RI (%)	with	19	-13.1	2.6	-2.9	4.1	0.200 ^e	
	no	19	-7.1	7.5	-1.5	3.4	0.200 ^e	0.071
WBC-RI (%)	with	19	-1.1	12.6	6.3	3.6	0.200 ^e	
	no	19	-0.1	14.7	7.3	3.9	0.200 ^e	0.224
RBC-RI (%)	with	19	-8.1	4.1	-1.9	2.8	0.200 ^e	
	no	19	-1.1	13.3	3.4	4.0	0.200 ^e	<0.0005
HGB-RI (%)	with	19	-7.5	3.8	-2.1	3.1	0.200 ^e	
	no	19	-2.2	12.5	3.1	4.0	0.093	<0.0005
HCT-RI (%)	with	19	-7.5	3.5	-1.6	2.6	0.067	
	no	19	-1.1	13.5	3.6	4.0	0.200 ^e	<0.0005
a.PLT (x10 ³ /µL)	with	19	116.0	232.0	166.5	33.9	0.200 ^e	
	no	19	143.0	292.0	210.8	37.6	0.200 ^e	<0.0005
PLT increase (%)		19	14.4	39.7	27.3	7.5		
PLT-RI decrease (%)		19	11.9	29.1	20.6	5.0		
PLCR-RI decrease (%)		19	3.5	32.3	19.5	7.5		

^aThe detailed results are reported in Tab.Ab. 4-2-1-2. ^bcWB was centrifuged with (with) the presence of RTH filter (by using RTH tubes; p.3-2-1) as well as without (no) the presence of RTH filter (by using Eppendorf tubes; p.3-2-2); ^cThe normality of distribution by Kolmogorov-Smirnov test; Lilliefors significance correction. ^dThe significance of difference by paired samples t-test. ^eThis is a lower bound of the true significance.

a.PLT= mean of the in-double measurements of PLT count for both RTH and Epp tubes after centrifugation; PLT increase= the increase of PLT count determined in Epp tubes after centrifugation compared with that in RTH tubes; PLT-RI decrease, PLCR-RI decrease= the decrease in PLT-RI and PLCR-RI respectively determined without RTH filter compared with that with the presence of RTH filter.

Fig. 4-2-1-2-1 to 6: Retention indices determined by the use of RTH tubes (RTH Filter) compared with Epp tubes (No Filter).

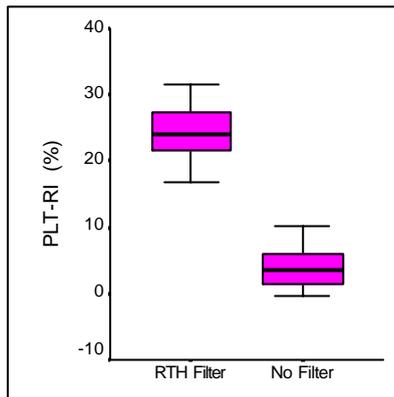


Fig. 4-2-1-2-1.

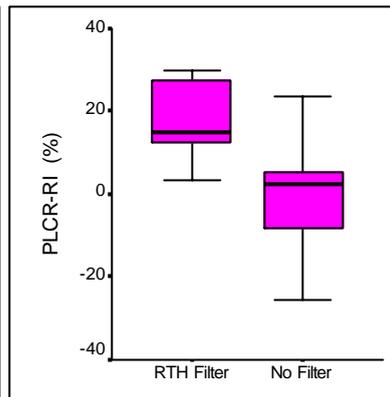


Fig. 4-2-1-2-2.

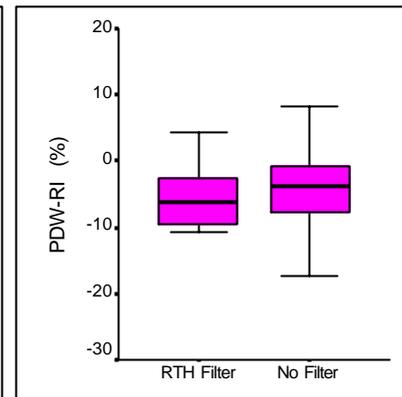


Fig. 4-2-1-2-3.

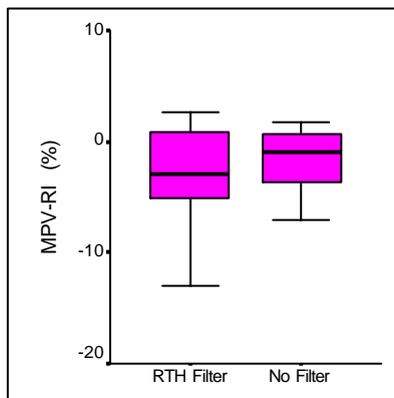


Fig. 4-2-1-2-4.

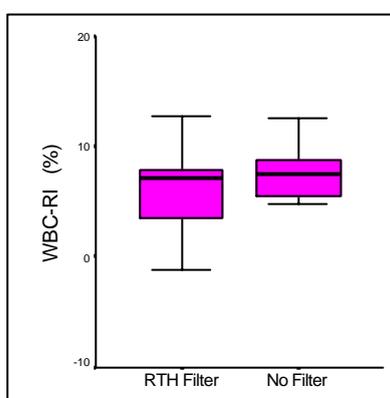


Fig. 4-2-1-2-5.

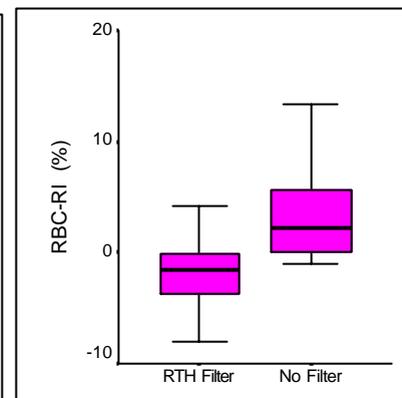


Fig. 4-2-1-2-6.

This study was performed on the cWB of 19 blood donors and the detailed results are reported in Tab.Ab.4-2-1-2 and the statistics of the key results are described in Tab.4-2-1-2. The distribution of RIs detected with and without RTH filter were mainly in line of normal assumption ($p > 0.05$; Tab.4-2-1-2). The levels of PLT-RI as well as PLCR-RI were significantly ($p < 0.0005$) lower in Epp than in RTH tubes (3.2 vs. 23.8%; and -1.2 vs. 18.4% respectively), as demonstrated in Fig.4-2-1-2-1 & 2. The mean of this decrease was estimated at 20.6% and 19.5% for PLT-RI and PLCR-RI respectively. No significant correlation was objected between PLT-RI values detected with and that without RTH filter ($p > 0.2$). PDW-RI and MPV-RI values in Epp tubes (no RTH filter) were elevated remarkably ($p > 0.05$) compared with that in RTH tubes (mean= 4.2% vs. 8.1% and 2.6% vs. 7.5% respectively), as illustrated in Fig.4-2-1-2-3 & 4. The mean and the variation of WBC-RI values in RTH tubes were slightly higher than that in Epp tubes (Fig.4-2-1-2-5). The negative mean of RBC-RI detected in RTH tubes (-1.9%) demonstrated a significant increase ($p < 0.0005$) and converted to a positive value (3.4%) in Epp tubes (Fig.4-2-1-2-6). The RIs (retention indices) of HGB as well as HCT in RTH and Epp tubes varied in parallel with RBC-RI, as described in Tab.4-2-1-2.

4-2-2. Specificity of RTH performed on citrated whole blood (cWB) compared with platelet isolated by gel-filtration (iPLT)

4-2-2-1. Aim and methodology of the pilot study “Sepharose” gel-filtered platelets

This study was designed to inspect the reliability of applying RTH on cWB in comparison with isolated PLT (iPLT; isolated from cWB by gel-filtration using Sepharose 2B; p.3-4-4) related to morphological control by PLT spreading. This study represented, therefore, a further step in the investigation of the specificity and the accuracy of RTH as a "simple" assay for screening the function of PLT in whole blood. The study was also performed as an early step to prepare a non-fixed PLT standard i.e., from native PLT.

4-2-2-1-1. Protocol carried out in the “Sepharose” pilot study

- 4-2-2-1-1-1. Blood is withdrawn from blood donors and collected into 10 mL isotonic citrated plastic tubes (10 mL SM; p.3-4-1).
- 4-2-2-1-1-2. RTH assay is performed on 0.5 mL cWB (SOP-3; p.4-1-2-5)⁶.
- 4-2-2-1-1-3. Citrated PRP (cPRP) is prepared from the remainder amount of cWB (SOP; p.3-4-2) and then transferred to a plastic tube. The time of centrifugation at 110 g⁶ was set on 30 min instead of 15 min (p.3-4-2) to obtain further amount of cPRP.
- 4-2-2-1-1-4. Platelet isolation using chromatography by "Sepharose 2B (gel-filtration) is applied on the “resting” cPRP.
- 4-2-2-1-1-5. The iPLT obtained by the Sepharose chromatography column (suspended in PLT buffer; p.3-3-6) are collected in 3 plastic tubes (T1-T3) and rested in 37°C water bath chamber for 30 min.
- 4-2-2-1-1-6. RTH assay is performed on 0.5 mL of the iPLT by the use of the collecting tube that bears the highest PLT count (mainly T2, occasionally T1; SOP; p.3-4-4-3-6).
- 4-2-2-1-1-7. Morphological control on RTH assay. PLT spreading⁷ was performed on both on cWB and iPLT before (b.F) and after (a.F) the passage of RTH filter. For the b.F control of cWB, preparations were done from the cWB in the original collecting tube (10 mL SM). The b.F control of iPLT was performed by use of the same collection tube on which RTH was performed (p.4-2-2-1-1-6).

Note 1: The b.F and a.F measurements were done at least twice and the mean values of the 1st and the 2nd measurements were used for calculating the retention indices (RI).

Note 2: PLT isolation was performed in the Dept. of experimental surgery. The available automatic cell counter “Coulter Act” did not allow to determine the whole CBC (e.g., PLCR, PDW, etc), but it was helpful for our main interest to measure PLT count for performing RTH as well as the RDW index for controlling the quality of the isolation of PLT. For samples 10-12, it was possible to perform CBC by use of

⁶ The centrifugation speed of the available centrifuge in the central laboratory of the Dep. of experimental surgery was specified to comply with the standard conditions (110 g) for preparing cPRP (p.3-4-2) as well as performing RTH (p.4-1-2-4-4-4). Considering the radius (r) of the used centrifuge and the equation of RCF (p.3-2-3), the centrifugation speed that is equivalent to 110 g was found to be 900 rpm.

⁷ By experiencing and evaluating PLT spreading, we found that the dilution performed before PLT spreading should consider the PLT count of the tested samples i.e., of cWB or iPLT before as well as after the passage of RTH filter and not the PLT count of the original withdrawn cWB, as mentioned in the original SOP of PLT spreading (p.3-4-5-2-3).

Sysmex K1000 counter in the Institute of Clinical Hemostaseology in order to gain further PLT size measurements i.e., PLCR and PDW.

4-2-2-1-2. Quality control of platelet isolation by gel-filtration using Sepharose 2B

The gel-filtration of PLT from cWB by using Sepharose 2B proved to deliver high quality of purely isolated PLT (iPLT). The PLT spreading test (Tab.4-2-2-3) showed that: **a.)** iPLT were intact, unstimulated, and disposing no Aggl or Aggr. **b.)** iPLT preserve their function which was demonstrated by the very well spread preparations. **c.)** In concordance with the regular "0" value of RDW in the iPLTsuspension (Tab.Ab.4-2-2-1-2-T1-T3), the PLT spreading showed no amounts of RBC or WBC regularly associated with iPLT (Tab.4-2-2-3). The significantly positive levels of the PLT-RI of iPLT reflect their ability to adhere to RTH filter and delivers further evidence on their integrity and function capabilities. The semi-quantitative technique of the isolation (p.3-4-4-3) allows collecting the iPLT in 3 tubes (T1, T2 & T3) and the highest recovery is supposed in the 2nd tube (T2). The detailed results of the repeated measurements on iPLT in the collection tubes (T1-T3) are described in Tab.Ab.4-2-2-1-2-T1-T3. Quantity assessment of the PLT isolation was done by comparing the PLT count obtained by the successive collection tubes of iPLT with that of original cWB, as shown in Fig.4-2-2-1-2. This figure demonstrated that the highest concentration of iPLT was found in T2. The only exception was sample No.3, where RTH assay and the morphologic control were applied on the first collection tube (T1) since it had a higher PLT count than T2. These findings together support at least the reliability of using Sepharose gel to isolate PLT for the research purposes.

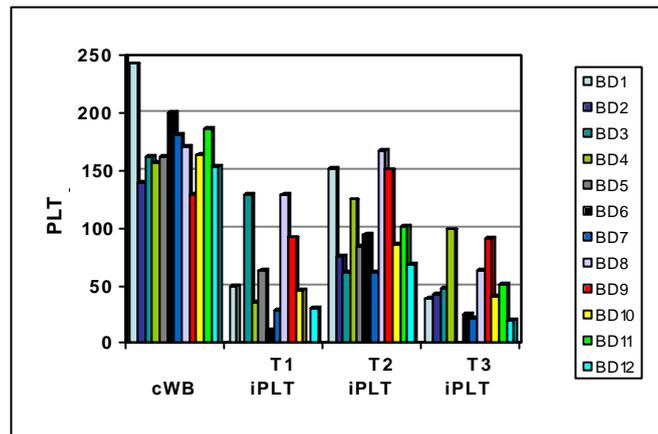


Fig. 4-2-2-1-2: Comparison of PLT count of the iPLT in the collection tubes (T1-T3) with that of the original cWB drawn from the different blood donors (BD).

4-2-2-1-3. Controlling the reproducibility of PLT count & PLT size measurements for both cWB and iPLT before and after passing RTH filter

PLT count and the parameters of PLT size distribution in cWB and iPLT before and after the passage of RTH filter were performed at least twice to assure the accuracy and the precision of the measurements. The times of repeating the measurements depended on the amount of the tested sample. The a.F measurements were repeated not more than 3 times since the a.F amount of the tested sample was less than 0.5 mL. Whereas the b.F amount (i.e., in the collection tube) was sufficient for further repetitions of the b.F measurements. The measurements performed on the different iPLT collection tubes were also repeated to assure the accuracy. The reproducibility of the whole measurements was acceptable in general, and the means of these measurements were considered for further calculations. The variability of PLT count in the different stages was acceptable (mean CV<3.4%), but it was higher in the a.F iPLT (up to 6.3%) due to their relatively low PLT counts (mean =45x10³/μL). The detailed results of controlling the reproducibility are described in Tab.Ab.4-2-2-1-3-1 to 4.

4-2-2-2. Results of RTH and distribution of PLT size measures for both cWB and iPLT

The “Sepharose” protocol (p.4-2-2-1-1) was applied on 12 blood donors and performed in 2 batches; 7 and 5 samples respectively. The key results of the 1st and the 2nd batch are summarized in Tab.4-2-2-2-1 and 2 respectively. The PLT-RI values of iPLT in the 1st batch compared with that of cWB (mean= 61.7% vs. 33.0%) demonstrated significant increases ranging from 40.6% to 181.4%: (mean PLT-RI increase= 92.8%; $p=0.018$ by Wilcoxon test), as illustrated in Fig.4-2-2-2-1.

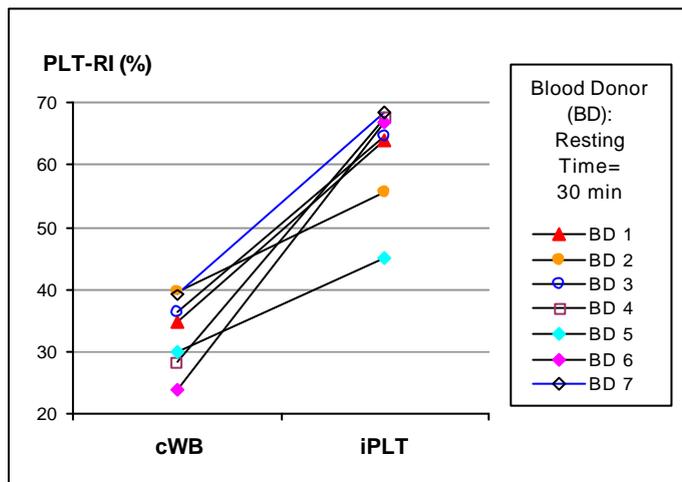


Fig. 4-2-2-2-1: PLT-RI increase in isolated PLT (iPLT) by gel-filtration compared with cWB.

Table 4-2-2-2-1: Key^a results of RTH measurements performed on cWB and iPLT in the first batch.

Serial	Sample ^b	PLT Count ($\times 10^3/\mu\text{L}$)		PLT-RI (%)	PLT-RI Increase (%)
		b.F	a.F		
1	cWB	243	159	34.6	
	iPLT	152	55	63.8	84.6
2	cWB	139	84	39.6	
	iPLT	76	34	55.6	40.6
3	cWB	162	104	36.1	
	iPLT	129	46	64.7	79.2
4	cWB	157	112	28.4	
	iPLT	125	41	67.5	137.3
5	cWB	163	115	29.8	
	iPLT	84	46	45.2	52.0
6	cWB	201	153	23.7	
	iPLT	95	32	66.7	181.4
7	cWB	182	111	39.1	
	iPLT	62	20	68.3	74.6

^a The results of PLT count described herein are the mean values of repeated measurements; a summary of the mean values of the other parameter (MPV, RDW) is described in Tab.Ab.4-2-2-2-1, whilst the details of the repeated measurements are described Tab.Ab.4-2-2-1-3-1 to 4.

^b The time of resting the gel-filtered platelets before applying RTH (p.4-2-2-1-1-5) was 30 min. iPLT= platelets isolated by gel-filtration; b.F= before passing RTH filter; a.F= after passing RTH filter; PLT-RI Increase= the increase of PLT-RI value in iPLT compared with that in cWB.

Table 4-2-2-2-2: Key^a results of RTH measurements performed on cWB and iPLT in the second batch.

Serial	Sample	Rest Time ^b (min)	PLT Count ($\times 10^3/\mu\text{L}$)		PLT-RI (%)	PLT-RI Increase (%)	PLCR (%)		PLCR-RI (%)	PLCR-RI Increase (%)
			b.F	a.F			b.F	a.F		
8	WB		171	131	23.4		NA	NA		
	iPLT	60	167	61	63.8	172.6	NA	NA		
9	WB		130	100	23.1		NA	NA		
	iPLT	30	151	76	49.5	114.5	NA	NA		
10	WB		164	107	34.8		20.3	24.6	20.7	
	iPLT	120	86	41	52.6	51.4	11.7	11.2	54.7	163.5
11	WB		186	141	24.5		23.3	22.9	25.8	
	iPLT	60	101	58	43.1	76.1	16.3	18.0	37.1	44.1
12	WB		153	109	29.1		17.7	18.3	26.7	
	iPLT	30	69	36	48.2	65.6	7.6	8.5	42.3	58.7

^a The results of PLT count described herein are the mean values of repeated measurements; a summary of the mean values of the other parameter (MPV, RDW) is described in Tab.Ab.4-2-2-2-2, whilst the details of the repeated measurements are described Tab.Ab.4-2-2-1-3-1 to 4.

^b The periods of resting the platelets isolated by gel-filtration iPLT before applying RTH (p.4-2-2-1-1-5).

iPLT= platelets isolated by gel-filtration; b.F= before passing RTH filter; a.F= after passing RTH filter; PLT-RI Increase and PLCR-RI Increase= the increase in iPLT compared with that in cWB; NA= not available in the menu of the used cell counter (p.4-2-2-1-1).

As an attempt to investigate whether this increase of PLT-RI values in iPLT compared with cWB was due to the activation of the iPLT after passing through Sepharose gel, increasing time for the resting periods of iPLT (bath chamber: 37 °C/ 30-120 min; p.4-2-2-1-1-5) were applied on the 2nd batch (samples 8 to 12) to assure iPLT deactivation before performing RTH, as described in Tab.4-2-2-2-2. The increase of PLT-RI in the 2nd batch (PLT-RI increase= 51.4-172.6%: mean= 96.1; Tab.4-2-2-2-2) was comparable to that found in the first one. By reviewing the results of the two batches combined (Tab.4-2-2-2-1 & 2), no significant correlation was objected between the periods of resting iPLT and the increase of PLT-RI ($p>0.5$; Spearman's rho). Thus, the results of PLT-RI in iPLT compared with that in cWB in batches 1 and 2 combined (samples 1-12: mean PLT-RI= 57.4% vs. 30.5%) were demonstrated in Fig.4-2-2-2-2 in order to illustrate the significance of PLT-RI increase (mean PLT-RI increase= 94.2%: $p=0.002$; Wilcoxon test). In parallel with the increase in PLT-RI, a similar sharp increase in PLCR-RI of iPLT (mean=44.7%) compared with that of cWB (mean=24.4%) was objected (PLCR-RI increase=44.1-163.5%: mean= 88.8%), as illustrated in Fig.4-2-2-2-2.

Fig. 4-2-2-2-2:
PLT-RI in cWB and PLT isolated by gel-filtration (iPLT) using increased resting periods for iPLT.

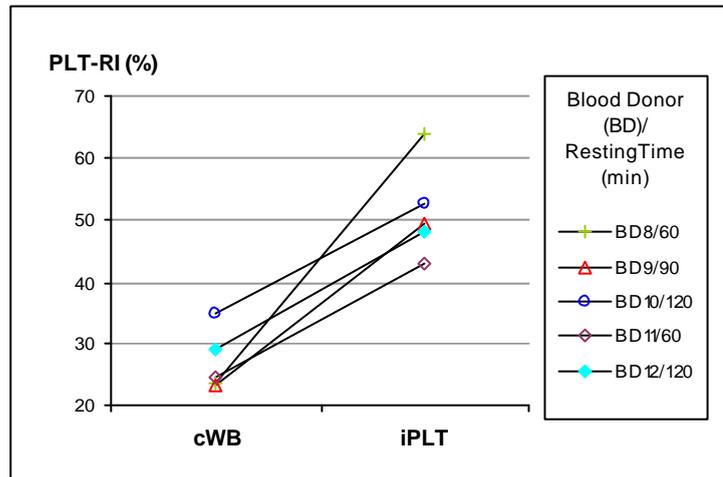
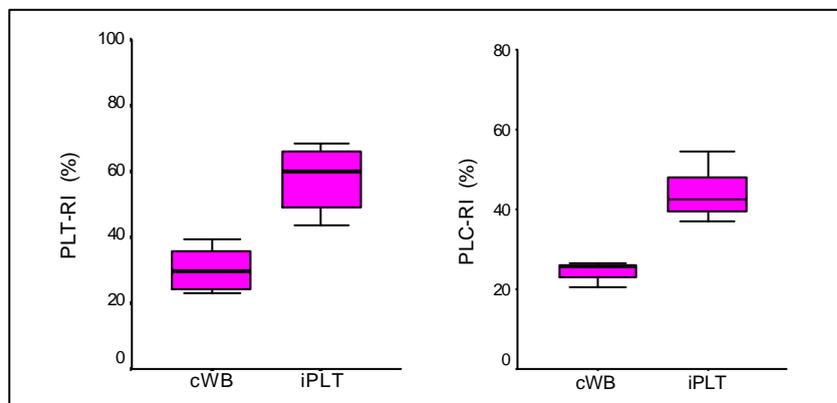


Fig. 4-2-2-2-2:
Increase of PLT retention in PLT isolated by gel filtration (iPLT) compared with cWB for the same blood donors.



The values of MPV-RI were remarkably higher in iPLT than in cWB (mean= 2.5% vs. 1.7%) whilst PDW-RI measurements demonstrated marked decrease in iPLT compared with cWB (mean= -2.6 vs. -1.1), as described in Tab.Ab.4-2-2-2-1 & 2.

4-2-2-3. PLT spreading for cWB and iPLT before and after passing RTH filter

The results of PLT spreading by the use of cWB and iPLT before (cWB-b.F; iPLT-b.F) and after (WB-a.F; iPLT-a.F) the passage of RTH filter are summarized in Tab.4-2-2-3. PLT spreading demonstrated, in general, normal findings in cWB-b.F with rather few aggregates (Aggr) and/or agglutinates (Aggl). The test of cWB-a.F presented non-spread PLT (inhibition) and few Aggr and/or Aggl, if any. PLT were well spread in iPLT-b.F and demonstrated few Aggl. The iPLT-a.F delivered light preparations that were thinner than those of cWB-a.F. The PLT in iPLT-a.F preparations were partly spread and demonstrated lot of Aggl. Intermediate forms of spread PLT were frequently found in iPLT-a.F. The PLT spreading showed occasionally disrupted PLT especially after passing RTH filter.

Table 4-2-2-3: Summary of the results of the PLT spreading performed on the samples 1-12.

Sample No.	cWB-b.F	cWB-a.F	iPLT-b.F	iPLT-a.F
1	Normal s. Aggr, Aggl -	Thin, not spread 70-80%, disrupted 10% rare s. Aggr & Aggl	Normal rare s. Aggl	Thin+, w. spread >80 % Aggl +++
2	Normal Aggr +	L. thin, not spread 40-50%, disrupted 30%; light Hyal Aggr -, Aggl-	w. spread > 90% disrupted 10%; s. Aggl	Thin, Normal; Inter+ disrupted 40% s. Aggl +++
3	Normal s. Aggr, Aggl -	Thin, not spread 70-80%, disrupted 20% rare s. Aggr & Aggl	L. thin Normal rare s. Aggl	Thin+, w. spread >80 % Aggl +++
4	w. spread 70% s. Aggr, Aggl	Thin not spread 60-70% Aggr, Aggl -	w. spread >75, Inter+ rare Aggl	Thin+ Normal; Inter+ Aggl +++
5	w. spread 80%; R 10-20% s. Aggr, & Aggl	L. thin not spread 40-60% rare s. Aggr, s. Aggl	w. spread > 90% s. Aggl	Thin Normal; Inter+ disrupted 30%, s. Aggl +++
6	Normal s. Aggr, Aggl -	Thin, not spread 60-70%; disrupted: 30-40% rare s. Aggr, s. Aggl	L. thin Normal; Inter+ s. Aggl	Thin+ not spread Aggl +++
7	L. thin w. spread >75% s. Aggr, Aggl	Thin not spread 70-80% rare s. Aggr & Aggl	w. spread > 75% s. Aggl	Thin Normal, disrupted 25%, Aggl +++
8	Normal s. Aggr & Aggl	Thin not spread 40-50% Aggr-, Aggl-	w. spread > 95% rare s. Aggl	L. thin Normal; Inter+ Aggl +++
9	Normal; Inter+ Aggr, Aggl -	Thin not spread 60-70% Aggr -, Aggl -	w. spread 90% s. Aggl	L. thin spread > 80% Aggl +++
10	L. thick w. spread >80% Aggr +, Aggl +	Thin not spread 60% Aggr-, Aggl +	w. spread >90% rare s. Aggl	L. thin Normal; Inter+ s. Aggl +++
11	w. spread > 90% Aggr, Aggl +	Thin not spread 60% Aggr -, Aggl +	w. spread 90% s. Aggl	spread: > 80% Aggl +++
12	w. spread > 80% Aggr +, Aggl	Thin, not spread 50-60% R 30%, Aggr -, Aggl++	w. spread 80% Aggl	Normal; Inter+ Aggl +++

cWB-b.F, cWB-a.F = tests applied on cWB before and after passing RTH filter respectively; iPLT-b.F, iPLT-a.F = tests applied on iPLT before and after passing RTH filter respectively; Normal= normal findings of PLT spreading; thin= thin preparation; L. thin= light thin preparation L. thick= light thick preparation; w spread = well spread; Aggr-, Aggr, Aggr+, Aggr++, Aggr+++ = no, few, increased, moderate, plenty of PLT aggregates respectively; Aggl-, Aggl, Aggl+, Aggl++, Aggl+++ = no, few, moderate, marked, and extensive PLT agglutinates respectively; s. Aggr= small aggregates; s..Aggl= small agglutinates; R= abnormal round PLT forms; Inter+= increased intermediate PLT forms; s= small; Hyal= hyalomere of PLT.

4-2-3. Quantity assessment of RTH assay and the materials trapped in RTH filter system

4-2-3-1. Aim and methodology of the study

4-2-3-1-1. Objective and design of the study: Identification of the weight retention index (wRI)

As a further step in the validation and standardization of the specificity and the accuracy of RTH assay, the amount of the blood used in RTH as well as that trapped by RTH filter system was quantitatively assessed by weight and volume measurements. The assessment was performed by using cWB from healthy blood donors and the normal level Eightcheck control (EC) and it was done in four axes:

- A.) Assessment of the blood amount used in RTH assay and the weight of RTH filter.
- B.) Establishing the reference range of the amounts of the blood trapped by RTH filter system by the use of cWB and investigating the “normality” of these values. For better evaluation and quantity control, a new retention index for estimating the percentage (%) of the amount of the blood trapped in the RTH filter was identified as follows:

$$\text{wRI (weight retention index \%)} = \frac{\text{Blood Trapped by RTH Filter} \times 100}{\text{Blood Used in RTH}}$$
- C.) Inspection of the probable correlation of the blood amounts trapped by RTH filter (wRI) with: a.) PLT-RI and the other RIs (retention indices) of RTH; and b) the weight of the blood used in RTH and the weight of RTH filter.
- D.) Investigation of the precision of the weight parameters including wRI by the use of cWB as well as EC. This axis was done as a part of the investigations performed in the study of the precision of RTH described in chapter p.4-2-4.

The RTH assay was performed according to the third SOP (SOP-3; p.4-1-2-5); where 500 µL of the tested material (cWB or EC) is pipetted into RTH tube and centrifuged at 110 g/ 5 min. Acceptable reproducible results of the blood amounts trapped by RTH filter indicate the reliability of the filter system under the standardized conditions (110 g/ 5 min). Otherwise, the protocol should be repeated with different conditions of centrifugation.

4-2-3-1-2. Methodology of the quantitative assessment

Eightcheck control stored at 2-8 °C was allowed to balance with room temperature before performing the measurements. The RTH measurements on cWB should be done within 120 min after blood withdrawal (acc. to SOP-3 of RTH; p.4-1-2-5). Both EC and cWB should be mixed gently and thoroughly before performing RTH. Two approaches were initially suggested for the quantitative assessments:

A.) Volume assessment The amounts were assessed by aspiration using an adjustable micropipette. The volume of the cWB after passing RTH filter (a.F size) could be roughly estimated as 470 µL. Using volume measurements (for the a.F amounts) proved, however, to be inaccurate since significant amounts of the blood may variably adhere to the inner wall of the lower part of RTH tube (p.3-2-1) after the aspiration by micropipette. It was decided, therefore, not to depend on the “volume measurements” for the quantitative assessment of RTH assay.

B.) Weight assessment The weight of the upper (with RTH filter) and the lower parts of RTH tube as well as the weight of RTH filter (p.3-2-1) were measured before (b.F) and after (a.F) the passage of the blood through RTH filter. The a.F weight measurements were performed directly after centrifugation (after performing the a.F measurements of RTH by the cell counter) to avoid evaporation of the blood trapped by RTH filter. A cover of parafilm was actually used in order to avoid such an error. The measurement of the amount used in RTH was calculated by subtracting the weight of the empty upper part of RTH tube from its weight after filling with blood (500 μ L). The weight of the amount of the blood trapped by RTH filter (Trapped cWB or EC) could be calculated by subtracting the b.F weight of RTH filter from its a.F weight. RTH filter should be carefully plugged out by a glass bar with a smooth surface that allows applying rather homogenous pressure on the filter while plugging and refixing it in order to preserve its integrity. The weight measurements were performed by using the facilities in the laboratory of the Dept. of Biology

4-2-3-2. Results of the quantity assessment of RTH by using cWB from blood donors

The study was applied on 31 blood donors. The detailed results of the determinations of the quantity assessments along with the measurements of RTH are reported in Tab.Ab.4-2-3-2. The statistics of the main determinations of the quantity assessment are described in the following table:

Table 4-2-3-2: Descriptive statistics^A and reference ranges of the weight parameters of RTH assay using cWB from healthy blood donors.

Weight Parameters	N	Range	Minimum	Maximum	Mean	SD (CV%)	Mean -2SD	Mean +2SD	KS Test ^B p	" ρ " with ^C PLT-RI
Added blood (mg)	31	7.4	502.6	510.0	505.5	1.9 (0.4)	501.7	509.3	0.078	0.059
RTH Filter (mg)	31	4.8	41.1	45.9	42.7	1.1 (2.6)	40.54	44.87	0.189	-0.296
Trapped cWB (mg)	31	4.2	21.1	25.3	23.1	1.1 (4.7)	20.95	25.24	0.200 ^D	0.151
wRI (%)	31	0.8	4.2	5.0	4.6	0.2 (4.7)	4.14	4.99	0.200 ^D	0.169

^AThe detailed results of the determinations of the quantity assessments and the RTH measurements are described in Tab.Ab.4-2-3-2.

^BTest of normality by Kolmogorov-Smirnov: significant at $p < 0.05$.

^C r = the correlation coefficient computed by using spearman's rho test; the correlations were statistically not significant ($p > 0.05$).

^DThis is a lower bound of the true significance.

The weight of the 500 μ L cWB used in RTH assay (mean= 505.6 mg) demonstrated low variance (CV=0.4%) and rather normal distribution ($p > 0.05$; KS test: Fig.4-2-3-2-1) with a slight skewness to the right (0.742). The weight of RTH filter (mean= 42.7 mg) showed also low variance (CV=2.6%) and normal distribution with the presence of few insignificant outliers ($p > 0.1$; KS test), as shown in Fig.4-2-3-2-2. The values of the weight of the blood trapped in RTH filter (mean= 23.1 mg) as well as wRI (mean= 4.6%) delivered comparably low variance (CV=4.7%) and were in line of normal assumption ($p > 0.1$; KS test), as illustrated in Fig.4-2-3-2-3 and 4. Due to the normal distribution of the weight parameters, their reference ranges were addressed depending on the theory of normal assumption defined as mean \pm 2SD (Tab.4-2-3-2). PLT-RI values were fairly proportional to cWB amounts added to RTH tubes ($r = 0.06$). No significant correlation was objected between the cWB amount trapped by RTH filter (wRI) and any of the RIs or the weight parameters of RTH ($p > 0.2$; Spearman's rho). Nevertheless, a slight positive correlation between PLT-RI and wRI values ($r = 0.107$) was observed. The values of wRI demonstrated remarkable

negative correlation with the weight of RTH filter ($r = -0.233$), as shown in Fig.4-2-3-2-5. The weight of RTH filter was also found to be negatively correlated with PLT-RI and with WBR-RI ($r = -0.296$; $r = -0.152$ respectively). These correlations were, however, not statistically significant ($p > 0.1$).

Fig. 4-2-3-2-1 to 4: Histograms of the weight parameters of RTH assay using cWB from healthy blood donors.

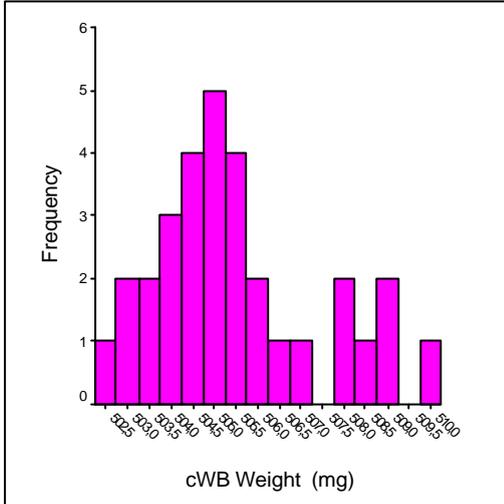


Fig. 4-2-3-2-1: Weight of the cWB used in RTH.

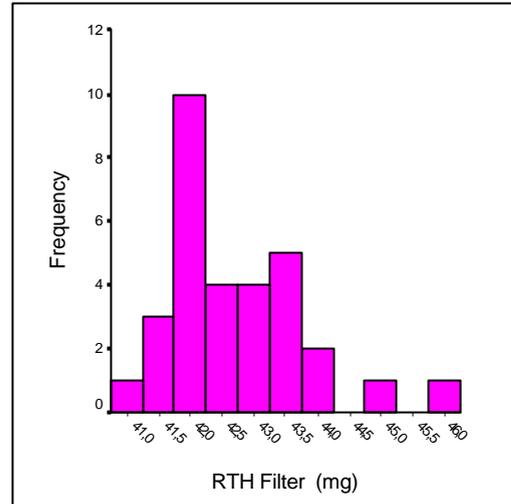


Fig. 4-2-3-2-2: Weight of RTH filter.

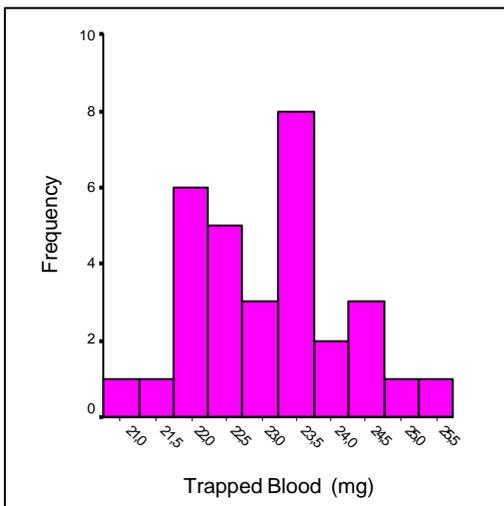


Fig.4-2-3-2-3: Weight of the cWB trapped in RTH filter.

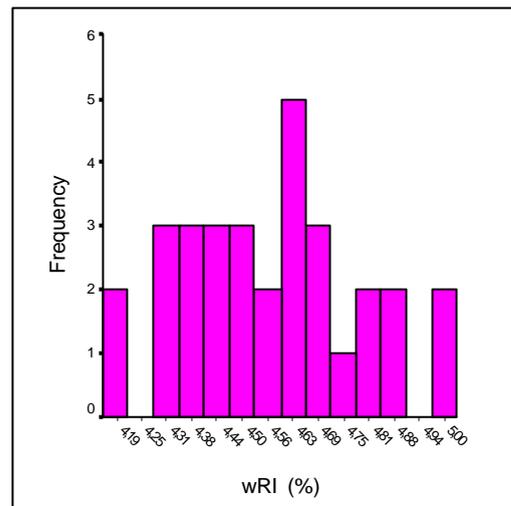
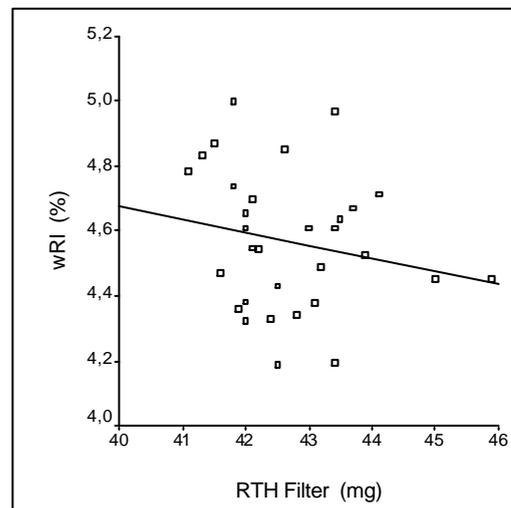


Fig. 4-2-3-2-4: Weight retention index (wRI).

Fig. 4-2-3-2-5: The weight retention index (wRI) by using cWB from healthy blood donors against the weight of RTH filter.



4-2-4. Precision of RTH assay

4-2-4-1. Aim and methodology of the study

After addressing the third SOP of RTH (SOP-3; p.4-1-2-5) and getting more experience in its performance, it was essential to evaluate the probable further improvement in the precision of RTH by using this SOP (SOP-3). The precision of PLT-RI and the other RIs was investigated in two axes:

A.) In-series precision: Two approaches were used to study the in-series precision of RTH; the first by using *fresh cWB* collected from healthy blood donors (BD; p.3-1), and the second by the use of normal level *Eightcheck control* (EC; p.3-3-2). For each cWB series, the b.F measurements of RTH were performed on the cWB in the blood collection tube. The cWB was then distributed into 10 RTH tubes (0.5 mL each) that were thereafter together centrifuged. After centrifugation, the a.F measurements were successively performed on the RTH tubes. For EC series, the b.F measurements were applied on the original tube of EC which was then distributed into 7 RTH tubes (0.5 mL each).

B.) Day-by-day precision: This study was performed on EC at different day intervals. The use of cWB from blood donors was not possible since it is not acceptable to perform RTH or any other PLT function assay on cWB after 180 min from blood collection [Thomas 2001].

In both axes, different Sysmex cell counters (K-1000, KX-21 and SF 3000) were used in order to inspect the probable improvement of using modern counters (Tab.4-2-4-2-1 & 2 and Tab.4-2-4-4). RTH was performed in-double and revealed no significant differences (Wilcoxon test) between the 1st and the 2nd measurements (SD of PLT-RI=1.7%). The mean values were, therefore, used to compute RIs and the statistics of RTH precision. The accuracy of the RIs of PLT and blood cells in cWB and EC was controlled by phase-contrast microscopy. In parallel to RIs precision, *quantity assessments* of the precision (both in-series and day-by-day) of the weight parameters of RTH were performed whenever the practical circumstances were convenient⁸. The methodology of the measurements of the weight parameters is described in the chapter of the quantity assessment of RTH (p.4-2-3-1). The weight parameters included: a.) the weight of the amount (500 µL) of the material tested by RTH (cWB and EC); b.) the weight of RTH filter; and c.) the weight of the material trapped by RTH filter and the weight retention index (wRI).

4-2-4-2. In-series precision performed on cWB from healthy blood donors and on the normal level Eightcheck control (EC)

The in-series precision was performed on cWB from 3 healthy BDs (PLT count=257, 195 and 229 x10³/µL respectively) and on 3 batches of the EC (PLT count= 189, 201 and 202 x10³/µL respectively). The descriptive statistics of the results of RTH precision are summarized in Tab.4-2-4-2-1 for cWB series and in Tab.4-2-4-2-2 for EC series. The distributions of PLT-RI as well as the other RIs were rather in line of normal assumption in both cWB and EC series (p>0.5; Shapiro-Wilk test). The mean values of the RIs of each cWB series varied due to the individual differences between the recruited blood donors, whereas the mean values of the RIs of the different EC series were comparable (p>0.05).

⁸ The weight measurements were performed by using the facilities in the laboratory of the Dept. of Biology away from the place of performing RTH measurements. Considerable delay (>30 min) in performing RTH assay due to the *heavy daily run* of the analyses in the Institute of Clinical Hämostaseology prevented therefore occasionally finishing the weight measurements within the time limit of 120 min (SOP-3; p.4-1-2-5). In such cases, the sample was excluded from the study.

Table 4-2-4-2-1: Descriptive statistics^a of the in-series precision of RTH measurements and the corresponding quantity assessments performed on cWB from healthy blood donors by using different models of Sysmex cell counters.

BD ^b No.	N	Statistics	PLT-RI	PLCR-RI	PDW-RI	MPV-RI	WBC-RI	RBC-RI	HGB-RI	HCT-RI	cWB Weight	RTH Filter	Trapped cWB	wRI
			(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(mg)	(mg)	(mg)
1.) Sysmex K-1000	10	Mean	33.9	31.9	2.5	1.7	5.6	-3.4	-3.0	-3.6	504.6	43.2	22.4	4.4
		SD (CV %)	4.1	4.6	3.7	2.4	2.0	1.5	1.3	1.5	1.1 (0.2)	1.3 (3.1)	0.6 (2.7)	0.1
		Lower Bound of 95% CI	31.0	28.6	-0.2	-0.1	4.1	-4.5	-3.9	-4.7	503.8	42.2	22.0	4.4
		Upper Bound of 95% CI	36.9	35.2	5.1	3.4	7.0	-2.3	-2.0	-2.5	505.4	44.2	22.8	4.5
2.) Sysmex KX-21	10	Mean	25.2	22.7	-5.3	-1.8	6.3	-3.4	-3.0	-3.4				
		SD (CV %)	2.5	4.9	3.3	1.5	2.2	1.0	1.1	1.0				
		Lower Bound of 95% CI	23.3	19.1	-7.7	-2.9	4.7	-4.1	-3.8	-4.1				
		Upper Bound of 95% CI	27.0	26.2	-3.0	-0.7	7.9	-2.7	-2.2	-2.7				
3.) Sysmex SF-3000	10	Mean	21.8	19.7	2.2	0.1	4.8	-4.2	-4.5	-4.2	504.8	43.1	22.8	4.5
		SD (CV %)	2.1	4.3	4.1	1.2	1.9	0.6	0.6	0.6	0.9 (0.2)	1.2 (2.9)	0.6 (2.6)	0.1
		Lower Bound of 95% CI	20.3	16.6	-0.8	-0.8	3.4	-4.6	-4.9	-4.7	504.2	42.2	22.4	4.4
		Upper Bound of 95% CI	23.3	22.9	5.1	0.9	6.2	-3.8	-4.0	-3.8	505.5	44.0	23.2	4.6
Total (n=30)		Correlation ^c with PLT-RI												
		Correlation Coefficient (r)	0.864	0.215	0.329	0.189	0.390	0.500	0.338	0.013	0.048	-0.364	-0.388	
		Sig. 2-tailed: (p value)	0.000	0.255	0.076	0.318	0.033	0.005	0.068	0.956	0.841	0.114	0.091	

^a detailed results of RTH measurements and the quantity assessments are described in Tab.Ab.4-2-4-2-1 and Tab.Ab.4-2-4-3-1 respectively. ^b the mutual correlations between PLT-RI and the other parameters calculated by Spearman's rho test. ^c BD= the serial number of the healthy blood donor (p.3-1) and the models of Sysmex cell counters used for RTH measurements; cWB Weight= the weight of the 500 µL blood (cWB) added to RTH tube to perform RTH assay; RTH Filter= the weight of RTH filter before adding blood; Trapped cWB= the weight of the blood trapped by RTH filter; wRI= the weight retention index of RTH i.e., the percentage (%) of the weight of the blood trapped in RTH filter (p.4-2-3-1-1-B); Lower and upper bound of 95% CI= the lower and the upper bounds of the 95% confidence intervals of the mean. Note: the accuracy of the RIs of PLT and blood cells was controlled by phase-contrast microscopy.

Table 4-2-4-2-2: Descriptive statistics^a of the in-series precision of RTH measurements and the corresponding quantity assessments performed on normal level Eightcheck control (EC) by using different models of Sysmex cell counters.

EC ^b No.	N	Statistics	PLT-RI	PLCR-RI	PDW-RI	MPV-RI	WBC-RI	RBC-RI	HGB-RI	HCT-RI	EC Weight	RTH Filter	Trapped EC	wRI
			(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(mg)	(mg)	(mg)
1.) Sysmex K-1000	7	Mean	2.3	10.3	0.9	1.2	9.7	-3.6	-2.7	-4.1	523.8	42.1	25.7	4.9
		SD (CV %)	1.9	5.6	2.5	1.2	1.4	0.7	0.4	0.8	1.7 (0.3)	0.4 (0.8)	0.8 (3.0)	0.1
		Lower Bound of 95% CI	0.57	5.15	-1.35	0.04	8.37	-4.30	-3.04	-4.80	522.2	41.7	25.0	4.8
		Upper Bound of 95% CI	4.1	15.4	3.2	2.3	11.0	-2.9	-2.4	-3.4	525.3	42.4	26.5	5.0
2.) Sysmex KX-21	7	Mean	2.3	10.7	5.1	1.2	13.1	-6.3	-5.5	-6.2	523.5	42.5	24.8	4.7
		SD (CV %)	2.9	4.3	2.4	1.2	2.2	7.8	6.8	7.2	1.1 (0.2)	0.7 (1.7)	0.9 (3.7)	0.2
		Lower Bound of 95% CI	-0.51	6.65	2.87	0.02	11.07	-13.53	-11.83	-12.83	522.5	41.9	23.9	4.6
		Upper Bound of 95% CI	5.1	14.7	7.3	2.3	15.1	0.9	0.8	0.4	524.5	43.2	25.6	4.9
3.) Sysmex KX-21	7	Mean	3.1	3.1	0.7	-0.2	11.4	-4.6	-6.5	-4.9	523.3	42.5	25.5	4.9
		SD (CV %)	2.2	8.5	1.0	1.8	3.0	2.8	4.8	2.8	0.6 (0.1)	0.8 (1.9)	0.7 (2.8)	0.1
		Lower Bound of 95% CI	1.02	-4.81	-0.23	-1.88	8.66	-7.21	-10.96	-7.44	522.7	41.7	24.9	4.8
		Upper Bound of 95% CI	5.1	11.0	1.6	1.4	14.2	-2.0	-2.1	-2.3	523.9	43.2	26.2	5.0
Total (n=30)		Correlation ^c with PLT-RI												
		Correlation Coefficient (r)	0.588	-0.042	0.419	-0.054	0.169	-0.162	0.149	0.005	0.225	0.238	0.258	
		Sig. 2-tailed: (p value)	0.005	0.855	0.058	0.818	0.463	0.483	0.519	0.984	0.328	0.299	0.259	

^a Detailed results of RTH measurements and the quantity assessments are Tab.Ab.4-2-4-2-2 and Tab.Ab.4-2-4-3-2 respectively. ^b The mutual correlations between PLT-RI and the other parameters calculated by Spearman's rho test. ^c The serial number of the tested normal level of Eightcheck control (EC) and the models of Sysmex cell counters used for RTH measurements. EC Weight= the weight of the 500 µL EC added to RTH tube to perform RTH assay; RTH Filter= the weight of RTH filter before adding EC; Trapped EC= the weight of EC trapped by RTH filter; wRI= the weight retention index of RTH i.e., the percentage (%) of the weight of the amount of EC trapped in RTH filter (p.4-2-3-1-1-B); Lower and upper bound of 95% CI= the lower and the upper bounds of the 95% confidence intervals of the mean. Note: the accuracy of the RIs of PLT and blood cells was controlled by phase-contrast microscopy.

The variation of PLT-RI in cWB series (SD=2.1-4.1) was significantly higher (p=0.002) than that of EC series (SD=1.9-3.0). This effect was not remarkable (p>0.05) for PLCR-RI where SD was rather comparable in both cWB and EC series (SD = 4.3-4.9 vs. 4.3-8.5). The mean values of PLT-RI as well as PLCR-RI were significantly lower (p<0.0005) in EC series compared with cWB ones (PLT-RI=2.3-3.1% vs. 21.8-29.9%; PLCR-RI= 3.1-10.3% vs. 19.2-30.5%), as shown in Fig.4-2-4-2-1 & 2. The resulting 95% confidence intervals (CI) of the means of PLT-RI and PLCR-RI, for both cWB and EC series, were

rather low (Tab. and Fig.4-2-4-2-1 & 2). The use of different models of Sysmex counters for RTH measurements revealed no significant differences for of the variations of PLT-RI and PLCR-RI in EC series (Fig.4-2-4-2-1 & 2). In contrast, the use of Sysmex KX-21 for cWB series delivered a remarkable decrease in the variation of PLT-RI compared with Sysmex K-1000 (SD=2.5 vs. 4.1). A more pronounced and statistically significant decrease ($p=0.01$) was obtained by using the available most modern model "Sysmex SF3000", as demonstrated in Fig.4-2-4-2-1. The variations (SD) of PDW-RI as well as MPW-RI were low in both cWB and EC series, and the mean values were rather comparable (Tab.4-2-4-2-1 & 2), as shown in Fig.4-2-4-2-3 & 4. Despite WBC-RI values were significantly higher in EC than in cWB (mean= 9.7-13.1 vs. 4.8-7.7%: $p<0.0005$), their variations were comparable (SD=1.4-3.0 vs. 1.9-2.2) and demonstrated small 95% CI, as illustrated in Fig.4-1-5-2-5. The mean values of the RIs of RBC, HGB, and HCT were comparable in both cWB and EC (Tab.4-2-4-2-1 & 2). Heavy tails were found in the 2nd and the 3^d series of RBC-RI in EC (Skewness= -0.63) causing overestimation of the variation of RBC-RI of these series (Fig.4-2-4-2-6). In parallel with RBC-RI, a similar effect was found in the same series for HGB-RI and HCT-RI. The other EC series as well as cWB series demonstrated low variation and small 95% CI, as illustrated in Fig.4-2-4-2-6.

The statistics of PLT-RI correlations with the other RIs in cWB and EC series are described in Tab.4-2-4-2-1 and 2 respectively. The patterns of the positive correlations in the cWB series were very similar to that found in the stage 4 of the improving protocol, as illustrated in Fig.4-2-4-2-7. The correlation of

Fig. 4-2-4-2-1 to 6: In-series precision of the retention indices of RTH assay performed on WB and on normal level Eightcheck control (EC) based on the 95% confidence intervals (CI) of the mean values using different models of Sysmex counters.

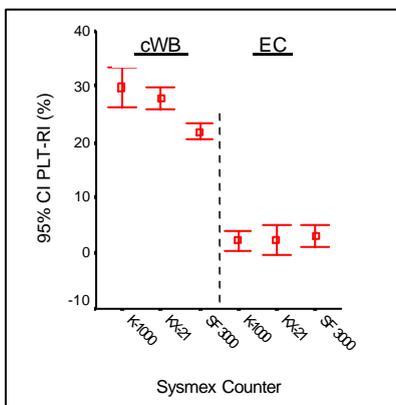


Fig. 4-2-4-2-1.

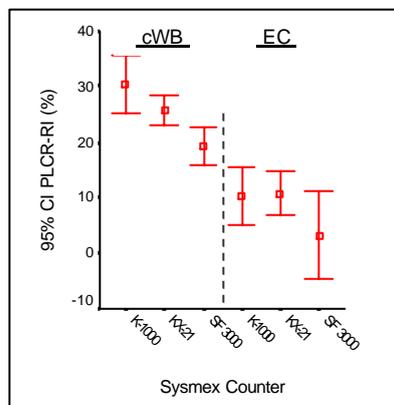


Fig. 4-2-4-2-2.

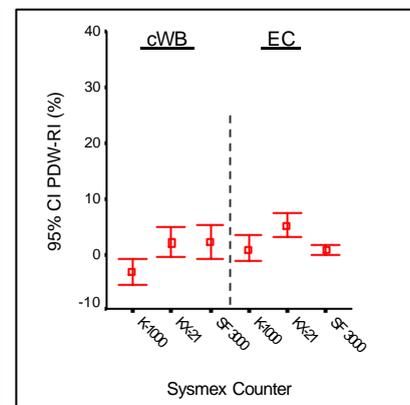


Fig. 4-2-4-2-3.

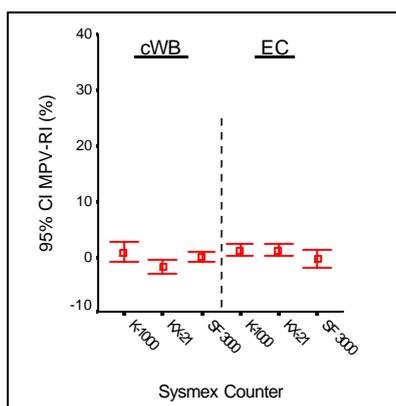


Fig. 4-2-4-2-4.

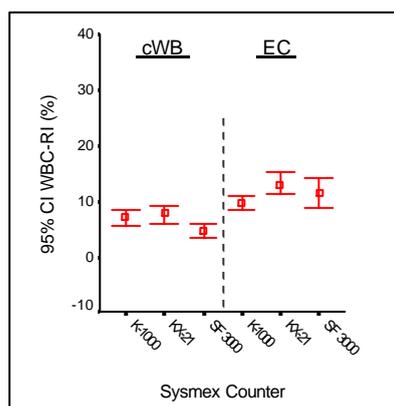


Fig. 4-2-4-2-5.

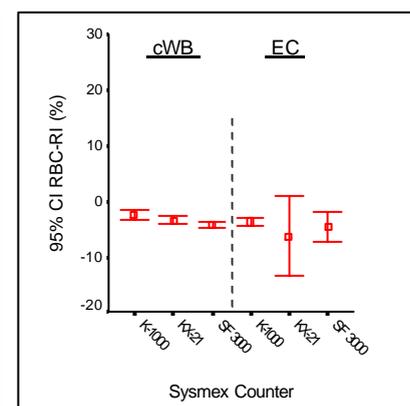
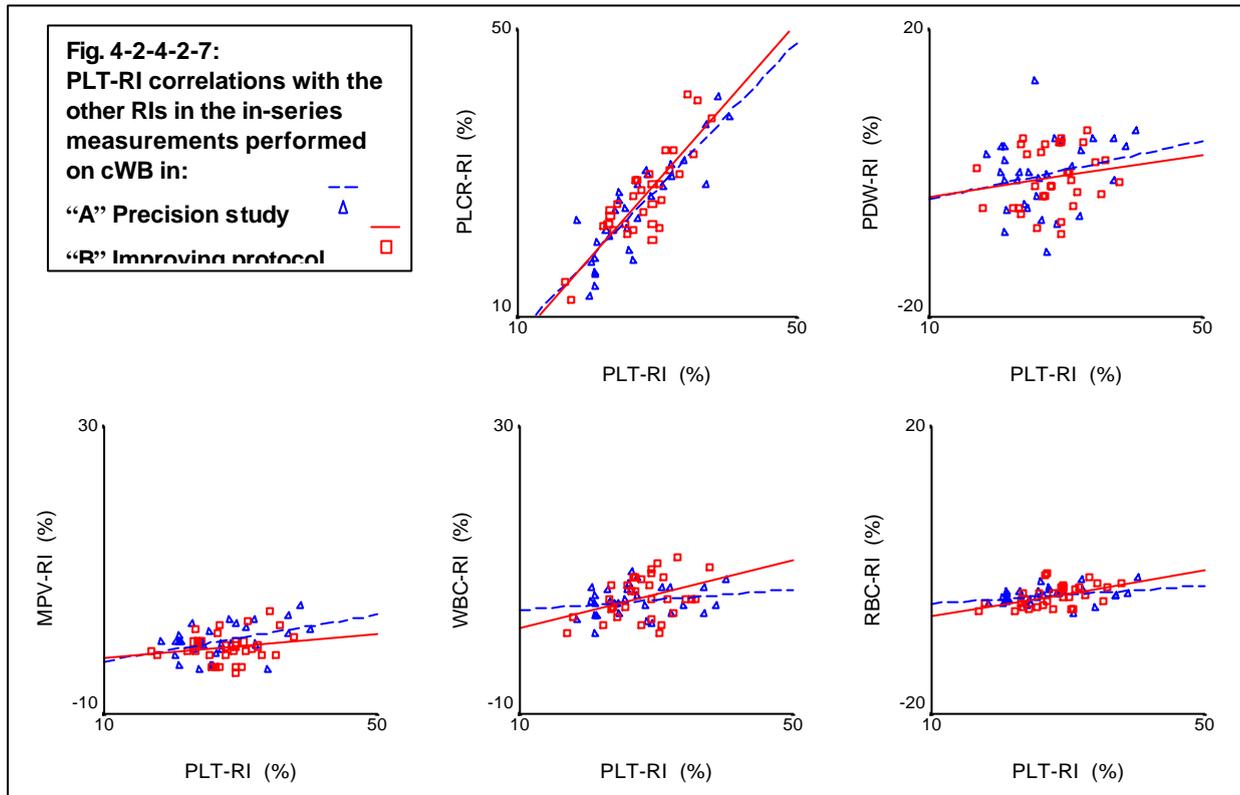


Fig. 4-2-4-2-6.

PLCR-RI with PLT-RI was statistically significant. The correlations between PLTR-RI and the other RIs in EC series varied from positive to negative, as described in Tab.4-2-4-2-2. None of these correlations was significant except the relation between PLT-RI and PLCR-RI (p=0.005).



4-2-4-3. Quantity assessments of the in-series precision performed on cWB and on normal level Eightcheck control (EC)

The statistics of the precision of RIs and the weight parameters of RTH are summarized in Tab.4-2-4-2-1 and 2 for cWB and EC series respectively. Shapiro-Wilk statistic implicated a normal distribution (p>0.05) for the values of these parameters within cWB and EC series. The values of the in-series measurements of the weight of RTH filter in both cWB and EC series were almost identical (mean= 42.1 to 43.2 g; CV=0.8-3.1%; Fig.4-2-4-3-1). The in-series measurements of the weight of the 500 µL cWB and EC used in RTH assay demonstrated low variation (CV=0.2-0.3%). The mean values of

Fig. 4-2-4-3-1 to 4: In series precision of the weight parameters of RTH assay performed on cWB and on the normal level Eightcheck control (EC) based on the 95% confidence intervals (CI) of the mean values using different models of Sysmex counters.

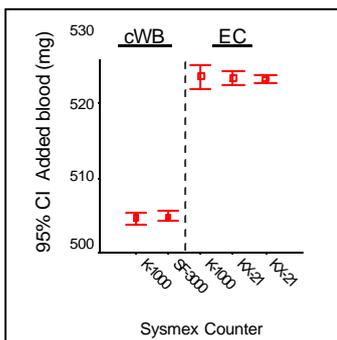


Fig. 4-2-4-3-1.

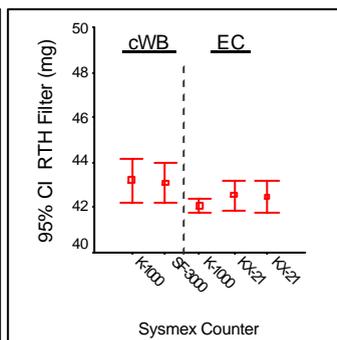


Fig. 4-2-4-3-2.

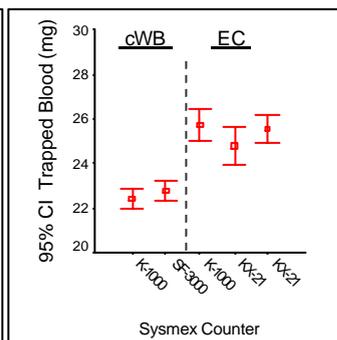


Fig. 4-2-4-3-3.

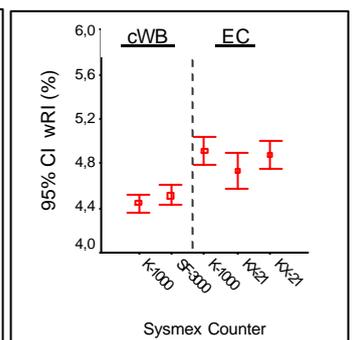


Fig. 4-2-4-3-4.

cWB series (504.6-504.8 g) were comparable and significantly ($p < 0.0005$) lower than that of EC series (523.3-523.8 g), as illustrated in Fig.4-2-4-3-2. The mean weight of the amounts trapped by RTH filter were significantly higher in EC series (24.8-25.7 g) than in cWB ones (22.4-22.8 g), as shown in Tab.4-2-4-3-3. Both cWB and EC series presented low variation ($CV = 2.6-3.7\%$). Similarly, the weight retention index (wRI) demonstrated low in-series variation for both cWB and EC ($SD = 0.1-0.2$). Furthermore, cWB had also significantly ($p < 0.0005$) higher mean values for EC series (4.7-4.9%) than for cWB ones (4.4-4.5%), as demonstrated in Fig.4-2-4-3-4.

4-2-4-4. Day-by-day precision performed on normal level Eightcheck control (EC)

The study of the day-by-day precision of RTH was performed on 2 batches of EC by the application of increasing time intervals ranging from 1 to 10 days, as described in the statistics of the results (Tab.4-2-4-2). The mean PLT counts of each batch were found to be 195 and $206 \times 10^3/\mu\text{L}$ respectively. The values of the day-by-day measurements of PLT-RI and other RIs in both EC batches were in line of normal assumption ($p > 0.1$; Shapiro-Wilk test). The mean values of PLT-RI and PLCR-RI in both batches were comparable to each other ($p > 0.3$; Fig.4-2-4-4-1 & 2) as well as to the mean values of EC detected in the study of the in-series precision of RTH (Fig.4-2-4-4-3 & 4). The use of Sysmex M-2000 in the 1st batch of EC led to a marked but insignificant decrease ($p > 0.05$; Levene test) in the day-by-day variations of PLT-RI ($SD = 1.5$) and PLCR-RI ($SD = 5.0$) compared with that delivered by using Sysmex KX-21 in the 2nd batch ($SD = 1.8$ and 8.8 respectively). In both cases, the variations were low and comparable ($p > 0.05$ Levene test) with that pronounced by EC in the in-series precision, as illustrated in Fig.4-2-4-4-3 & 4.

Table 4-2-4-4: Descriptive statistics^a of the day-by-day precision of RTH assay and the corresponding quantity assessments performed on normal level Eightcheck control (EC) using different models of Sysmex cell counters.

EC ^b No.	Statistics		PLT-RI	PLCR-RI	PDW-RI	MPV-RI	WBC-RI	RBC-RI	HGB-RI	HCT-RI	EC Weight	RTH Filter	Trapped EC	wRI
	N		(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(mg)	(mg)	(mg)	%
1.) Sysmex M-2000	5	Mean	2.9	8.4	1.4	2.6	12.9	-3.1	-2.1	-3.0	523.8	42.1	25.7	4.9
		SD (CV %)	1.5	5.0	2.2	1.0	2.9	0.5	1.2	0.6	1.7 (0.3)	0.4 (0.8)	0.8 (3.0)	0.1
		Lower Bound of 95% CI	1.0	2.2	-7.9	-0.7	9.3	-3.7	-3.7	-3.8	522.2	41.7	25.0	4.8
		Upper Bound of 95% CI	4.8	14.6	6.5	4.8	16.6	-2.4	-0.6	-2.3	525.3	42.4	26.5	5.0
2.) Sysmex KX-21	6	Mean	2.4	4.1	-3.4	1.4	9.8	-2.9	-4.4	-3.4	42.7	523.7	24.8	4.7
		SD	1.8	8.8	3.0	2.6	3.3	1.1	1.3	0.6	1.3 (2.9)	3.8 (0.7)	1.0 (4.1)	0.2
		Lower Bound of 95% CI	-0.51	6.65	2.87	0.02	11.07	-13.53	-11.83	-12.83	522.5	41.9	23.9	4.6
		Upper Bound of 95% CI	5.1	14.7	7.3	2.3	15.1	0.9	0.8	0.4	524.5	43.2	25.6	4.9

^aDetailed results in of RTH measurements and the quantity assessments are Tab.Ab.4-2-4-4 and Tab.4-2-4-5 respectively; ^bEC No.: the serial number of the tested normal level of Eightcheck control (EC) and the models of Sysmex cell counters used for RTH measurements; increasing time intervals were applied: 1, 2, 3, 9 and 10 days on the first EC, and 1, 2, 3, 4, 5 and 8 days on the second EC.

EC Weight= the weight of the 500 μL of EC added to RTH tube to perform RTH assay; RTH Filter= the weight of RTH filter before adding EC; Trapped EC= the weight of EC trapped by RTH filter; wRI= the weight retention index of RTH (the percentage (%) of the weight of the amount of EC trapped in RTH filter; p.4-2-3-1-1-B); Lower and upper bound of 95% CI= the lower and the upper bounds of the 95% confidence intervals of the mean. *Note:* the accuracy of the RIs of PLT and blood cells was controlled by phase-contrast microscopy.

No significant differences were found between the means ($p > 0.1$) or the variations ($p > 0.1$) of the day-by-day measurements of PDW-RI, MPV-RI and WBC-RI performed on both EC batches. The findings of these RIs in the day-by-day precision were actually comparable to that of EC expressed by the in-series precision, as demonstrated in Fig.4-2-4-4-5, 6 and 7. For both EC batches, the values of RBC-RI demonstrated comparable variations ($p > 0.1$; Levens' test) with rather similar mean values ($p > 0.01$; t-test). As illustrated in Fig.4-2-4-4-8, the day-by-day variation was as low as that found in the

1st EC series of the in-series precision and much lower than the high variation of the 2nd and the 3rd series which demonstrated heavy trails (p.4-2-4-2). The variations of HGB-RI and HCT-RI were comparable to that of RBC-RI (Tab.4-2-4-4).

Fig. 4-2-4-4-1:
Day-by-day precision of PLT-RI performed on normal level Eightcheck control (EC).

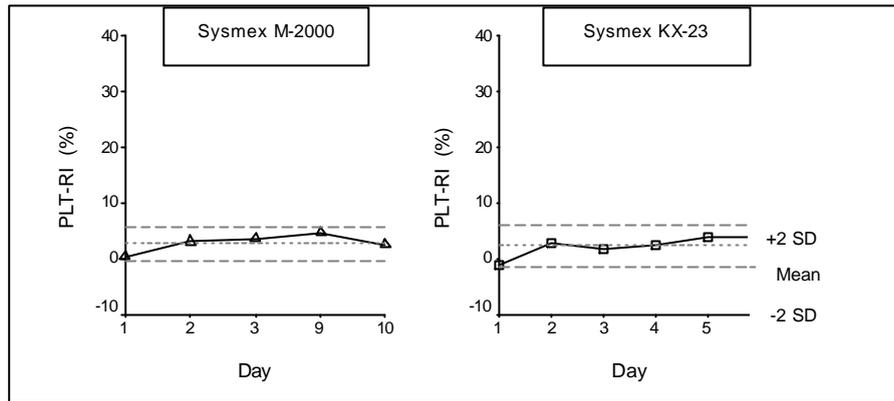


Fig. 4-2-4-4-2:
Day-by-day precision of PLCR-RI performed on normal level Eightcheck control (EC).

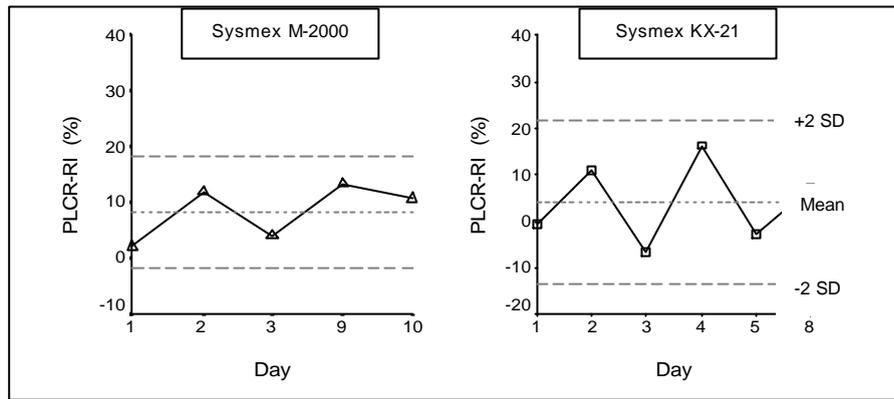


Fig. 4-2-4-4-3 to 8: Day-by-day precision (Day) of the retention indices of RTH assay performed on normal level Eightcheck control (EC) based on the 95% confidence intervals (CI) using different models of Sysmex counters compared with that of the in-series precision (Series)

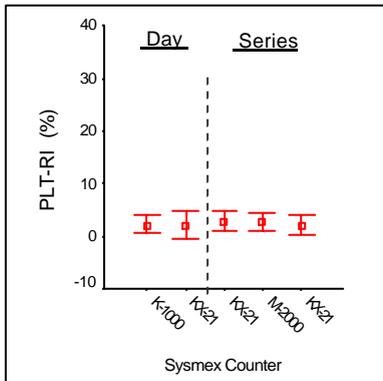


Fig. 4-2-4-4-3.

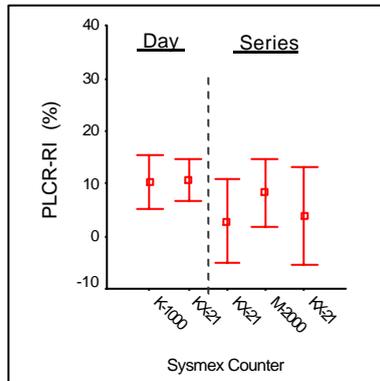


Fig. 4-2-4-4-4.

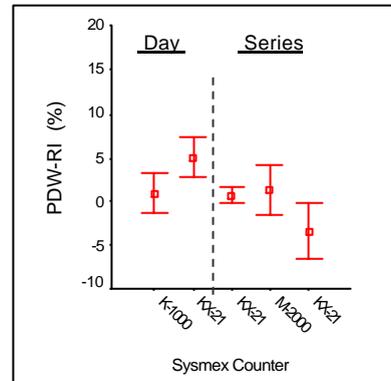


Fig. 4-2-4-4-5.

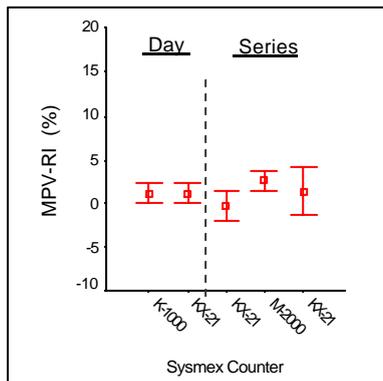


Fig. 4-2-4-4-6.

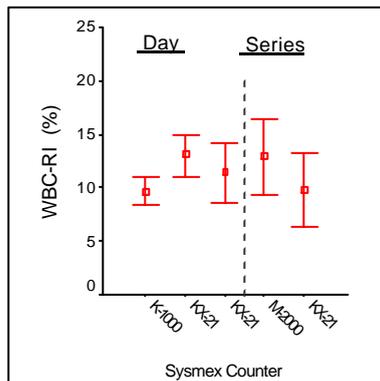


Fig. 4-2-4-4-7.

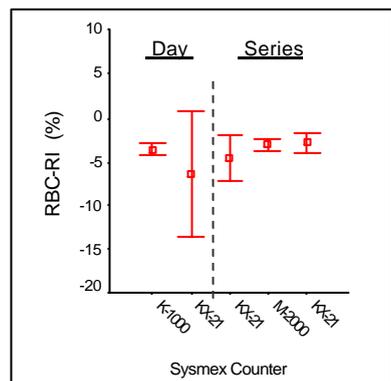


Fig. 4-2-4-4-8.

4-2-4-5. Quantity assessment of the day-by-day precision performed on normal level Eightcheck control (EC)

The quantity assessment of the study of the day-by-day precision of RTH was performed on the second batch of EC of this study. The statistics of the results are summarized Tab.4-2-4-4. The values of the weight parameters were in line of normal assumption ($p>0.1$; Shapiro-Wilk). The mean values of each of the weight of RTH filter (42.7 g), the weight of the 500 μ L EC used in RTH assay (523.2 g), and the weight of the EC amount trapped by RTH filter (24.8 g) were quite comparable (Anova test: $p>0.1$) to that delivered by the different series EC of the in-series precision (mean values= 42.4 g, 523.5 g, 25.3 g respectively). The variations of these parameters observed in the day-by-day quantity assessments (CV=1.3%, 0.6%, 4.1% respectively) were low but slightly higher than the low variation of the in-series one (CV= 0.6%, 0.2%, 3.2% respectively). Nevertheless, the differences were not statistically significant ($p>0.05$; Levene test), as demonstrated in Fig.4-2-4-5-1, 2, and 3.

The day-by-day variations of wRI were quite low (mean=4.7%: SD=0.20; Fig.4-2-4-5-5) and comparable with that demonstrated by the in-series assessments (mean=4.8%. SD=0.15), as illustrated in Fig.4-2-4-5-5. Statistically, no significant differences in the means (Anova test: $p>0.1$) or in the variations (Levene test: $p>0.1$) of wRI were objected between the in-series and the day-by-day measurements, as shown by Fig.4-1-2-5-4.

Fig. 4-2-4-5-1 to 4: Day-by-day precision (Day) of the weight parameters of RTH assay performed on the normal level Eightcheck control (EC) based on the 95% confidence intervals (CI) using different models of Sysmex counters compared with that of the in-series precision (Series).

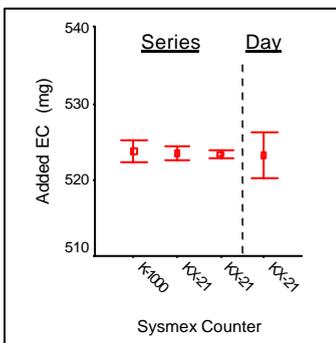


Fig. 4-2-4-5-1.

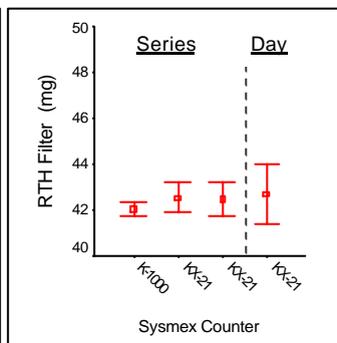


Fig. 4-2-4-3-2.

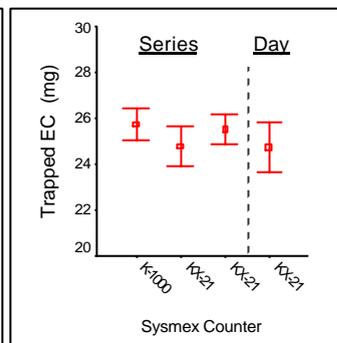


Fig. 4-2-4-3-3.

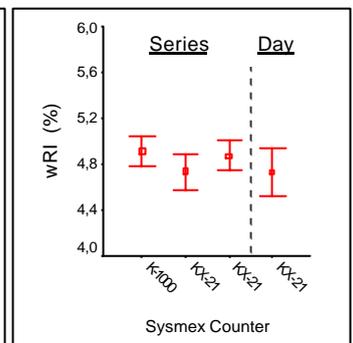
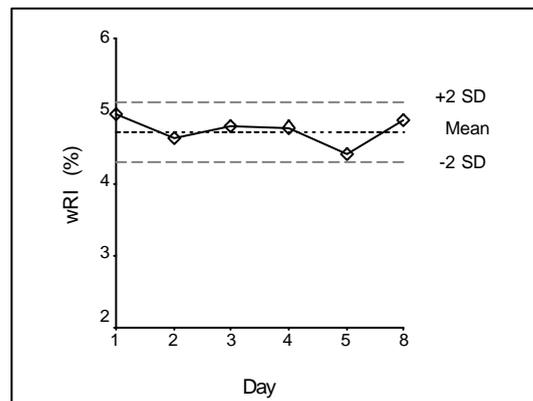


Fig. 4-2-4-3-4.

Fig. 4-2-4-5-5: Day-by-day precision of wRI performed on Eightcheck control (EC).



4-2-5. Validation of a newly developed PLT standard by using RTH compared with the control of phase-contrast microscopy

4-2-5-1. Aim and methodology of the study: Developing a convenient PLT standard

The standardized PLT preparation supplied by Sysmex (Eightcheck control; p.3-2-1) was used in the control of the precision of RTH. It turned out that this standard is not optimal as a reference control in the sense of correctness since it delivered very low PLT-RI value (mean=2.6) with low variance (SD=2.1) reflecting the unacceptably reduced function of the PLT in this standard (p.4-2-4-2 & 4). For this particular purpose, we tried to develop our standard PLT preparation in the Institute of Clinical Hemostaseology with the kind cooperation of Prof. Morgenstern in the Dept. of Biology. *The morphological acceptance criteria* of our intended PLT standard are: **A.)** The standard should compose of unstimulated PLT i.e., intact discocytes forms. **B.)** The number of discocytes with small pseudopodia (echinodiscocytes) should not exceed 10%. **C.)** Fully activated PLT (with long pseudopodia or with an activated shape) should not exist in PLT suspension of the standard. **D.)** The PLT should be separately suspended in the PLT preparation with no aggregation (Aggr) or agglutination (Aggl). **E.)** Only few traces of WBC and/or RBC are allowed to be presented in the standard. For the morphological control, the phase-contrast microscopy was used.

On the other hand, the preparation of the PLT standard was regarded as a further inspection of the reliability of using RTH assay in research for assessing PLT function specifically and accurately in comparison with CBC (complete blood count; including PLT count) and in due of morphological control. The PLT standard was first planned to be prepared by employing the gel-filtered PLT (obtained by using Sepharose 2B; p.4-1-1-1). This approach was found, however, to be unacceptable (p.5-4) and it was decided therefore to try another approach by preparing unstimulated PLT standard from cPRP.

4-2-5-1-1. Initial formula for preparing unstimulated PLT standard

- 4-2-5-1-1-1. Prepare 25 mL cPRP from apparently healthy blood donors (SOP; p.3-4-2).
- 4-2-5-1-1-2. Fixation with 40 μ L glutardialdehyde (25% for electron microscopy, p.3-3-12)/ mL suspension (cPRP).
- 4-2-5-1-1-3. First control on the PLT morphology using phase-contrast microscope (p.3-5-2).
- 4-2-5-1-1-4. Centrifugation: 550 g/ 20 min at room temperature.
- 4-2-5-1-1-5. Removing of the supernatant.
- 4-2-5-1-1-6. Covering of the pellet with PBS⁹ containing NaN₃ (0.1%) at 4°C.
- 4-2-5-1-1-7. Resuspension (stirring).
- 4-2-5-1-1-8. Second control on the PLT morphology using phase-contrast microscope (p.3-5-2).

4-2-5-1-2. Developing the PLT standard under morphological control: Final formula of the preparation of the PLT standard

Taking into consideration the successive experiments in order to improve the initial protocol of the standard and the resulting modification (described in details in Ab.4-2-5-1), the final protocol of preparing the PLT standard that meets the morphological criteria (p.4-2-5-2-A to C) was addressed as follows:

⁹ PBS (phosphate buffer saline): NaCl: 8000 mg, KCl: 200 mg, Na₂HPO₄: 1000 mg, NaH₂PO₄: 150 mg, KH₂PO₄: 200 mg, D.W: S.Q. 1000 mL. Adjust at pH 7.4-7.6. Store at 4°C.

- 4-2-5-1-2-1. For each *lot of the PLT standard*, cPRP is prepared from not more than *one*¹⁰ healthy blood donor (SOP; p. 3-3-2).
- 4-2-5-1-2-2. *PLT deactivation and morphological control*¹⁰. Rest cPRP in 37°C bath-chamber for 30 min sooner after preparation. cPRP was not accepted for the next steps unless it fulfills the morphological criteria by phase-contrast microscopy (p.4-2-5-1-A to C).
- 4-2-5-1-2-3. *Fixation* with 150 µL/ mL cPRP by a solution containing:
- | | |
|--|-------|
| 100 mg EDTA in 0.8% NaCl-solution ¹¹ | 10 mL |
| Glutardialdehyde ¹² (25% for electron microscopy) | 1 mL |
| 0.1 M Phosphate buffer ¹³ pH 7.4 | 4 mL |
- 4-2-5-1-2-4. *Morphological control* of the PLT by phase-contrast microscopy (p.4-2-5-1-A to C).
- 4-2-5-1-2-5. Centrifugation¹⁴: 220 g/ 20 min at room temperature.
- 4-2-5-1-2-6. *Removing of the supernatant immediately*¹⁵ after centrifugation (with suitable syringe).
- 4-2-5-1-2-7. *Resuspension*. The pellet should be *sooner* covered with PBS¹⁶ (pH=7.4) containing NaN₃ (0.1%), *stirred thoroughly* (40-50 sec) on a vibrator to deliver homogenous suspension with no visible particles (PLT agglutinates). The resulting suspension is the final “PLT standard” which should be stored at 4°C. *Note*: The ratio PRP:PBS =1:1 delivers the normal level standard, while the ratio 3:5 delivers the low level standard
- 4-2-5-1-2-8. *Morphological control* of PLT by phase-contrast microscopy (p.4-2-5-1-A to C).

Note 1: Non-thrombogenic plastic slides were used for the microscopic examinations to avoid possible PLT activation or agglutination by contact with glass surface.

Note 2: The preparations excluding the “washing” steps (p.4-2-5-1-2-5 to 8) were defined as the standards of “unwashed” PLT (Suw), while the standards of “washed” PLT (Sw) included these steps.

¹⁰ The morphologic control of the cPRP (prepared from more than one blood donor) before the fixation step (p.4-2-5-1-1-1) revealed rare WBC with no significant traces of RBC, but PLT were unacceptably hyper-stimulated (presence of PLT aggregates and agglutinates, PLT discocytes with small pseudopodia >10%). It was decided therefore to: A.) prepare cPRP from no more than one blood donor to avoid probable incompatibility; B.) cPRP should be rested in water-bath to assure PLT deactivation due to pre-analytical causes and/or after being disturbed by the centrifugation and cPRP preparation. C.) PLT in the collected cPRP should be controlled morphologically before using it for preparing the standard.

¹¹ Isotonic EDTA was added to avoid unacceptable agglutinations of the fixed PLT.

¹² Lower concentration of glutardialdehyde, compared with the initial formula (p.4-2-5-1-1-2), was used to avoid cPRP gelation.

¹³ Cacodylate buffer (0.1 M: recommended for good staining especially for electron microscopy) was initially used, but then it was substituted with phosphate buffer (0.1M) since it is convenient for phase-contrast microscopy, and moreover not toxic and with less costs. The formula of the phosphate buffer (0.1M) is as follows:

Stock solution I:	14.2 g NaH ₂ PO ₄ /1000 mL D.W.
Stock solution II:	13.6 g KH ₂ PO ₄ /1000 mL D.W.
Mixing ratio:	7:3 (stock solution I:II)

Adjust at pH=7.4. Stable for weeks when stored at 4°C.

¹⁴ Lower relative centrifugal forces (RCF; compared with the initial formula p. 4-2-5-1-1-4), were experimented to wash fixed PLT and our addressed centrifugation condition proved to substantially reduce PLT agglutination of the washed PLT.

¹⁵ Any delay in removing the supernatant and resuspending PLT (p.4-2-5-2-3-7) would increase the possibility of PLT agglutinations in the washed PLT.

¹⁶ An inclusion of 10g. EDTA/1000 to the PBS (p.4-2-5-1-1-6) was considered to avoid the tendency of agglutination by time throughout the few hours after preparation. The final formula of PBS was:

EDTA: 10g, NaCl: 8000 mg, KCl: 200 mg, Na₂HPO₄: 1000 mg, NaH₂PO₄: 150 mg, KH₂PO₄:200 mg, D.W: S.Q. 1000 mL. Adjust at pH 7.4-7.6. Stored at 4°C. *Note.* NaN₃ should be added to PBS (0.1%; mg/mL) just before use to avoid bacterial and fungal contamination in the PLT standard.

4-2-5-1-3. Methodology of the successive lots of the PLT standard

Depending on the “final” formula of the PLT standard (p.4-2-5-1-2), three batches of the standard were produced. *The First lot* composed of normal (N.Sw-1) and low level (L.Sw-1) standards of washed PLT (p.4-2-5-1-2); each was distributed into aliquots of 600 μL . *The second lot* included one normal level standard of unwashed PLT (Suw-2) that was prepared and stored in one tube. Considering the significant improvement in the stability of the standard of unwashed PLT demonstrated by Suw-2 compared with that of the washed PLT (Sw-1) delivered by the first lot (tables 4-2-5-3 & 4), normal level standards of washed (Sw-3) and unwashed (Suw-3) PLT were prepared from the same cPRP in the 3^d lot of the standard for further inspection of this improvement. Each of these standards was stored in one tube, sealed firmly, and stored in refrigerator ($<8^{\circ}\text{C}$). PLT count of the cPRP from which the standards of the first lot were prepared was $364 \times 10^3/\mu\text{L}$. Since the standard include an addition of 150 μL fixation solution / mL cPRP (p.4-2-5-1-2-3), the expected value of PLT count for N.Sw-1 and L.Sw-1 were $364 \times 0.85 = 318 \times 10^3/\mu\text{L}$ and $364 \times 0.85 \times 3/5 = 191 \times 10^3/\mu\text{L}$ respectively. Similarly, the expected value of the PLT count of the second and the third lot of the standard were $413 \times 10^3/\mu\text{L}$ (486×0.85) and $396 \times 10^3/\mu\text{L}$ (467×0.85) respectively.

4-2-5-1-4. Methodology of evaluating the precision of PLT standards morphologically and by the use of RTH assay

The stability and the precision of the different lots of the PLT standard were investigated. The study of the in-series precision composed of performing CBC (complete blood count), whereas the study of the day-by day precision included additionally the morphological examination by phase-contrast microscopy. The measurements of CBC and RTH in the study of the precisions (in-series and day-by-day) were carried out in-double. The detailed results, figures and tables are described in Ab.4-2-5-2. Only the key results are summarized in this chapter. The standards were allowed to balance with room temperature and then mixed thoroughly before applying any control (for the first lot, only one aliquot was used for each one time control).

The in-series precision was studied on N.Sw-1 and on Suw-2 as examples for the washed and unwashed PLT standards respectively. *The day-by-day precision* was investigated in each of the three lots of the PLT standard. The control was performed initially from day to day and then with increasing time intervals as often as the quantity of the standards allowed. Having reasonable insight about the precision of the PLT standard by the control performed on the 1st and the 2^d lots, we step ahead to use RTH assay for evaluating PLT function of the 3^d lot of the standard. Due to the practical conditions of the work, the measurements of the in-series as well as on the first day of the day-by-day precision were generally possible to be performed only few (2-4) hours after preparing the standard, whilst the microscopic control in these studies was performed within 60 min after the preparation.

4-2-5-2. In-series precision of the PLT standard

Table 4-2-5-2: Controlling the in series precision^a of the parameters of the PLT standards.

Standard Lots ^b	Statistics	PLT (x10 ³ /μL)	PDW (fL)	MPV (fL)	PLCR (%)	WBC (x10 ³ /μL)	RBC (x10 ⁶ /μL)	HGB (g/dL)	HCT (%)
1 st Standard of Washed PLT (N.Sw-1) ^c	Mean	88	16.6	10.8	34.4	6.2	0.03	0.1	0.0
	SD	1.7	0.2	0.1	0.4	0.2	0.005	0.1	0.0
	CV (%) ^d	1.90	0.92	1.31	1.21	2.47			
2 nd Standard of Unwashed PLT (Suw-2)	Mean	427	12.0	10.0	26.7	0.2	0.07	0.1	0.1
	SD	5.9	0.2	0.1	1.2	0.03	0.11	0.04	0.03
	CV (%) ^d	1.37	1.32	1.08	4.66				

^aThe detailed measurements are described in Tab.Ab.4-2-5-2. ^bThe measurements of the first and the second standard were performed 3 and 4 hours after their preparation respectively. ^cThe normal level (N.Sw-1) of the 1st lot of the standard and not the low (L.Sw-1) was considered for the comparison with the 2nd lot since only normal level standard (Suw-2) was prepared in the 2nd lot. ^dDue to the low mean value of the traces of these cells, the high CVs are meaningless and were therefore omitted.

4-2-5-3. Day-by-day precision of the first lot of PLT standard

The PLT counts of the normal (N.Sw-1) and low level (L.Sw-1) washed PLT standards measured on the 1st day (87 and 61 x10³/μL respectively; Tab.4-2-5-3-1) were much lower than expected β18 and 191x10³/μL respectively; p.4-2-1-3). This significant and early decline in PLT count of the N.Sw-1 and L.Sw-1 (72 and 68% respectively) was followed by remarkable stability during the first 6 day control (CV=7% and 15% respectively). The next control showed a trend of PLT decrease by time that was more pronounced in the normal level (Fig.4-2-5-3-1). In parallel, WBC count increased significantly up the 1st day control despite only rare WBC could be objected microscopically (Tab.4-2-5-3-2). The microscopic control revealed no increase in WBC but significant increases in Aggl were observed and were rather proportional to the decreases of PLT as well as to the increases in WBC; some of those Aggl demonstrated WBC size-like particles. WBC increased with close association to the decrease of PLT up to a cut point where the decline began, as illustrated in Fig.4-2-5-3-2. No significant variation was observed in PLCR or in PLT size distribution other than a slight decrease in MPV and PLCR, as further illustrated in Ab.4-2-5-2; Fig.4-2-5-3-3 and 4.

Table 4-2-5-3-1: Summary of the key* results of the day-by-day control on the first washed PLT standards.

PLT Standard	Serial No.	Time (day)	PLT (x10 ³ /μL)	WBC (x10 ³ /μL)	RBC (x10 ⁶ /μL)
Normal Level Standard (N.Sw-1): PRP:PBS= (1:1)	1	1**	87	6.3	0.03
	2	2	91	7.5	0.02
	3	3	92	5.8	0.02
	4	4	103	5.3	0.03
	5	5	93	5.9	0.03
	6	6	82	6.9	0.03
	7	27	66	9.0	0.02
	8	34	53	5.1	0.02
	9	51	58	8.6	0.02
	10	76	74	7.1	0.02
	11	83	47	8.8	0.02
Low Level Standard (L.Sw-1): PRP:PBS= (3:5)	1	1**	61	4.1	0.02
	2	2	61	4.6	0.02
	3	3	59	4.5	0.02
	4	4	67	4.2	0.02
	5	5	61	4.3	0.02
	6	6	44	4.2	0.01
	7	27	43	5.9	0.01
	8	34	41	5.3	0.01
	9	51	42	5.5	0.01
	10	76	15	1.8	0.01
	11	83	50	8.6	0.02

*The detailed results of the CBC measurements are described in Tab.Ab.4-2-5-3; **The measurements were applied 4 hours after preparing the standard, whilst the microscopic examination of the standard was performed within 30 min after its preparation.

Table 4-2-5-3-2: Day-by-day control on the first washed PLT standards by phase-contrast microscopy*.

Serial No.	Time Intervals (day)	Normal Level Standard (N.Sw -1)	Low Level Standard (L.Sw -1)
		PRP:PBS = 3:3	PRP:PBS = 3:5
1	1	PLT+++ , f RBC, r WBC Aggl: <5%: s >90%	PLT++ , f RBC, r WBC Aggl: <5%: s >90%
2	2	PLT+ , r RBC Aggl: 65%: s 35%, b 10%	PLT+ , r RBC Aggl: 65%: s 20%, b 15-20%
3	3	PLT+ , r RBC Aggl: 65%: s 40%, b 15-20%	PLT+ , r RBC Aggl: 70%: s 25%, b 15%
4	4	PLT+ , r RBC Aggl: 60%: s 40%, b 20%	PLT+ , r RBC Aggl: 65%: s 25-30%, b 15%
5	5	PLT+ , r RBC Aggl: 65%: s 40%, b 15-20%	PLT+ , r RBC Aggl: 65-70%: s 20%, b 20%
6	6	PLT+ , r RBC Aggl: 70%: s 35, b 15-20%	PLT+ , r RBC Aggl: 75%: s 25%, b 20-25%
7	27	PLT+ , r RBC Aggl: 80%: s 40%, 5%	PLT+ , r RBC Aggl: 80%: s 25%, b 5-10%
8	34	PLT+ , r RBC Aggl: 80-90%: s 10%, b 60%	PLT+ , r RBC Aggl: 80%: s 10%, b 25-30%
9	51	PLT+ , r RBC Aggl: 80%: s 30%, b 15%	PLT+ , r RBC Aggl: 80%: s 5%, b 25-30%
10	76	PLT+ , r RBC Aggl: 70-80%: s 30%, b 15-20%	PLT+ , r RBC Aggl: >90%: s 5%, b 75%
11	83	PLT+ , r RBC Aggl: 80-90%: s 10%, b 20%	PLT+ , r RBC Aggl: 70-80%: s <5%

* For all the examined preparations, the PLT standards preserved the intact unstimulated discocyte forms of PLT with less than 10% echinodiscocytes disposing no long pseudopodia or fully activated shapes.

PLT+, PLT++, PLT+++ = low, moderate and high PLT concentration respectively; f= few; r= rare; Aggl = the percentage of agglutinated PLT; s, b: the percentage of PLT forming small (2-4 PLT) and big Aggl (>18 PLT) respectively.

The microscopic control of the PLT standards (Tab.4-2-5-3-2) showed the intact unstimulated discocyte forms of PLT with less than 10% echinodiscocytes disposing no long pseudopodia or activated shapes.

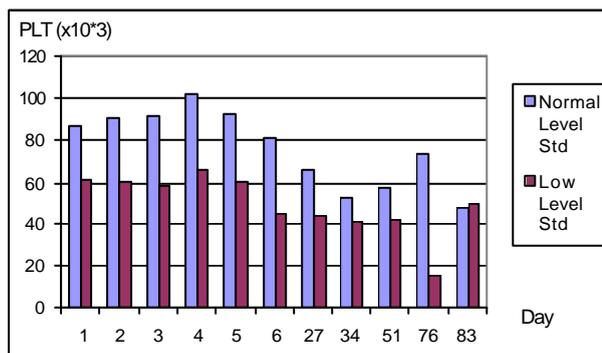


Fig. 4-2-5-3-1: Day-by-day precision of the PLT count in the first standards of washed PLT.

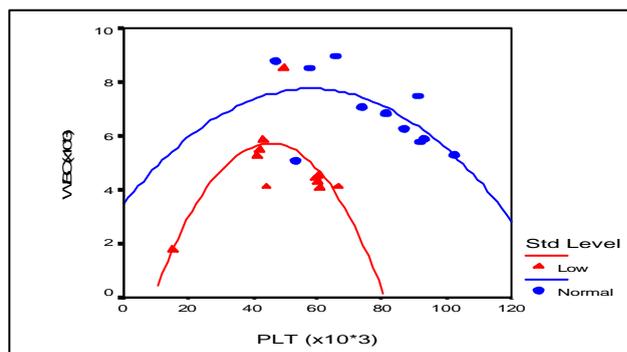


Fig. 4-2-5-3-2: Correlation between PLT and WBC count in the day-by-day control of the first standards of washed PLT.

4-2-5-4. Day-by-day precision of the second lot of PLT standard

The PLT count on the 1st day (429 x10³/μL; Tab.4-2-5-4-1) was comparable to the expected value (413 x10³/μL; p.4-5-2-1-3). PLT demonstrated later significant decrease by time (r=-0.94; p<0.0005; Fig.4-2-5-4-1). On the 2nd day, PLT declined remarkably (11%) and then showed evident stability within

the next 6 days ($366-359 \times 10^3/\mu\text{L}$). PLT continued to decrease by time and stepped below $300 \times 10^3/\mu\text{L}$ (i.e., >30% decrease) after 33 days. WBC increased by time in close association with PLT decrease ($r=-0.94$, $p<0.0005$) and the increase in the Aggl observed microscopically (Tab.4-2-5-4-2). PLCR and PLT size distribution demonstrated rather stable features by time (Ab.4-2-5-2; Fig.4-2-5-4-3 & 4).

Table 4-2-5-4-1: Summary* of the key results of the day-by-day control on the second unwashed PLT standard (Suw-2).

Serial No.	Time (day)	PLT ($\times 10^3/\mu\text{L}$)	WBC ($\times 10^3/\mu\text{L}$)	RBC ($\times 10^6/\mu\text{L}$)
1	1**	429	0.2	0.03
2	2	366	1.0	0.05
3	5	359	1.5	0.03
4	8	330	2.7	0.04
5	12	331	3.2	0.03
6	15	332	4.3	0.03
7	18	310	4.6	0.03
8	21	307	6.1	0.03
9	26	312	6.6	0.03
10	29	307	7.9	0.03
11	33	279	12.1	0.03
12	38	280	12.4	0.03

* The detailed results of the CBC measurements are described in Tab.Ab.4-2-5-4. **The measurements were applied 3 hours after preparing the standard, whilst the microscopic examination of the standard was performed within 30 min after its preparation.

Morphologically, the PLT preserved their intact unstimulated discocytes forms, no WBC was found, and only rare traces of RBC were encountered (Tab.4-2-5-4-2).

Table 4-2-5-4-2: Day-by-day morphological control on the second unwashed PLT standard (Suw-2) by phase-contrast microscopy*.

Serial No.	Time (day)	Microscopic Examination
1	1	PLT+++; r RBC Aggl: <1%: s >97%
2	2	PLT+++; r RBC Aggl: 10%: s 80%
3	5	PLT+++; r RBC Aggl: 10-15%: s 75%
4	8	PLT+++; r RBC Aggl: 20%: s 65%
5	12	PLT+++; r RBC Aggl: 20%: s 60%
6	15	PLT+++; r RBC Aggl: 20%: s 50-60%
7	18	PLT+++; r RBC Aggl: 25%: s 50%
8	21	PLT+++; r RBC Aggl: 25%: s 40%
9	26	PLT+++; r RBC Aggl: 25%: s 35%
10	29	PLT+++; r RBC Aggl: 25%: s 25%
11	33	PLT+++; r RBC Aggl: 30-35%: s 10-20%
12	38	PLT+++; r RBC Aggl: 30-35%: s 5%

* For all the examined preparations, the unwashed PLT standard preserved the intact unstimulated discocyte forms of PLT (<10% echinodiscocytes, no long pseudopodia or fully activated shapes). PLT+++ = high PLT concentration; r= rare; Aggl= the percentage of agglutinated PLT; s, b: the percentage of PLT forming small (2-4 PLT) and big Aggl (>18 PLT) respectively.

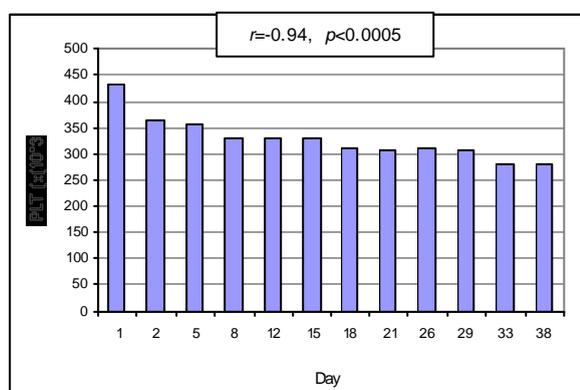


Fig. 4-2-5-4-1: Day-by-day precision of the PLT count in the second lot of the standard of unwashed PLT (Suw-2).

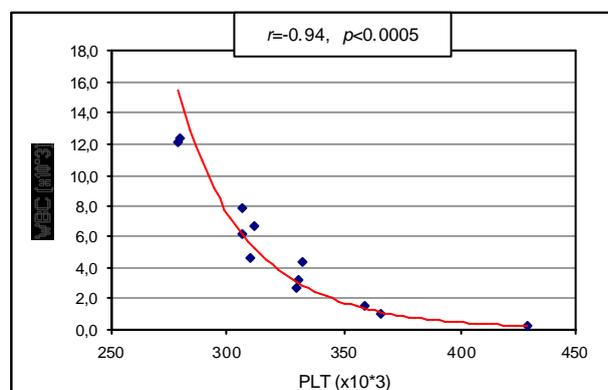


Fig. 4-2-5-4-2: Correlation between PLT and WBC in the day-by-day control of the second unwashed PLT standard.

4-2-5-5. Day-by-day precision of the third lot of PLT standard

In comparison with the standard of the unwashed PLT (Suw-3), the washed PLT standard (Sw-3) revealed much lower ($p < 0.0005$) PLT levels and demonstrated significantly more evident ($p = 0.005$) decrease in PLT by time, as described in Tab.4-2-5-5 and illustrated in Fig.4-2-5-5-1. On the 1st day (few hours after preparation), the Sw-3 delivered a PLT count ($90 \times 10^3/\mu\text{L}$; Tab.4-2-5-5-1) that was much lower (77%) than the expected value ($396 \times 10^3/\mu\text{L}$; p.4-5-2-1-3). On the 2nd day, PLT declined sharply and then preserved rather stable range ($58-68 \times 10^3/\mu\text{L}$) within the next 30 days. The Suw-3 showed rather stable PLT count within the first 17 days ($373-411 \times 10^3/\mu\text{L}$) that ranged about the expected value ($396 \times 10^3/\mu\text{L}$). PLT began to decrease significantly later, where it stepped down $252 \times 10^3/\mu\text{L}$ (i.e., >33% decrease) on the 31st day. WBC levels in the washed standard (Sw-3) were much higher than those in the unwashed one (Suw-3: $p = 0.005$; Fig.4-2-5-5-2). The increase of WBC was closely correlated with PLT decrease ($r = -0.669$, $p = 0.002$; Fig.4-2-5-5-3) and was positively correlated with the PLT agglutination observed microscopically (Tab.4-2-5-5-2). This correlation was more pronounced in the unwashed standard (Suw-3). The parameters of PLT size distribution of the washed standard (Sw-3) could not be recognized by the automatic counter, whilst it was determinable for the unwashed standard (Suw-3) and presented a remarkable decrease by time (Ab.4-2-5-2; Fig.4-2-5-5-4 and 5).

Table 4-2-5-5-1: Summary^a of the key results of the day-by-day control of the third lot of PLT standard using RTH.

Standard Type	Serial No.	Time (day)	PLT ($\times 10^3/\mu\text{L}$)		PLCR (%)		WBC ($\times 10^3/\mu\text{L}$)		RBC ($\times 10^6/\mu\text{L}$)		PLT-RI (%)	PLCR-RI (%)	WBC-RI (%)
			b.F	a.F	b.F	a.F	b.F	a.F	b.F	a.F			
Washed PLT Standard (Sw-3)	1 ^b	1	90	58	NR	NR	4.5	3.0	0.05	0.01	35.6	NR	33.3
	2	3	59	87	NR	NR	4.3	2.4	0.02	0.01	-47.5 ^d	NR	44.2
	3	6	64	53	28.9	NR	4.2	2.5	0.01	0.01	17.2	NR	40.5
	4	8	65	55	NR	NR	4.5	2.9	0.02	0.01	15.4	NR	35.6
	5	11	68	61	NR	NR	5.0	2.8	0.02	0.01	10.3	NR	44.0
	6	14	61	49	29.2	NR	4.0	2.3	0.01	0.01	19.7	NR	42.5
	7	17	66	55	NR	NR	4.5	2.6	0.02	0.01	16.0	NR	42.7
	8	22	66	53	28.8	NR	3.7	1.9	0.01	0.03	19.7	NR	48.6
	9 ^c	31	58		NR		3.8		0.10				
	Mean										19.1	NR	41.4
Unwashed PLT Standard (Suw-3)	1 ^b	1	377	356	39.2	37.9	0.8	0.8	0.6	0.04	5.6	8.7	20.0
	3	3	411	356	35.8	34.9	0.9	0.9	0.5	0.04	13.4	15.6	44.4
	3	6	387	364	34.8	34.2	0.9	0.9	0.5	0.03	5.9	7.6	44.4
	4	8	380	371	33.3	33.3	1.0	0.9	0.9	0.03	2.4	2.4	10.0
	5	11	373	290	33.6	32.9	1.4	1.4	0.9	0.03	22.3	23.9	35.7
	6	14	386	362	32.2	31.3	1.8	1.8	1.1	0.03	6.2	9	38.9
	7	17	380	364	31.0	30.0	3.1	3.1	1.4	0.04	4.2	7.3	54.8
	8	22	345	359	31.0	28.0	3.6	3.6	1.3	0.03	-4.1 ^d	6.0	63.9
	9 ^c	31	252		28.7		9.3	9.3		0.01			
	Mean										8.6	10.0	39.0

^aThe detailed results of RTH measurements are described in Tab.Ab.4-2-5-5. ^bThe measurements were applied 2 hours after preparing the standard, whilst the microscopic examination was performed within 30 min after its preparation. ^cThe amount was too little for performing a.F measurements and was partly gelled. ^dThe negative PLT-RI values were dropped out from the mean because they are attributed to PLT degradation and not to a real retention to the PLT, as explained in the discussion (p.5-1-2-7-A-3-c). b.F= RTH measurements performed on the PLT standard before centrifugation and passing RTH filter (in the collection tube); a.F= RTH measurements performed on the PLT standard after centrifugation and passing RTH filter (in the lower part of RTH tube); NR= not readable by the automatic cell reader.

Performing RTH revealed significantly ($p=0.01$) higher PLT-RI for the washed standard (Sw-3: mean=19.1%; range 10.3 to 35.6%) compared with the unwashed standard (Suw-3: mean=8.6%; range 2.4 to 22.3%), as demonstrated in Fig.4-2-5-5-4. Contradictory could be interpreted microscopically by the degradations of Aggl after passing RTH filter, as explained in p.5-1-2-7-3-B.

WBC-RI levels were remarkably high in both the washed (Sw-3) and the unwashed (Suw-3) standards (mean WBC-RI= 41.4% and 39.0%; range= 33.3-48.6% & 10-63.9% respectively). WBC-RI seemed to increase by time; especially in the unwashed standard (Suw-3: Fig.4-2-5-5-5). PLCR-RI of the unwashed standard (Suw-3) was significantly correlated with PLT-RI ($r=0.952$, $p<0.0005$), as shown in Fig.4-2-5-5-6.

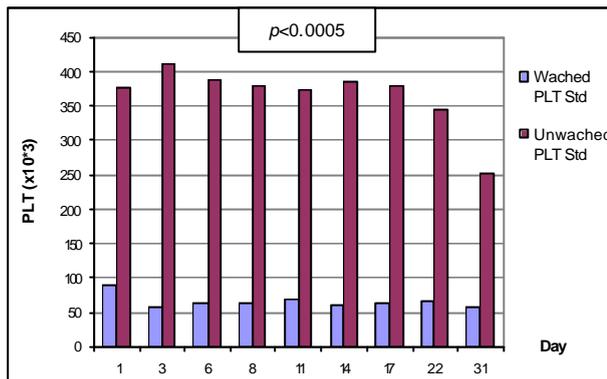


Fig. 4-2-5-5-1: Day-by-day precision of the PLT count in the third lot of the PLT standards.

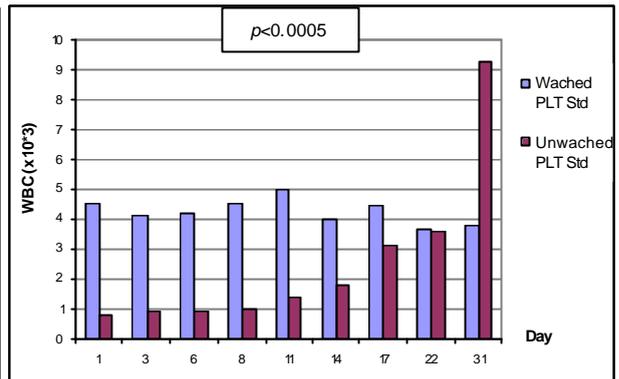


Fig. 4-2-5-5-2: Day-by-day precision of the WBC count in the third lot of the PLT standards.

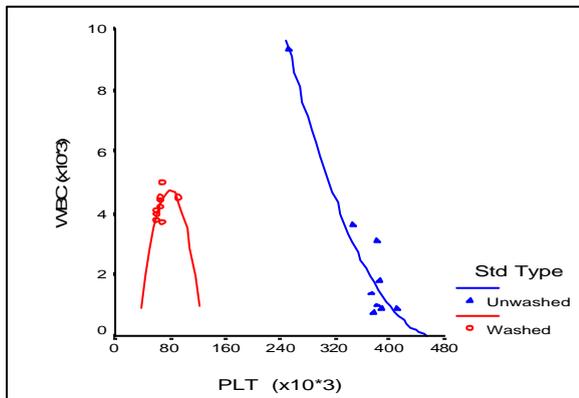


Fig. 4-2-5-5-3: Correlation between PLT and WBC in the day-by-day control of the third lot of the PLT standards.

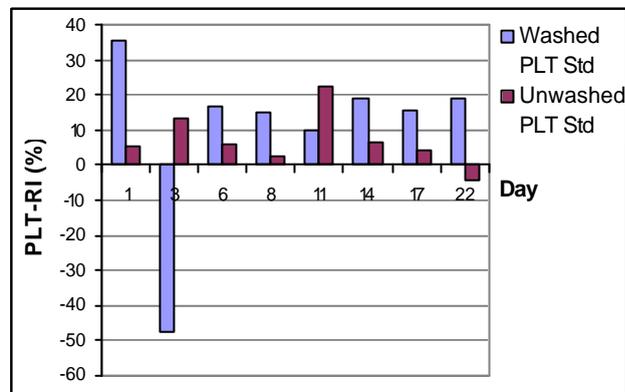


Fig. 4-2-5-5-4: Day-by-day control of PLT-RI in the third lot of the PLT standards.

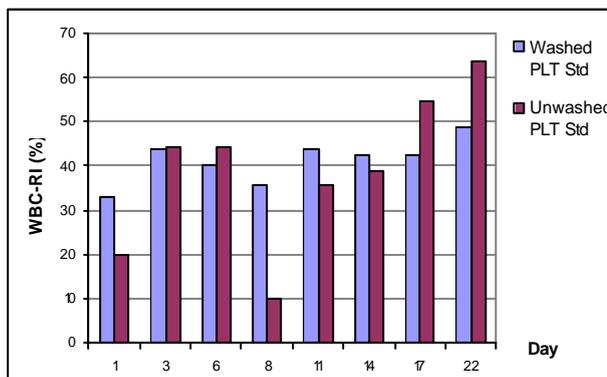


Fig. 4-2-5-5-5: Day-by-day control of WBC-RI in the third lot of the PLT standards.

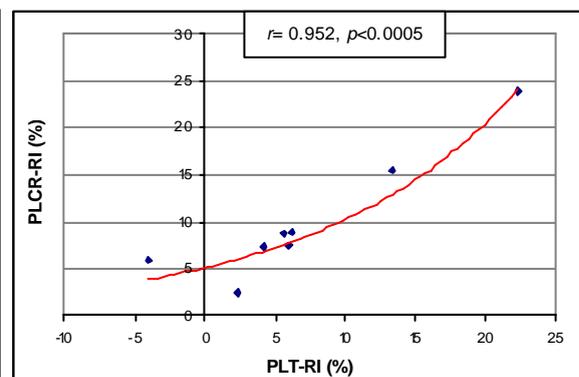


Fig. 4-2-5-5-6: Correlation between PLCR-RI and PLT-RI in the day-by day control of the third lot of the unwashed PLT standard (Suw-3).

The microscopic control of both the washed (Sw-3) and the unwashed (Suw-3) PLT standards showed permanently intact unstimulated discocytes forms of PLT (<10% echinodiscocytes, no long pseudopodia or activated shapes). No traces of leucocytes could be objected and only rare erythrocytes were counted (Tab.4-2-5-5-2).

Table 4-2-5-5-2: Day-by-day morphological control on the third lot of PLT standards by phase-contrast microscopy*.

Serial No.	Time (day)	The Washed PLT Standard (Sw -3)		The Unwashed PLT Standard (Suw -3)	
		b.F	a.F	b.F	a.F
1	1	PLT+, r RBC Aggl: 75%: s 10%, b 65%	PLT+, r RBC Aggl: 15-20%: s 10-20%	PLT+++, r RBC Aggl: 2-5%: s 90%	PLT+++, r RBC Aggl: <3%: s >90%, b<2%
2	3	PLT+, r RBC Aggl: 85%: s 10%, b 70%	PLT+, r RBC Aggl: 15% s 40-50%, b<5%	PLT+++, r RBC Aggl: <1%: s 90%	PLT+++, r RBC Aggl: <3%: s >90%, b<2%
3	6	PLT+, r RBC Aggl: 80%: s 10%, b 70%	PLT+, r RBC Aggl: 25-30%: s 20%	PLT+++, r RBC Aggl: 2-5%: s 90%	PLT+++, r RBC Aggl: 2-4%: s >90%, b<2%
4	8	PLT+, r RBC Aggl: 80%: s 10%, b 70	PLT+, r RBC Aggl: 30%: s 20%	PLT+++, r RBC Aggl: 5%: s 80%	PLT+++, r RBC Aggl: 5%: s 90%, b <2%
5	11	PLT+, r RBC Aggl: 80%: s 10%, b 65%	PLT+, r RBC Aggl: 25%: s 20%	PLT+++, r RBC Aggl: 5-10%: s 75%	PLT+++, r RBC Aggl: 5%: s 85% , , b<2%
6	14	PLT+, r RBC Aggl: 80-85%: s 10%, b 70%	PLT+, r RBC Aggl: 25-30%: s 20%	PLT+++, r RBC Aggl: 5%: s 50-60%	PLT+++, r RBC Aggl: 5-10%: s 65%, b 5%
7	17	PLT+, r RBC Aggl: 80%: s 10%, b 70%	PLT+, r RBC Aggl: 25-30%: s 15-20%	PLT+++, r RBC Aggl: 5-10%: s 30%	PLT+++, r RBC Aggl: 5%: s 65%, b<2%
8*	22	PLT+, r RBC Aggl: 80%: s10%, b 70-75%	PLT+, r RBC Aggl: 25%: s 15%	PLT+++, r RBC Aggl: 15%: s 40%	PLT+++, r RBC Aggl: 5-10%: s 65%, , b<2%
9	31	PLT+, r RBC Aggl: 85%: s 10%, b 70-75%		PLT+++, r RBC Aggl: 35%: s 20%	

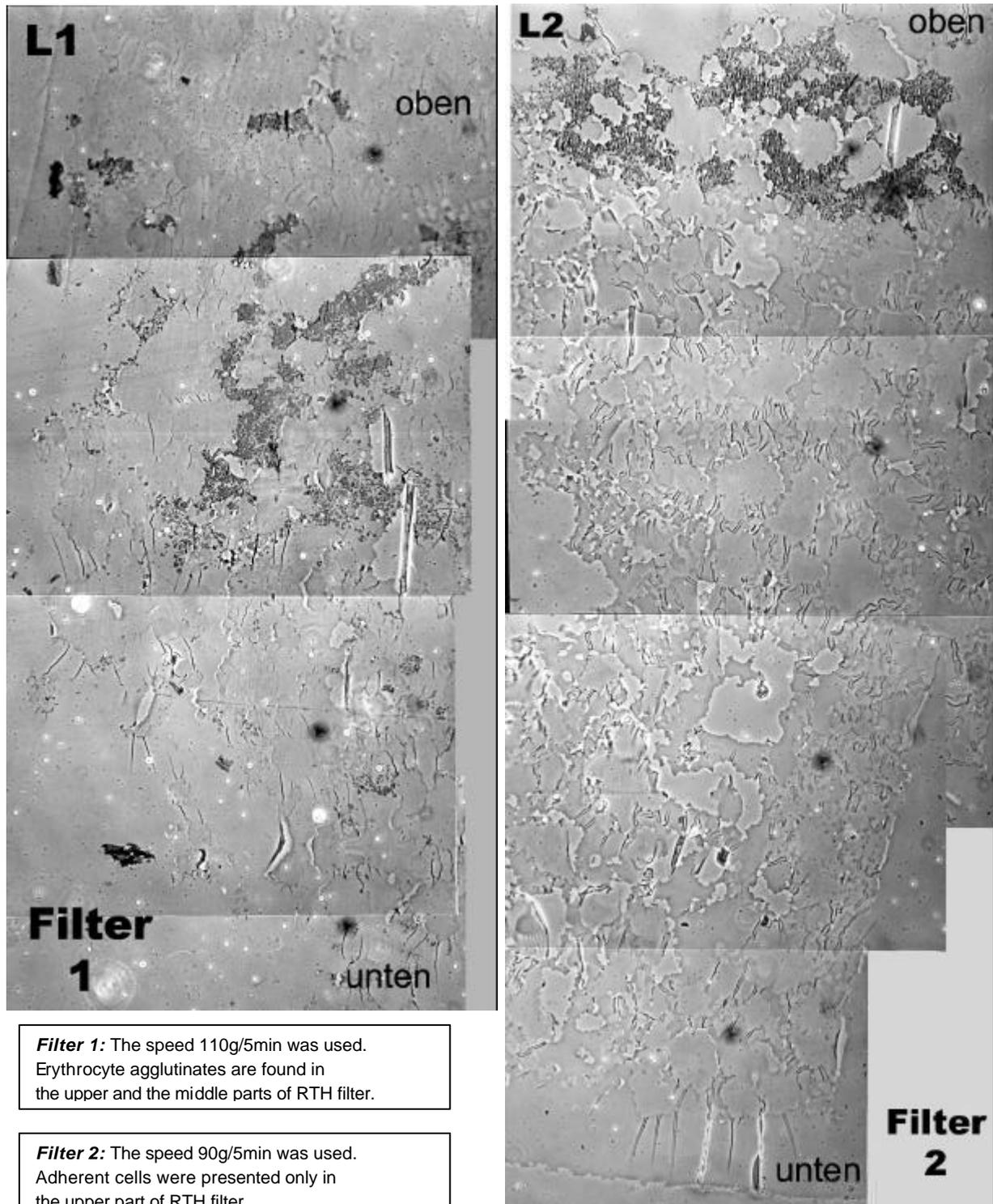
* For all the examined preparations, the PLT standards preserved the intact unstimulated discocyte forms of PLT (<10% echinodiscocytes, no long pseudopodia or fully activated shapes). PLT+++ = high PLT concentration in the microscope field; r= rare; Aggl = the percentage of agglutinated PLT; s, b: the percentage of PLT forming small (2-4 PLT) and big Aggl (>18 PLT) respectively.

4-2-6. Morphological characterization of the interaction of platelets and blood cells with RTH filter

4-2-6-1. Aim and methodology of the study

This study was designed to further evaluate the retention phenomena for PLT and blood cells as well as to characterize the modalities of their interactions with the surfaces of the inter-connected micro

Fig. 4-2-6-2-1: Phase-contrast microscopy of RTH filter sections obtained by applying different centrifugal speeds.



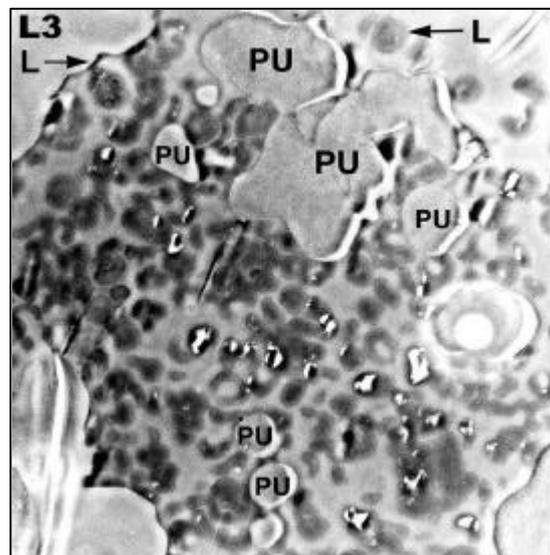
pores of the polyurethane filter of RTH (PU; p.3-2-1). RTH assay (SOP-3; p.4-1-2-5) was performed on cWB drawn from 2 healthy blood donors (sample 1: PLT count= $278 \times 10^3/\mu\text{L}$; PLT-RI=32%, sample 2: PLT count= $236 \times 10^3/\mu\text{L}$; PLT-RI=28%) Semi- and ultra-thin fixed sections of RTH filter were prepared afterwards and examined by phase-contrast besides scanning (SEM) and transmission (TEM) electron microscopy, as described in the methods (p.3-5-3). Two samples of cWB were used for performing RTH and for each two rotational speeds were used for the centrifugation of RTH tubes: 110 g and 90 g. Increasing time intervals were used for the centrifugation of RTH tube by 110 g (5, 10 and 15 min). Only of the key results of the electron microscopy of RTH filter by using our standard condition 110 g/ 5 min are summarized herein because: a.) Using the speed 110 g resulted in acceptable differentiation of blood cells in the filter compared with limited one by using the speed 90 g (described in p.4-2-6-2), and b.) The application of 110 g with intervals higher than 5 min revealed no further improvement in the distribution of blood cells in RTH filter. On the other hand, due to the findings revealed by phase-contrast microscopy (p.4-2-6-2) blood cells were more available in the upper than in the middle or the lower parts of RTH filter. The results of the electron microscopy presented here are therefore focused on the sections of the upper part of RTH filter, unless otherwise is mentioned.

The protocol of this study was also performed on 4 samples of the normal level Eightcheck control (EC) and on 2 samples of cPRP, and the results were published elsewhere [Nemeh 2005, 2005, 2006]. The key results are summarized here also since they are of vital significance for understanding the specificity of RTH assay. Actually, this study represented a further step for the morphological assessment of the specificity and the accuracy of RTH assay for screening the function of PLT (p.5-2-7, 9).

4-2-6-2. Phase-contrast microscopic examinations of RTH filter by using cWB

By the use of the centrifugal speed 90 g/ 5 min, the blood cells (agglutinated erythrocytes, leukocytes and PLT aggregates) were found only in the mesh of the upper parts of RTH filter (Fig.4-2-6-2-1). Using 110 g/ 5 min, the blood cells reached significantly deeper levels. They were observed in the mesh of the upper and the middle parts of the filter demonstrating lower density in the later, as shown in Fig.4-2-6-2-1. In both cases, only few blood cells were objected in the lower part of RTH filter. The Fig.4-2-6-2-2 presents an example of higher magnification of the middle part of RTH filter by using the centrifugal speed i.e., 110 g/ 5 min.

Fig. 4-2-6-2-2: Phase-contrast microscopy of a section in the middle part of RTH filter (110g/5min). Among the erythrocytes, two leukocytes were objected (L). PU= polyurethane.



4-2-6-3. Electron microscopic examinations of RTH filter by using cWB

The surfaces of the filter could always be recognized (Fig.4-2-6-3-1 to 3). The bulging of the sintered polyurethane (PU) material of the filter results in a mesh of interconnected micropores or canals with various widths. The filter structure forms therefore a labyrinth-like mesh that resembles the inter-cellular matrix.

A.) Platelets findings:

PLT aggregates on the filter were extensively objected by SEM (Fig.4-2-6-3-a-1), while significantly less leucocytes and erythrocytes adhered on it. The platelets adhered mainly on the surfaces of the upper part of the filter and spread extremely forming wall bound aggregates that could mostly bridge between the opposite surfaces of the filter pores, as shown in Fig.4-2-6-3-a-2. Individually adherent and/or non-spread PLT were occasionally encountered Fig.4-2-6-3-a-3. The density of adherent and aggregated PLT is related with their activation and retention value as well as their count before the passage of the filter. The TEM showed adhering and wide-spreading PLT with prolonged pseudopodia building very thin layers covering the PU surface of the most upper part of the filter (Fig.4-2-6-3-a-4). The adherent PLT developed a network of aggregates that were filled with plasma and mostly bridging the opposite PU surfaces (Fig.4-2-6-3-a-1, 2 & 5). The characteristic PLT-contacts objected in the endothelium were clearly built

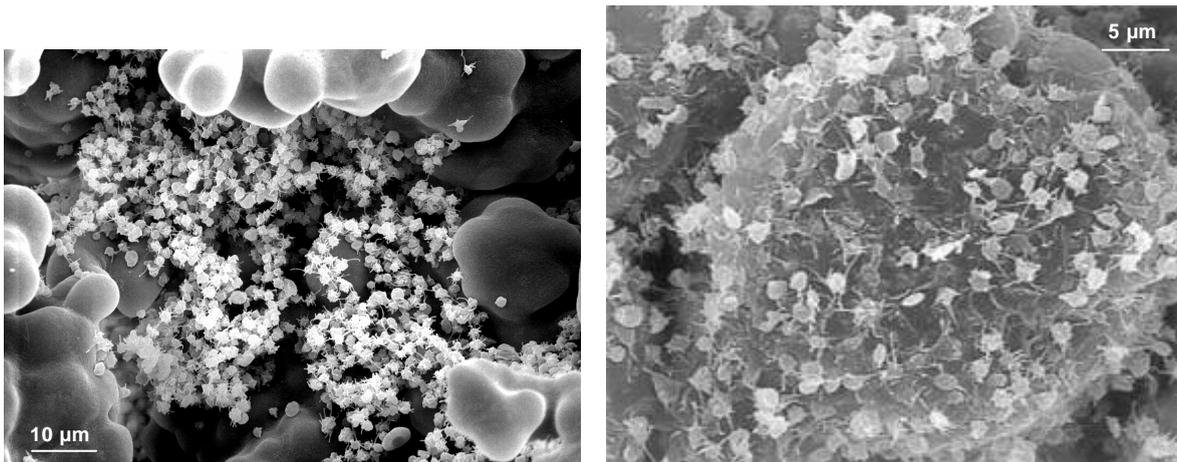


Fig.4-2-6-3-a-1: SEM showing extensive platelet adherence and aggregation on the surfaces of the porex filter. (PLT count= $278 \times 10^3/\mu\text{L}$; PLT-Rl=32%). SEM= scanning electron microscopy.

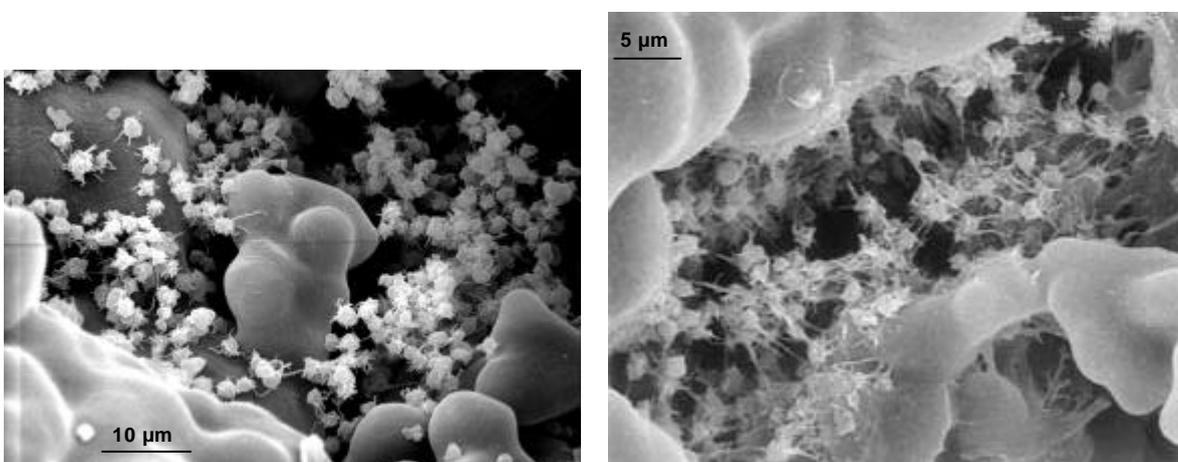


Fig.4-2-6-3-a-2: SEM showing wall-bound PLT aggregates bridging the opposite surfaces of the filter pores. (PLT count= $236 \times 10^3/\mu\text{L}$; PLT-Rl=28%). SEM= scanning electron microscopy.

by PLT aggregates demonstrating: (a) connecting tight contacts and (b) bridges of fibrinogen molecules, as shown in Fig.4-2-6-3-a-6. PLT borders could not always be recognized implicating irreversible aggregates (Fig.4-2-6-3-a-2 & 5). Free aggregates were encountered in the upper and in the middle parts of the filter (Fig.4-2-6-3-a-7). Other signs of PLT activation were evident in forming contractile cytoskeleton associated with shape changes and moderate degranulation/secretion (Fig.4-2-6-3-a-8). No contact between the platelets and leukocytes or erythrocytes was objected.

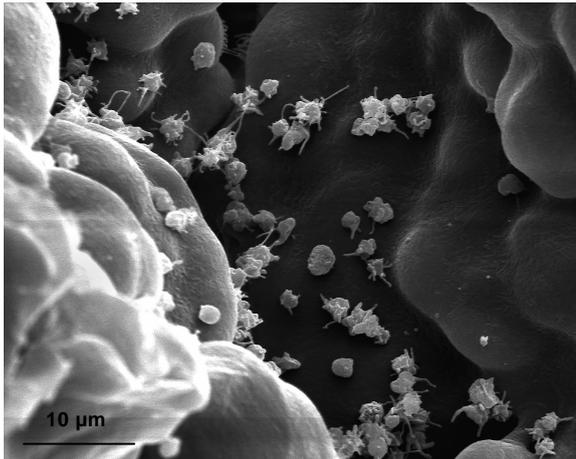


Fig.4-2-6-3-a-3: SEM showing occasional single and/or nonspread adherent PLT. (PLT count=236x10³/μL; PLT-RI=28%). SEM= scanning electron microscopy.

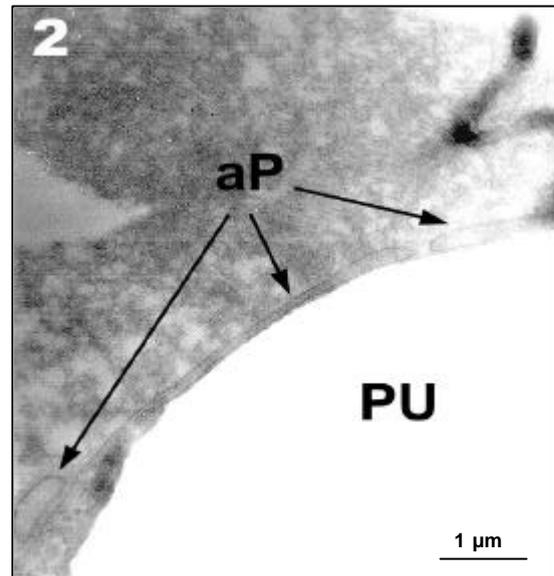


Fig. 4-2-6-3-a-4: TEM showing adherent PLT (aP) spread extremely and degranulated building a very thin layer on the polyurethane (PU) surface of the filter. TEM= transmission electron microscopy.

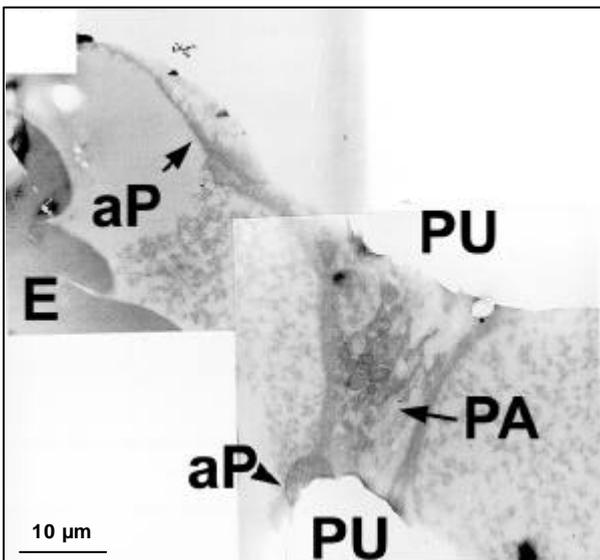


Fig. 4-2-6-3-a-5: TEM showing adherent (aP) and aggregated (PA) platelets bridging a small pore (PU) of RTH filter. TEM= transmission electron microscopy. E = erythrocytes.

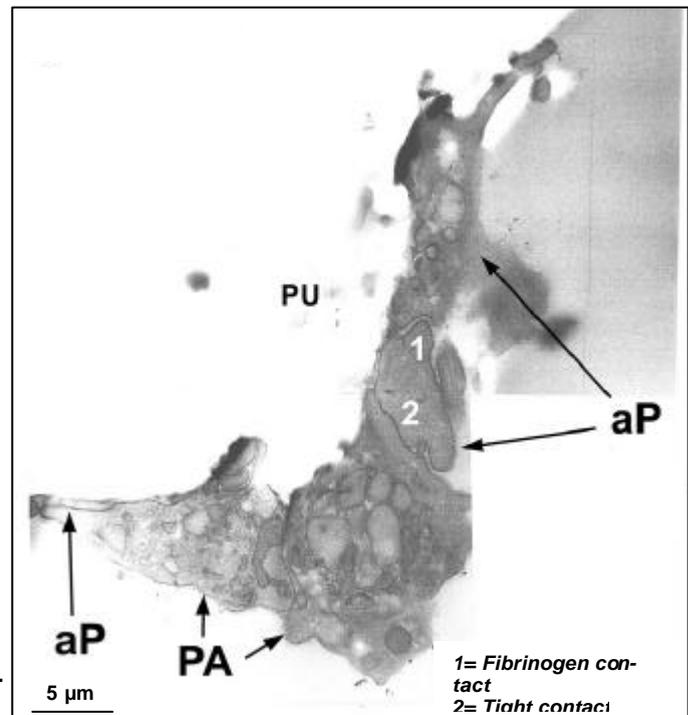


Fig. 4-2-6-3-a-6: Adherent (aP) and aggregated (PA) platelets on the polyurethane surface (PU). The characteristic fibrinogen-mediated contacts (1) and the "tight contacts" (2) are demonstrated.

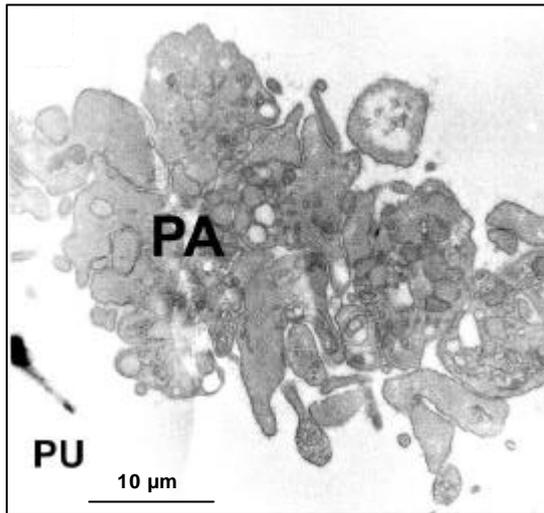


Fig. 4-2-6-3-a-7: TEM demonstrate free PLT aggregate (PA) in the middle part of RTH filter. TEM= transmission electron microscopy; PU= polyurethane.

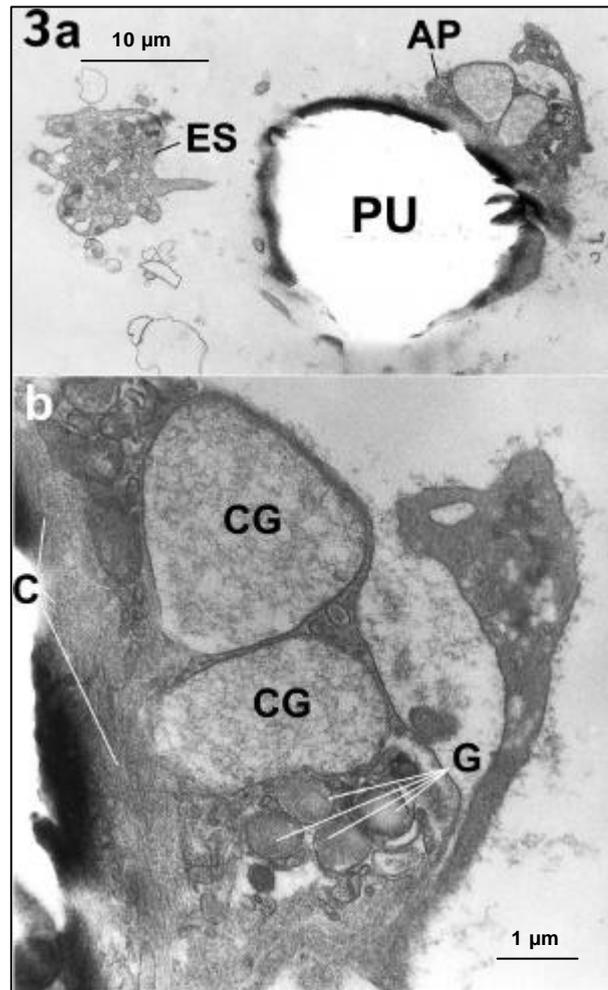
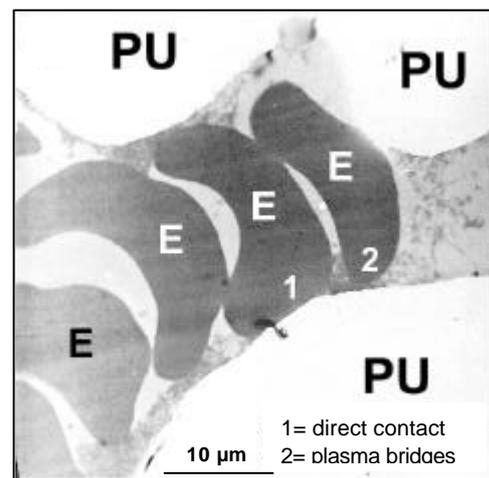


Fig. 4-2-6-3-a-8: TEM demonstrating PLT degranulation. In part (a) PLT adheres (AP) on the polyurethane surface of a small pore of the filter. The magnification in part (b) shows the micro-filament of the cytoskeleton (C) in the adherence area. Granules during the secretion are distinguished beside the unchanged alpha granules (G). EC= Echinocytes (activated PLT); TEM= transmission electron microscopy.

B.) Erythrocytes findings:

Big erythrocyte agglutinates were objected in the upper part of the filter where the coin-roll (rouleaux) formations were frequently seen. The erythrocytes adhered directly on the polyurethane (PU) surface via membrane contact (Fig.4-2-6-3-b). Most of the erythrocytes retained blood plasma layers between the cells and the PU-surfaces (plasma bridges; Fig4-2-6-3-b).

Fig. 4-2-6-3-b: Coin-rolls forms of erythrocytes (E) in the upper part of RTH filter showing direct membrane contact with the polyurethane surface PU (1). Most of them retain plasma layers between the cells and PU (2).



C.) Leukocytes findings:

Monocytes (Fig.4-2-6-3-c-1) and neutrophil granulocytes (Fig.4-2-6-3-c-2) adhered on the PU surface of RTH filter, but they were also found among the erythrocytes. Whilst it was possible to find contacts between the adherent monocytes and erythrocytes (Fig.4-2-6-3-c-1), no such contacts were observed from the granulocytes. No contacts between the PLT and leukocytes could be objected.

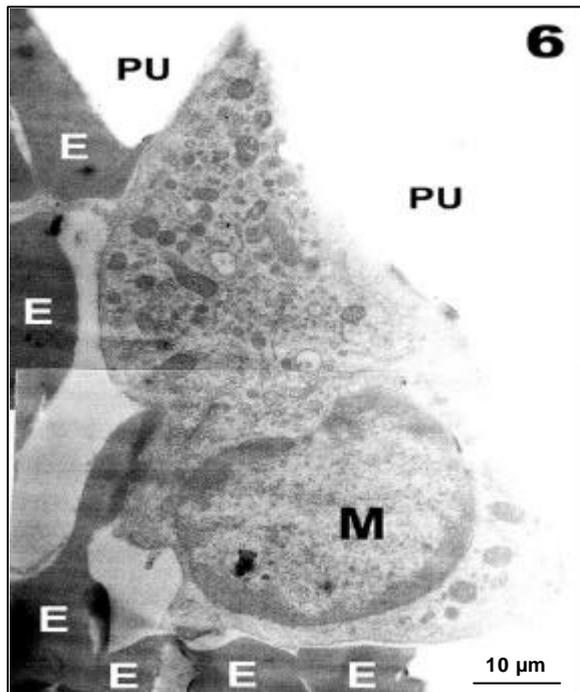


Fig. 4-2-6-3-c-1: Adherent leukocytes on the polyurethane surface of RTH filter (PU). A monocyte (M) and an erythrocyte (E, above) adhere on PU. The erythrocytes (E) demonstrate partly close contacts to the monocytes.

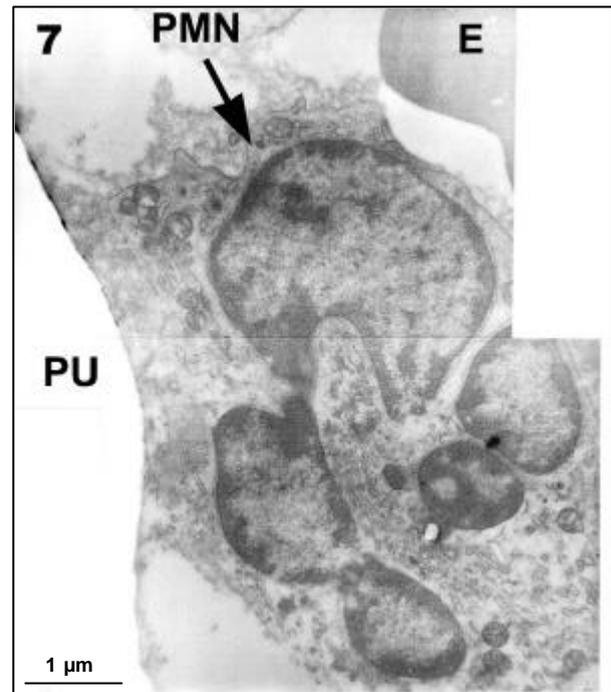
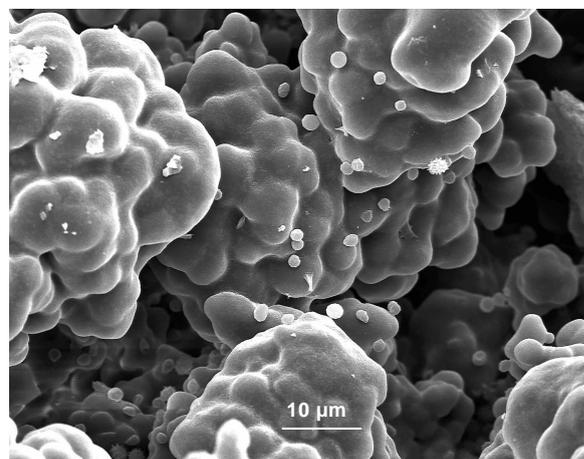


Fig. 4-2-6-3-c-2: A granulocyte (PMN) adhere on the polyurethane surface of RTH filter (PU). The cell was fully degranulated; no contact with other cells was objected.

4-2-6-4. Electron and phase-contrast microscopy of RTH filter by using Eightcheck control (EC)

The prefixed PLT in the normal level Eightcheck control (EC) were mainly found in the mesh of the upper part of RTH filter. The concentration of PLT decreased in deeper parts. Examining the upper part by SEM showed that PLT were spherical in shape, clumping in the canal lumen and adhered rarely on its PU surfaces (Fig.4-2-6-4-1). Examining the filter by TEM showed PLT agglutinates composing of degranulated echinospherocytes. The PLT in these agglutinates were connected via "PLT selective contacts" (PC) and demonstrated no real adherence on the PU surface of RTH filter (Fig.4-2-6-4-1). Occasionally, some PLT agglutinates presented focal contacts with the PU surface of the filter (Fig.4-2-6-4-2). Agglutinated leukocytes and erythrocytes were objected in low rate comparable to that encountered in cWB.

Fig. 4-2-6-4-1: SEM showing partly degranulated echinospherocytes connected via focal contacts (FC*) by which they can adhere on the PU surface of RTH filter.
ML= membrane lesion.



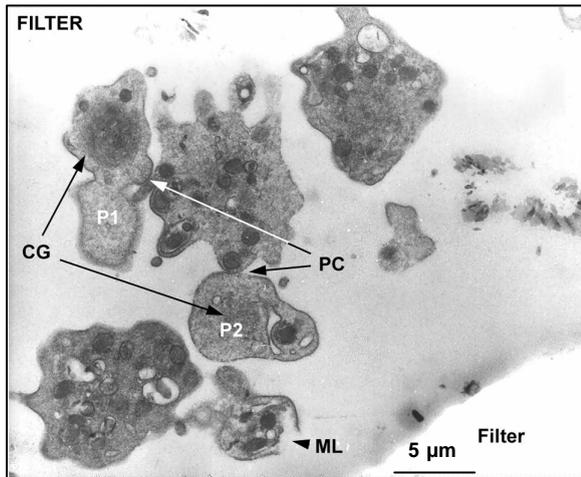


Fig. 4-2-6-4-2: TEM showing degranulated echinospherocytes (P1 and P 2) connected via platelet selective contacts (PC) with centralized contractile gel (CG) and not adherent on the filter surface. ML= membrane lesion.

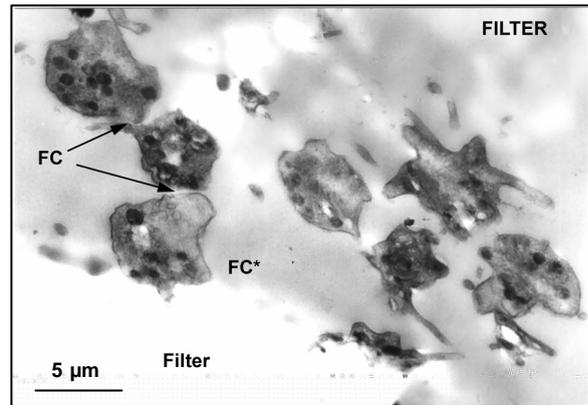


Fig. 4-2-6-4-3: TEM showing partly degranulated echinospherocytes connected via focal contacts (FC*) by which they can adhere on the PU surface of RTH filter. ML= membrane lesion.

4-3. Reference ranges of the retention indices (RIs) of RTH assay measured in cWB

4-3-1. Aim and design of the study

The main aim of this study was to establish the reference ranges of the “main” retention indices (PLT-RI and PLCR-RI) of RTH assay performed on cWB from healthy individuals. The reference ranges of the “secondary” retention indices (PDW-RI, MPV-RI, WBC-RI, RBC-RI, HGB-RI, & HCT-RI) were in parallel established to provide more insight into the effect of RTH filter. In addition, the study was designed to investigate the correlations between the different retention indices (RIs) in healthy people. Our consequent question was whether the identification of such probable correlations along with addressing the reference ranges of the secondary RIs may together help for:

- A.) Enlarging the clinical significance of RTH assay by the use of cWB.
- B.) Playing the role of additional discriminators to the accuracy and the right interpretation of the main RIs i.e., PLT-RI and PLCR-RI, and indicating the possible errors in these indices.

Apparently healthy blood donors (BD) were recruited from the blood bank (p.3-5-1). The SOP-2 of RTH (p.4-1-1-7) was initially used on 20 healthy individuals. After addressing the significant improvement in RTH precision and accuracy by the application of SOP-3 in comparison with SOP-2 (Tab.5-1-3) and the acceptable results of the validation of SOP-3 (p.5-2), we decided to put apart the results of SOP-2 and to apply SOP-3 for the study of the reference ranges of RTH. Thus, RTH assay was performed on 73 blood donors by the application of SOP-3. The accuracy of PLT and blood cell retention indices and the normal integrity of PLT were controlled by phase-contrast microscopy (p.3-5-2). The results of 3 BD were dropped since they were far away from the statistical distribution of the other results (Tab.Ab.4-3-1-1). The study of the reference range included therefore at least 70 blood donors.

Most of RTH measurements were performed in-double and the means of the resulting values were used to establish the normal ranges and to perform the different investigations in this study. The reference sample was assigned into 3 subgroups according to the mode of performing the measurements before (b.F) and after (a.F) passing RTH filter (Tab.4-3-4):

- 1.) 2b.F:2a.F = two b.F as well as a.F measurements (n= 31).
- 2.) 2b.F:1a.F = two b.F measurements and one a.F measurement (n= 18).
- 3.) 1b.F:1a.F = one b.F as well as a.F measurement (n= 21).

The compatibility of the results of these three modes of measurements and the probable improvements by a specific mode was investigated. On the other hand, RTH measurements were initially performed by using the automatic cell counter “Sysmex M 2000”. For further assurance of the reliability, more modern counters i.e., Sysmex KX-21 and Sysmex SF 3000 were used later (Tab.4-3-5) and the probable improvements in the accuracy and the precision of the measurements were inspected.

4-3-2. Establishing the reference ranges of the retentions indices of RTH

The detailed measurements of the reference ranges of the retention indices (RIs) of RTH are reported in Tab.Ab.4-3-2. The median, the percentiles, and the 90% reference range of the different RIs (RI) of RTH in cWB are described in Tab.4-3-2-1. The formal test of normal distribution (Kolmogorov Smirnov test)

confirmed the normal assumption for PLT-RI ($p=0.2$) as well as the other RIs ($p>0.05$). But the normality of RBC-RI, HGB-RI and HCT-RI was rather critical, as shown in Tab.4-3-2-1. The distributions of the different RIs were demonstrated by histograms. PLT-RI values trended to be skewed to the right (Fig.4-3-2-1). The distribution of PLCR-RI, PDW-RI, MPV-RI and WBC-RI was in line with normal assumption (Fig.4-3-2-2 to 4). Few high outliers were found in PDW-RI and in MPW-RI. The values of RBC-RI, HGB-RI and HCT-RI presented heavy tails, as illustrated in Fig.4-3-2-5 to 7.

Table 4-3-2-1: The 90% reference ranges^a of RTH based on empirical percentiles.

Retention Index (RI)	N	Median (%)	90% Reference Range (%)	Percentiles (%)							KS ^b (p)
				5	10	25	50	75	90	95	
PLT-RI	70	25.3	15.5 to 38.3	15.5	17.3	20.9	25.3	28.9	32.0	38.3	0.200 ^c
PLCR-RI	70	19.6	4.8 to 35.1	4.8	7.7	13.8	19.6	27.8	33.1	35.1	0.200 ^c
PDW-RI	70	-5.3	-19.0 to 4.0	-19.0	-13.8	-9.5	-5.3	-1.5	2.5	4.0	0.200 ^c
MPV-RI	70	-2.3	-8.2 to 3.2	-8.2	-6.3	-4.3	-2.3	0.7	2.1	3.2	0.200 ^c
WBC-RI	70	4.8	-2.7 to 11.7	-2.7	-2.1	1.2	4.8	7.3	8.9	11.7	0.200 ^c
RBC-RI	70	-3.6	-8.7 to 4.3	-8.7	-7.6	-5.3	-3.6	-1.2	0.9	4.3	0.054
HGB-RI	70	-3.9	-10.2 to 4.6	-10.2	-7.9	-5.1	-3.9	-1.7	1.5	4.6	0.049
HCT-RI	70	-3.2	-9.0 to 3.9	-9.0	-7.5	-5.0	-3.2	-1.0	0.8	3.9	0.064

^aThe detailed measurements of the reference ranges of RTH are described in Tab.Ab.4-3-2.

^bTest of normality by Kolmogorov-Smirnov; Lilliefors significance correction; significant when $p<0.05$. ^cThis is a lower bound of the true significance.

Fig. 4-3-2-1 to 8: Histograms of the distributions of the retention indices of RTH in healthy individuals (n=70).

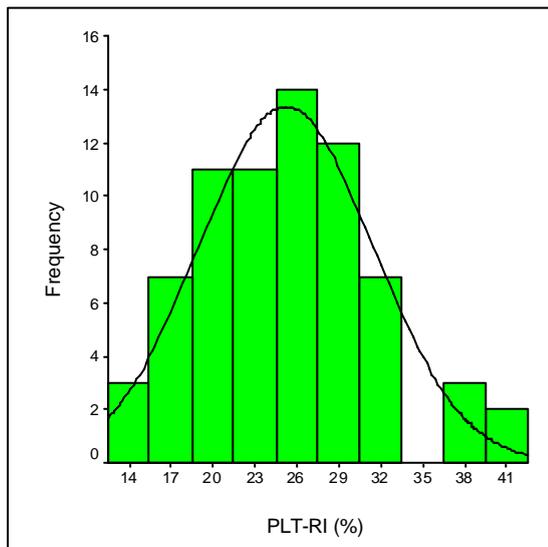


Fig. 4-3-2-1: PLT-RI reference values.

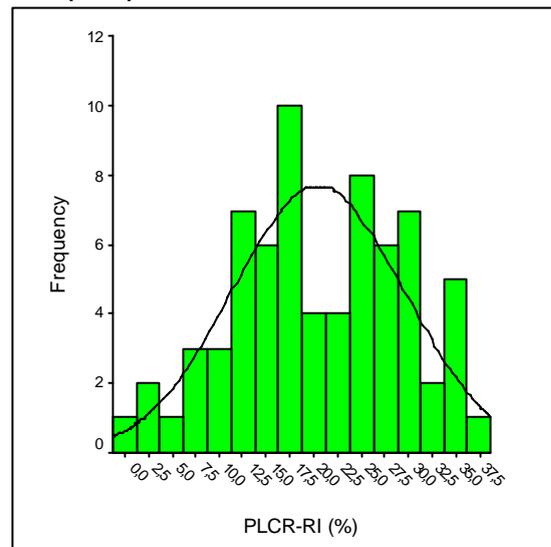


Fig. 4-3-2-2: PLCR-RI reference values.

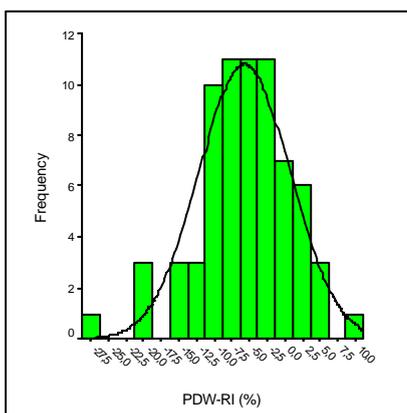


Fig. 4-3-2-3.

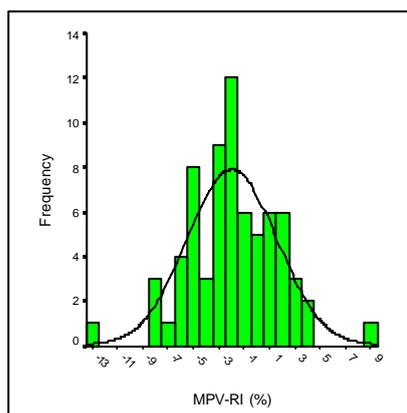


Fig. 4-3-2-4.

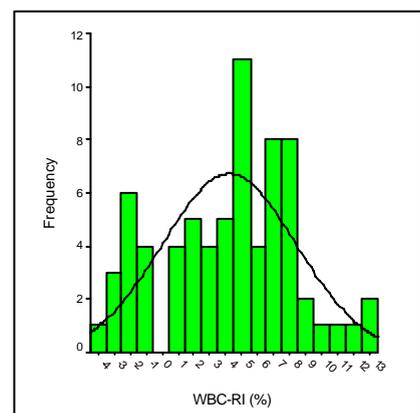


Fig. 4-3-2-5.

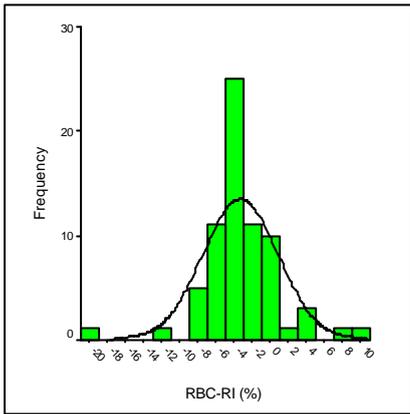


Fig. 4-3-2-6.

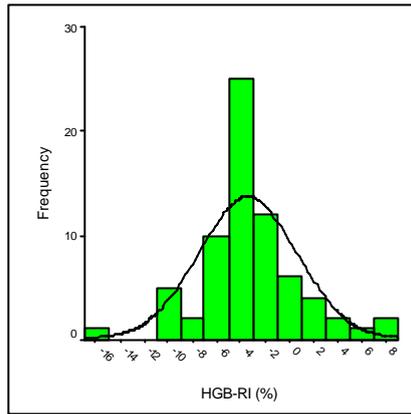


Fig. 4-3-2-7.

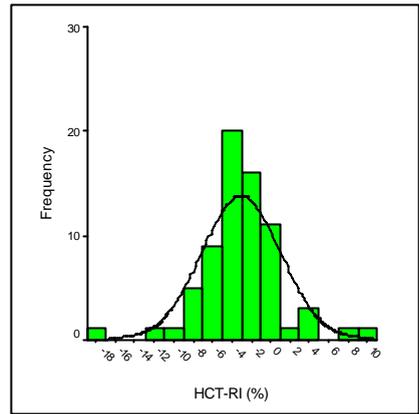


Fig. 4-3-2-8.

Further inspection of the distribution of the RIs was therefore performed by Q-Q Plot test which demonstrated an acceptable normal distribution for PLT-RI as well as for the other RIs as shown in Fig.4-3-2-9 to 12. Actually the skewness in PLT-RI was low (0.489) and within acceptable range (Std error = 0.287).

Fig. 4-3-2-9 to 16: Test of normality of the retention indices of RTH in healthy individuals by Q-Q Plot test.

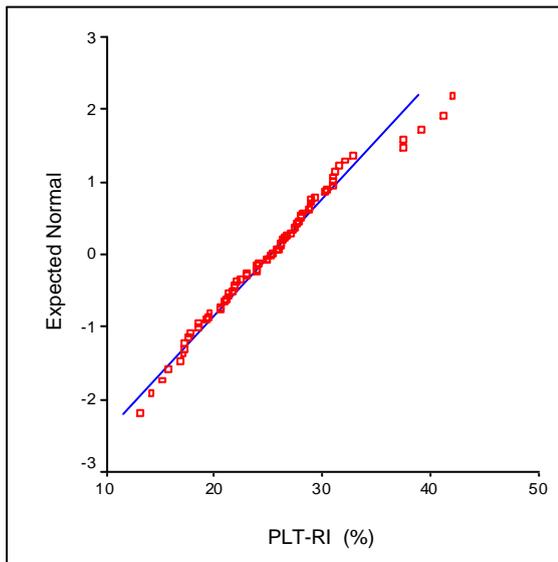


Fig. 4-3-2-9: Normal Q-Q plot of PLT-RI.

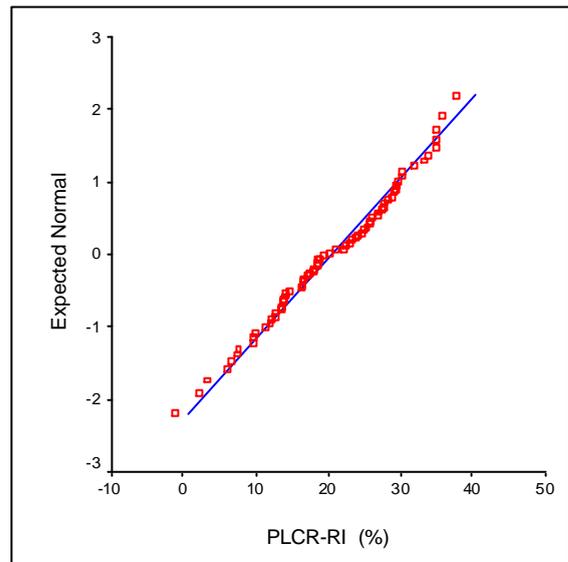


Fig. 4-3-2-10: Normal Q-Q plot of PLCR-RI.

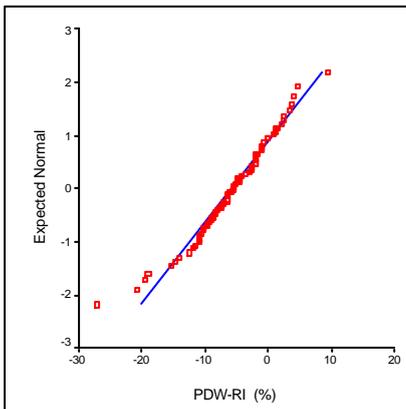


Fig. 4-3-2-11.

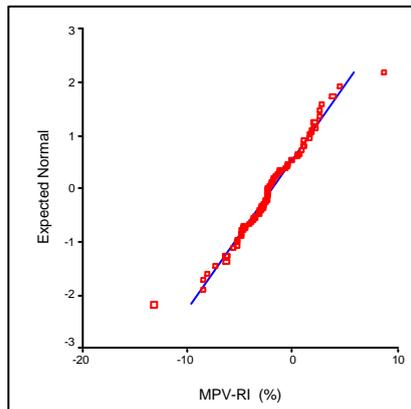


Fig. 4-3-2-12.

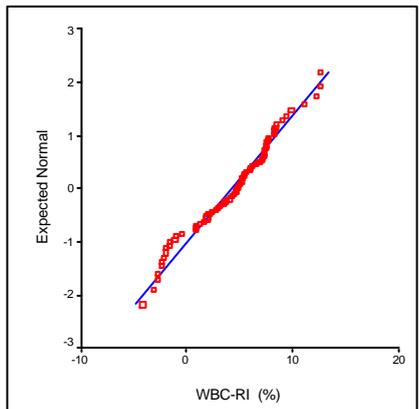


Fig. 4-3-2-13.

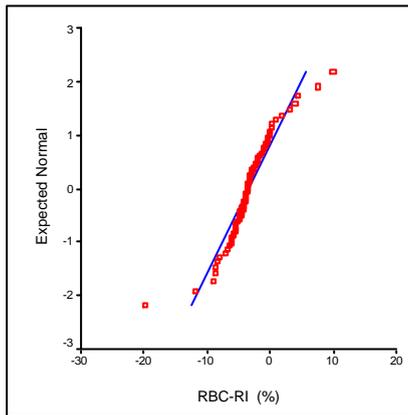


Fig. 4-3-2-14.

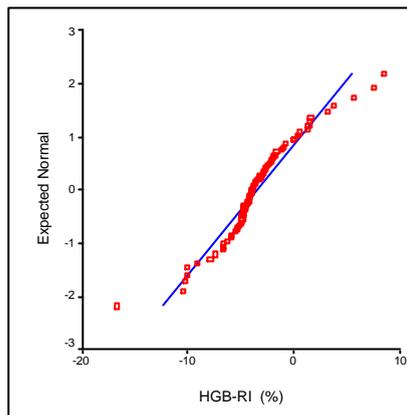


Fig. 4-3-2-15.

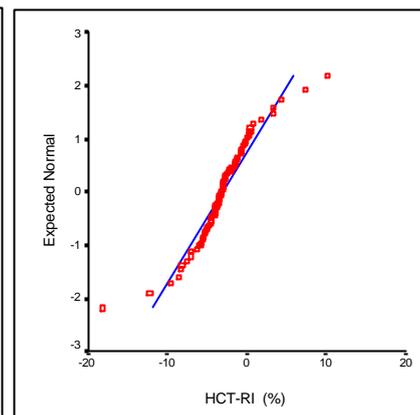


Fig. 4-3-2-16.

Table 4-3-2-2: Reference range of RTH based on normal theory with the mutual correlation with PLT-RI.

Retention Index (RI)	N	Mean (%)	SD	Mean -2SD (%)	Mean +2SD (%)	95% Confidence Intervals for Mean (%)		Correlation* with PLT-RI "r"
						Lower Bound	Upper Bound	
PLT-RI	70	25.22	6.25	12.72	37.72	23.73	26.71	-
PLCR-RI	70	20.45	9.05	2.35	38.55	18.29	22.60	0.689
PDW-RI	70	-5.64	6.46	-18.56	7.28	-7.18	-4.10	-0.074
MPV-RI	70	-1.97	3.52	-9.01	5.07	-2.81	-1.13	-0.021
WBC-RI	70	4.21	4.14	-4.07	12.49	3.23	5.20	0.183
RBC-RI	70	-3.25	4.13	-11.51	5.01	-4.24	-2.27	0.062

*r = the correlation coefficient computed by using spearman's rho test; the correlations were statistically not significant ($p > 0.05$) except for PLCR-RI ($p < 0.0005$).

These results combined implicated the rightness of considering PLT-RI as well as the other RIs to be distributed in line of normal assumption. Taking this into consideration, the reference range of PLT-RI and the other RIs could be acceptably expressed by using the 95% reference ranges based on normal theory and defined as mean \pm 2SD, as described in Tab.4-3-2-2. Interestingly, the PLT-RI values demonstrated inverse correlation ($r = -0.22$) with the PLT count measured before the passage of RTH filter. The statistical significance of this correlation was rather critical ($p = 0.68$).

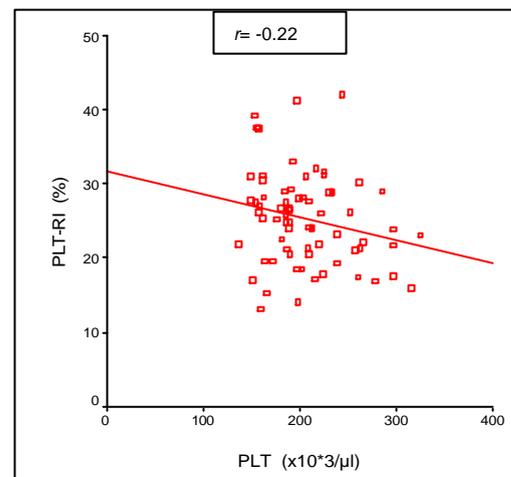


Fig. 4-3-2-17: PLT-RI against PLT count in healthy individuals (n=70) with line of marked negative correlation.

4-3-3. Investigation of hypothetic mutual correlations between PLT-RI and other RIs

The correlations between the different RIs are illustrated in Fig.4-3-3-1. Statistically, Spearman's rho test was used to inspect the significance of these correlations. The main retention indices i.e., PLT-RI and PLCR-RI, demonstrated significant positive mutual correlation ($r = 0.702$, $p < 0.0005$), as shown in Fig.4-3-3-2. Lower significant correlation was observed between PLCR-RI and both of PDW-RI ($r = 0.396$, $p = 0.001$) and MPV-RI ($r = 0.592$, $p < 0.0005$), as demonstrated in Fig.4-3-3-3 & 4. No significant correlation was noted between PLT-RI and PDW-RI and MPV-RI ($p > 0.05$). Nevertheless PDW-RI in parallel with MPV-RI seemed to be slightly negatively correlated with PLT-RI (Fig.4-3-3-5 & 6). WBC-RI values were

insignificantly but positively correlated with PLT-RI ($r=0.183$; $p=0.129$: Fig.4-3-3-7). RBC-RI has no correlation with PLT-RI ($r=0.062$; $p=0.609$: Fig.4-3-3-5). HGB-RI and HCT-RI values varied in parallel with RBC-RI ($p<0.0005$) and they followed the same pattern of insignificant correlation with PLT-RI ($p>0.05$) as RBC-RI did (Fig.4-3-3-1).

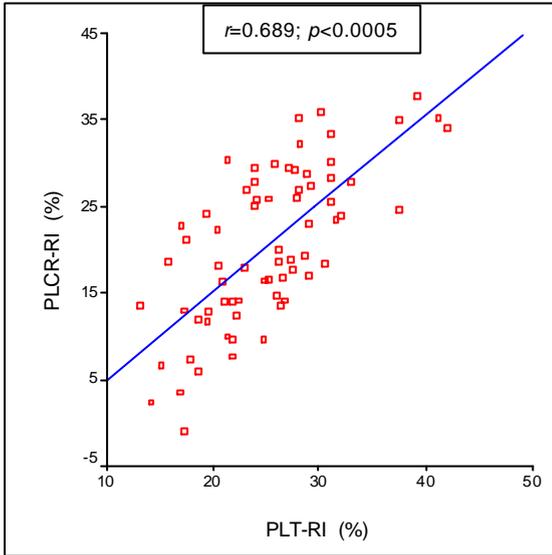


Fig. 4-3-3-2: PLCR-RI against PLT-RI with line of best fit.

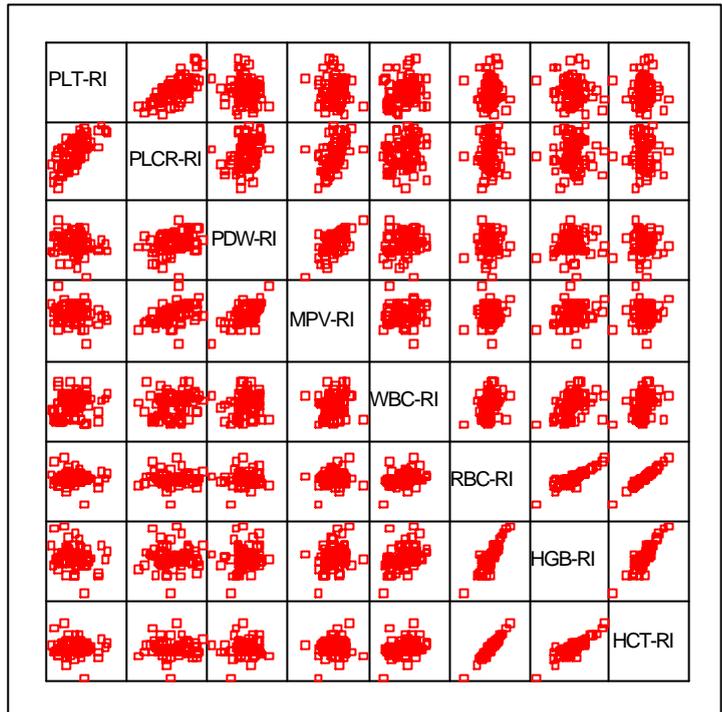


Fig. 4-3-3-1: Scatter matrix of the mutual correlations between the different retention indices of RTH.

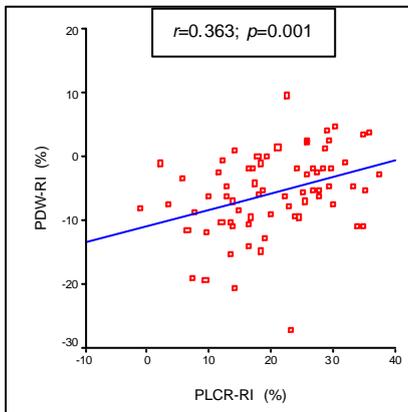


Fig. 4-3-3-3: PDW-RI against PLCR-RI.

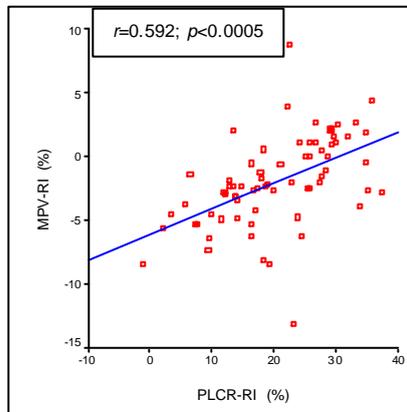


Fig. 4-3-3-4: MPV-RI against PLCR-RI.

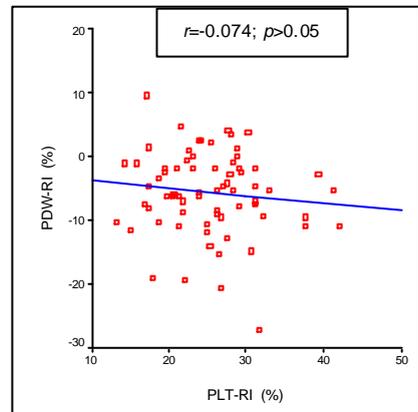


Fig. 4-3-3-5: PDW-RI against PLT-RI.

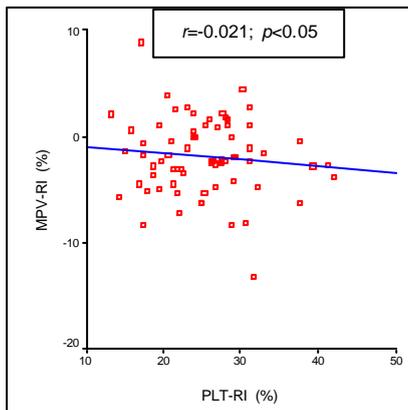


Fig. 4-3-3-6: MPV-RI against PLT-RI.

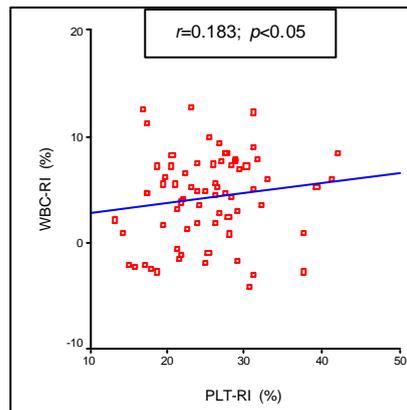


Fig. 4-3-3-7: WBC-RI against PLT-RI.

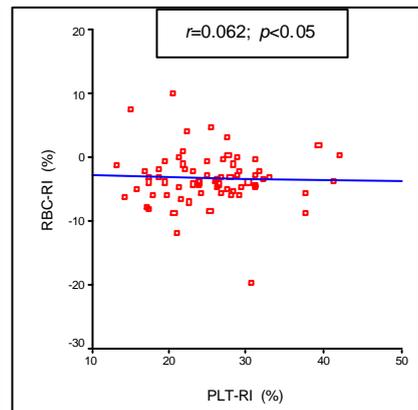


Fig. 4-3-3-8: RBC-RI against PLT-RI.

Furthermore, a comparison between the values of PLT-RI in blood donors with positive RBC-RI versus those with negative RBC-RI revealed no significant difference in the means of PLT-RI ($p=0.62$; Mann Whitney test) as well as their variances, (Tab.4-3-3-1).

Table 4-3-3: Comparison of PLT-RI in healthy individuals with positive and negative RBC-RI.

PLT-RI	RBC-RI	N	Mean (%)	SD	Std. Error of Mean	Equality of Mean (p^A)	Equality of Variances (p^B)
Subgroup	Positive	10	27.01	8.26	2.61	0.46	0.317
	Negative	60	24.92	5.88	0.76		

^AStudents t-test for equality of means adjusted for unequal variances, $p=0.62$ Mann Whitney U-Test.

^B Levene test for equality of variances.

4-3-4. Investigation on the modality of RTH measurements

The means and standard deviations of the 3 subgroups representing the different modes of measurements (p.4-3-1) are given in Tab.4-3-4. The differences of the mean values of RIs among these subgroups were not significant except for WBC-RI ($p=0.029$). Pairwise comparisons (Fishers protected

Table 4-3-4: Descriptive statistics of the different subgroups of the modes of RTH measurements.

Retention Index (RI)	Subgroup ^a	N	Mean (%)	SD	Equality of Means (p^b)	Homogeneity of Variances (p^c)
PLT-RI	2b.F:2a.F	31	26.70	7.02	0.165	0.259
	b.F2:a.F1	18	23.29	4.29		
	1b.F:1a.F	21	24.69	6.17		
	Total	70	25.22	6.25		
PLCR-RI	2b.F:2a.F	31	21.77	8.73	0.511	0.158
	b.F2:a.F1	18	18.73	10.45		
	1b.F:1a.F	21	19.96	8.37		
	Total	70	20.44	9.05		
PDW-RI	2b.F:2a.F	31	-5.33	6.37	0.868	0.960
	b.F2:a.F1	18	-5.44	7.04		
	1b.F:1a.F	21	-6.27	6.35		
	Total	70	-5.64	6.46		
MPV-RI	2b.F:2a.F	31	-1.63	3.26	0.781	0.371
	b.F2:a.F1	18	-2.28	4.23		
	1b.F:1a.F	21	-2.19	3.35		
	Total	70	-1.97	3.52		
WBC-RI	2b.F:2a.F	31	3.01	4.14	0.029	0.503
	b.F2:a.F1	18	6.24	4.01		
	1b.F:1a.F	21	4.25	3.73		
	Total	70	4.21	4.14		
RBC-RI	2b.F:2a.F	31	-3.71	3.80	0.203	0.652
	b.F2:a.F1	18	-1.75	2.81		
	1b.F:1a.F	21	-3.85	5.27		
	Total	70	-3.25	4.13		
HGB-RI	2b.F:2a.F	31	-4.35	4.14	0.097	0.723
	b.F2:a.F1	18	-1.75	2.73		
	1b.F:1a.F	21	-3.45	4.63		
	Total	70	-3.41	4.07		
HCT-RI	2b.F:2a.F	31	-3.64	3.88	0.163	0.636
	b.F2:a.F1	18	-1.49	2.66		
	1b.F:1a.F	21	-3.54	5.00		
	Total	70	-3.06	4.05		

^a2b.F:2a.F = two measurements before the passage of the filter (2b.F) and two measurements afterwards (2 a.F); b.F2:a.F1 = two measurements before the passage of the filter (2b.F) and one measurement afterwards (1a.F); 1b.F:1a.F = one measurements before the passage of the filter (1b.F) and one measurements afterwards (1a.F). ^bAnova Fisher test protected least significant difference of variances based on the mean values. ^cLevene test for homogeneity of variances.

least significant difference test) revealed marked difference ($p= 0.067$) between each of the modes using one a.F measurement (subgroups 2b.F:1a.F and 1a.F:1a.F) and that using two a.F measurements (subgroups 2b.F:2a.F). The variations (SD) of PLT-RI values were remarkably higher in the subgroup of 2a.F measurements than 1a.F measurement, as described in Tab.4-3-4). Nevertheless, the test of the homogeneity of the variances in the different subgroups revealed no significant difference ($p>0.05$; Levene test), as described in Tab.4-3-4).

4-3-5. Effect of using different automatic cell counters

No significant difference was observed between the means of the main retention indices of RTH i.e., PLT-RI and PLCR-R by using different Sysmex cell counters, while remarkable differences were pronounced by the other RIs (Tab.4-3-5). Levene's test of homogeneity of variances showed no significant differences between the different models of Sysmex cell counters, as described in Tab.4-3-5).

Table 4-3-5: Descriptive statistics of the use of different models of Sysmex cell counters

Retention Index (RI)	Sysmex Model	N	Mean (%)	SD	Equality of Means (p)*	Homogeneity of Variances (p)**
PLT-RI	M 2000	22	26.14	7.70	0.289	0.121
	KX 21	19	26.30	5.66		
	SF 3000	29	23.81	5.27		
	Total	70	25.22	6.25		
PLCR-RI	M 2000	22	22.54	9.54	0.237	0.343
	KX 21	19	21.24	7.22		
	SF 3000	29	18.34	9.57		
	Total	70	20.44	9.05		
PDW-RI	M 2000	22	-6.12	6.42	0.563	0.631
	KX 21	19	-4.27	6.58		
	SF 3000	29	-6.18	6.50		
	Total	70	-5.64	6.46		
MPV-RI	M 2000	22	-1.76	3.21	0.859	0.903
	KX 21	19	-1.78	3.82		
	SF 3000	29	-2.25	3.64		
	Total	70	-1.97	3.52		
WBC-RI	M 2000	22	4.19	3.51	0.004	0.196
	KX 21	19	1.82	4.51		
	SF 3000	29	5.80	3.67		
	Total	70	4.21	4.14		
RBC-RI	M 2000	22	-3.17	3.94	0.050	0.894
	KX 21	19	-5.08	4.38		
	SF 3000	29	-2.12	3.80		
	Total	70	-3.25	4.13		
HGB-RI	M 2000	22	-3.56	4.13	0.020	0.819
	KX 21	19	-5.34	4.23		
	SF 3000	29	-2.03	3.47		
	Total	70	-3.41	4.07		
HCT-RI	M 2000	22	-3.32	4.12	0.036	0.784
	KX 21	19	-4.76	4.08		
	SF 3000	29	-1.74	3.62		
	Total	70	-3.06	4.05		

* Anova Fisher test for analyzing the significance of variances based on the mean values.

** Levene test for homogeneity of variances.

V. DISCUSSION

5-1. Basic developments improving the methodology, reliability and standardization of the procedures of RTH assay

The developments improving the methodology, the reliability and the standardization of the first “SOP” of RTH assay (SOP-1; p.3-4-3-2) were done in two stages. The identifications and modifications of the first stages (p.4-1-1) were addressed to the second SOP of RTH (SOP-2; p.4-1-1-7). The investigations on the reliability of SOP-2, performed in the improving protocol (p.4-1-2), led to further improvements that were addressed to the third SOP of RTH (SOP-3; p.4-1-2-5).

5-1-1. Identifications and modifications on SOP-1 addressed to SOP-2 of the RTH assay

5-1-1-1. Applying RTH assay on cWB instead of cPRP in the clinical practice

Previous studies of RTH were mainly focused on the use of cPRP [Krischek 2000, Schenk 2001, and Nickels 2003]. Our study was planned to step ahead in the validation of RTH performed on cPRP. We suggested, however, using cWB instead of cPRP in the clinical practice because of the following reasons:

- A. Using cWB reflects the actual physiological status more accurately than cPRP and prevents artifact variability related to PLT activation due to the separation steps [Moris 2001, Franchini 2005].
- B. Preparing cPRP by centrifugation leads to an unstandardized *gradient* of blood cell concentrations (mainly PLT) that vary according to the distance from the bottom of the collecting tube. Sampling from cPRP would lead therefore to variable results of RTH.
- C. Sampling from cPRP implicates variability in PLT function attributed to PLT gradient in the plasma after centrifugation and the consequent diverse modalities of PLT interaction with the medium which differ inter-individually and also according to the concentrations of the blood components varying due to the gradient effect. Our investigations on the PLT isolated by gel-filtration (iPLT) compared with cWB as well as on the standards of washed fixed PLT compared with the unwashed PLT enhance these conclusions and demonstrated significant PLT predisposition to hyperactivation by partial absence of normal blood medium (p.5-2-3) which underlines the superiority of cWB. Such hyperactivation is especially serious because it increases the probability of the occurrence of PLT particles phenomenon after the passage of RTH filter which can cause major destroy in the reliability of RTH measurements, as described in p.5-1-2-2 and p.5-1-2-7-C.
- D. The blood amount required for performing RTH could be minimized to 0.5 mL instead of at least >1.5 mL in the case of cPRP (cPRP preparation; p.3-4-2).
- E. Using cWB is easier and at least save the time of the preparation of cPRP (>15 min; p.3-4-2).

In addition to the advantages of using cWB (points D and E), the points A to C underline the probable variations implicated by using cPRP. Such variations may be simply avoided by the use of cWB. It was decided therefore to consider cWB as the tested material of choice for performing RTH assay in the

clinical practice. The objective of our study was consequently to validate, improve and standardize RTH assay as a simple assay for measuring PLT function in cWB.

5-1-1-2. Standardizing the centrifugation conditions of RTH tube at 110 g/ 5 min

As mentioned in p.3-4-3-2-2-5, the centrifugation conditions of RTH tube varied extensively between 79-160 g for 5-16 min [Wienefoet 2003]. No standard conditions were specified, and the most common centrifugal forces (gravitational forces) used in previous RTH studies ranged between 80-120 g [Wieding 2004]. In addition, these conditions were even not clearly defined in other studies [Wieding 2001]. Our suggestion to adapt the speed 110 g/ 5 min as a standardized condition for the centrifugation of RTH tube (p.4-1-1-1) proved to help in delivering reliable SOP (SOP-3) and significant RTH values in regard of the followings:

A.) The morphological characterization of the interaction of PLT and blood cells with RTH filter (p.4-2-6-2) proved that: (1) By using the force 90 g, the PLT distribution was limited mainly in the upper part of RTH filter (Fig.4-2-6-2-1). Whilst the use of 110 g delivers convenient distribution of the platelets in the filter as their adherence extend acceptably from the upper until the middle-lower parts of the mesh of RTH filter. Using higher “g” forces would therefore result in major increase of the PLT forced out of the filter and thus disrupt the significance of the retention indices. (2) The application of 110 g with intervals higher than 5 min did not improve the distribution of PLT and blood cells in RTH filter. It is not justified, therefore, to use higher intervals which would undesirably prolong the turnaround time of the test.

B.) The quantity assessment of SOP-3 proved that applying 110 g/ 5 min lead to high precision for the percentage of the amount of cWB as well as Eightcheck control trapped in RTH filter (wRI= the weight retention index: CV<4%; Tab.4-2-4-2-1 and 2). This study revealed also significant reference range of wRI in cWB (mean \pm 2SD=4.6 \pm 0.4 %; Tab.4-2-3-2 and Fig.4-2-3-2-1).

C.) Adapting this condition could deliver a well standardized and reliable procedure (SOP-3; p.4-1-2-5) implicating acceptable accuracy and specificity (p.5-2) as well as precision (p.5-3).

D.) Delivering a reliable and meaningful reference range of PLT-RI with acceptable biovariability (mean \pm 2SD= 25.2 \pm 12.5; Tab.4-3-2-2, Fig.4-3-2-1) and adequate margins for assessing the pathological variations. Higher rotational speeds (>110 g) would consequently deliver reference ranges that are too low (<12%) for assessing the hypo-retention and PLT dysfunction. In addition, our investigation to lower speeds (e.g., 90 g) delivered too high PLT-RI values (mean=73%; Schenk 2002) implicating too small margins for the assessing PLT hyperactivity and pathological thrombophilic variations which was the main aim of developing RTH [Wenzel 200, Schenk 2002, Wieding 2004].

5-1-1-3. Excluding the addition of the reactivating reagent (CaCl₂ with r-Hirudin)

This modification, initially suggested upon our consideration that this addition may induce unacceptable variability was further supported by our investigations (p.4-1-1-2) which showed significant variances in the measurements of RTH assay induced by the addition of this reagent as follows: **A.)** The correlation between the reactivating reagent and PLT-RI was variable and converted from positive to negative at a non precisely identified cut-point (Fig.4-1-1-2-B-1). **B.)** The response of PLT function (PLT-RI) to the reactivating reagent varied inter-individually (Fig.4-1-1-2-B-1). **C.)** Similar effect was objected for the response of

large PLT reflected by the variation of PLCR-RI (Fig.4-1-1-2-B-2). The addition of CaCl₂ as blank (SOP-1; p.3-4-3-2-2-2) according to Wenzel 2000 is not justified since RI was to be interpreted as a relative value. It was decided therefore to exclude the addition (50 µL) of the reactivating reagent in order to avoid the variable response of PLT.

5-1-1-4. Standardizing the amount passing RTH filter: Applying the measurements before the passage of RTH filter on the collection tube instead of RTH tube

As described in the results of improving the first SOP of RTH (p.4-1-1-3), the application of the b.F measurements on the tested material in the original collecting tube delivered a sufficient quantity for the b.F and the a.F in-double measurements (100-400 µL per measurement). The amount should pass RTH filter (b.F amount) was standardized and fixed at 500 µL. By considering the unstandardized marked positive correlation of PLT-RI with the b.F amount (p.4-2-3-2), this modification eliminates the variability attributed to the variances in the amounts aspirated from the different cell counters (100-400µL) for performing the b.F measurements on the material added to RTH tubes. This is especially critical for comparative studies e.g., inter-Lab investigations. Moreover this modification along with the exclusion of the addition of 50 µL of the reactivating reagent (p.5-1-1-3) increases the amount of the tested materials that are supposed to pass RTH filter allowing for further controls (e.g., PLT isolation and PLT spreading; p.4-2-2) on these materials after passing RTH filter. In addition, resting the blood in the collection tubes for a standardized time (1-2 min) on a mechanical rotator allows the PLT – disturbed after blood withdrawal – to calm down and ensure the homogeneity of the tested sample leading to more reliable b.F measurements. In contrast, applying the b.F measurements on the blood added into RTH tubes after standing it or after the incubation step (up to 10 min: SOP-1; p.3-4-3-2-2-3) implicates variant rates of blood sedimentation and heterogeneity that result in variances of PLT count and CBC (b.F measurements). Further support for this modification was provided by our study of “blood mixing effect” which showed that PLT are significantly less opposed to hyperactivation in big tubes by inconvenient mixing than in smaller Epp tubes (Fig.4-1-2-4-2-b), as explained in p.5-1-3-A.

**5-1-1-5. Standardizing the contact time of cWB with RTH filter:
Exclusion of the incubation step**

The justification of resting the disturbed tested material (cPRP) in the RTH tube before performing the b.F measurements (incubation step up to 10 min; p.4-1-1-4) in earlier RTH procedures was no longer rational after our modification: (a) Using the cWB instead of cPRP. This prevents PLT activation during the preparation steps (p.5-1-1-1). (b) Applying the b.F measurements on the tested materials in the collection tube instead of RTH tubes (p.5-1-1-4). In addition, the RTH filter is not completely athrombogenic (p.5-2-6). Excluding the incubation step can thus avoid: 1.) possible PLT activation during the contact time with the filter which can falsely increase PLT-RI, and 2.) the variability resulting from variant grades of PLT activation attributed to different periods of incubation and to the inter-individual variability of PLT response to such activation. Thus, the exclusion of the incubation step standardizes the time of the contact between the tested material and RTH filter and improve the reliability of RTH assay.

5-1-1-6. Using new tips for the successive measurements of RTH assay

The investigation on the effect of using one tip for successive pipetting from the same sample (in-double or in-series measurements) revealed significant increase in the variation of the blood amount added into RTH tubes compared with using new tips (101.4%; Tab.4-1-1-5-B). The quantity assessment showed a marked unstandardized correlation between PLT-RI and the b.F amount (p.4-2-3-2). We do suggest therefore using only new tips in order to avoid such unstandardized variation in the measurements.

5-1-1-7. Basic improvement in the significance of PLCR-RI by using the corrected formula

Using the absolute values of large PLT in the corrected formula of PLCR-RI instead of its percentages has resulted in basic improvement in the significance of PLCR-RI and its interaction with the other RIs of RTH (p.4-1-1-2). This was obvious in the results of the reference ranges of RTH determined on 70 healthy blood donors (p.4-3). Using the corrected formula delivered a meaningful reference range of PLCR-RI values demonstrating a significant correlation with PLT-RI ($p < 0.0005$, $r = 0.689$; Fig.5-1-1-7). Interestingly, the systemically lower levels of PLCR-RI compared with PLT-RI (mean = 20.5% vs. 25.2%; Tab.4-3-2-3) suggests that the large PLT are less active than the normal sized PLT and are retained therefore less effectively on RTH filter. Defining these important observations was not possible by using the original formula where PLCR-RI values reflected no remarkable correlation with PLT-RI ($p > 0.1$) and delivered negative reference range (mean = -6.4%) with no clinical significance, as shown in Fig.5-1-1-7. Actually, the same pattern of strong correlation between PLCR-RI and PLT-RI was objected by using the corrected formula in the diverse investigations of our study e.g., RTH precision (p.4-2-4-2).

The measurements of PLCR-RI were quite beneficial throughout our investigations for developing and validating RTH (p.5-1-2-4-B and p.5-1-2-5) and could provide significant insights into the specificity and the clinical significance of RTH assay, as further explained in p.5-4-4-3. Addressing the significance and the implications of PLCR-RI was only possible by using the corrected formula. An evident example is the crucial role of the corrected formula, in contrast to the original one, in defining the errors of RTH measurements and validating the correction associated with using the new standard vortex (Fig.5-1-2-5-C), as described in p.5-1-2-5-C. In addition, the PLCR-RI measurements based on the corrected formula was also beneficial for setting a useful indicator (RI-Diff; Tab.4-1-2-2-2) for the interpretation and the quality assurance of RTH assay (p.5-4-6-1-d).

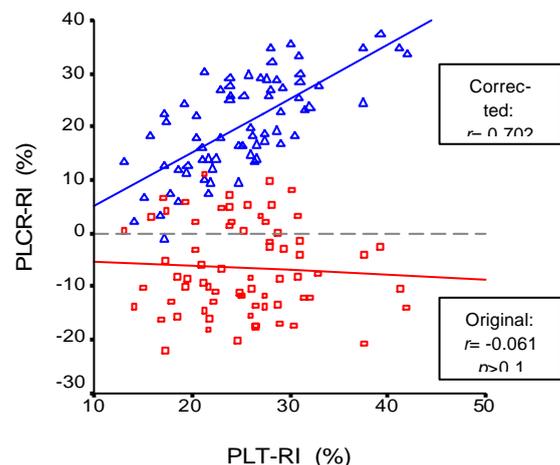


Fig. 5-1-1-7: Correlation between PLCR-RI and PLT-RI in healthy individuals (n=70) using the original and the corrected formulas of PLCR-RI.

5-1-2. Identifications and modifications on SOP-2 addressed to SOP-3 of the RTH assay

5-1-2-1. Standardizing the time of performing RTH assay

As described in p.4-1-2-2 (Tab.4-1-2-2-2), the use of different time intervals in stages 1-3 of the improving protocol (15-147 min after the blood withdrawal) failed to reduce the unacceptably significant in-series increase of PLT-RI values (increase by time up to 61%: $p < 0.0005$; Fig.4-1-2-2-2-2). The substantial correction of PLT-RI increase by time in stage 4 (Fig.4-1-2-3-2-2) by using the new standardized vortex approach, in spite applying different time intervals (29-118 min), indicates that this increase was mainly initiated by the inconvenient vortex method applied in stages 1-3 (vortex 3 min; SOP-1; p.4-1-1-7), as described as follows: the PLT, preactivated by shear forces while passing through RTH filter, seemed to be further over-stimulated by inconvenient vortex leading to PLT aggregates (Aggr) that increase by time. This results in relevant decrease of the a.F PLT count and consequently in relevant increase of PLT-RI, as further explained in the "PLT particles" phenomenon (p.5-1-2-7-B). This explanation interprets the controversy of PLT-RI values measured in sample 6a and 6b. The substantially higher time intervals used in sample 6b (118-147 min) compared with 6a (50-86 min) were expected to be associated with higher in-series increase and variation of PLT-RI. But the PLT-RI measurements revealed substantially higher in-series increase (s.Incr= 61.4% vs. 6.1%: $p < 0.005$; Fig.4-1-2-2-2-2) and variation (SD=20.7 vs. 4.3; Fig.4-1-2-2-2-1) in sample 6a versus 6b. Using relatively lower time intervals in sample 6a allowed recording the significant in-series increase in Aggr formation by time. Whilst by using very high intervals in sample 6b, the formation of such Aggr reached almost its highest rate before starting with the a.F measurements and thus only slight increase and variation by time (in-series) could be recorded. The higher mean of PLT-RI pronounced by sample 6b compared with 6a (86% vs. 78%) support this interpretation. Another evidence on the interpretation of the increase of PLT-RI by time (in-series) is that the correction of PLT-RI increase in stage 4 was associated with significant reduction ($p < 0.0005$) of the highly elevated levels of PLT-RI and their high variation in stages 1-3 to rational levels of PLT-RI with acceptable variation in stage 4 (mean= 62.5% vs. 27.9%, SD= 13.8 vs. 4.7) in spite using different time intervals in this stage (29-118 min), as illustrated in Fig.4-1-2-2-2-1. Actually, by applying the standardized vortex in stage 4, the time had no influence or correlation with PLT-RI values within intervals <120 min, as demonstrated in Fig.4-1-2-3-2-2. Performing RTH with higher intervals (>120 min) may lead to unacceptable decrease in PLT-RI that could be attributed to the gradual loss of PLT function by time (Fig.4-1-2-3-2-2). It was decided, therefore, to consider this time window in SOP-3 (p.4-1-2-5) as a standard limit for performing RTH.

5-1-2-2. "PLT particles phenomenon": A major source of errors in blood cell counting and RIs measurements

As described in the previous section (p.5-1-2-1), applying different time intervals in stages 1-3 of the improving protocol failed to decrease the unacceptably high in-series increase of PLT-RI (up to 61%; Fig.4-1-2-2-2-2) as well as to reduce the resulting elevated levels of PLT-RI (up to 82%) and their high variation (SD=14; Fig.4-1-2-2-2-1). Three main observations in stages 1-3 triggered us to hypothesize the

“PLT particles” phenomenon and to inspect its relevance by investigating the influence of mixing the a.F blood by vortex on the reliability of PLT-RI measurements (p.4-1-2-3 and 4). This was an *essential step in the improvement of RTH as it was attempt to find a rational interpretation of the unacceptable errors and to help in finding the appropriate solutions*. The related observations are:

- A.)** The b.F measurements of PLT delivered low variations (mean CV=1.1%; Tab.4-1-2-2-2). While the significant variations were restricted in the a.F measurements; where RTH tubes were vortexed inconveniently (3 min) according to the original SOP of RTH assay (SOP-1; p.3-4-3-2-2-6).
- B.)** The significant horizontal increases in PLT-RI of the same RTH tube (up to 23.2%; Tab.4-1-2-2-2); due to the significant decrease in PLT count of the second horizontal (in-double) measurement compared with the first one, were not correlated with the time intervals ($p>0.5$) and could not be attributed to the differences within each series of RTH tubes (Tab.4-1-2-2-2, Fig.4-1-2-3-2-2).
- C.)** The significant inverse correlation between the remarkably negative levels of WBC-RI and the irrationally elevated levels of PLT-RI in stages 1-3 ($r= -0.82$; $p<0.0005$; Fig.4-1-2-3-2-4).

The hypothesis of “PLT particles” phenomenon could be initially outlined as follows:

“Platelets (PLT) in cWB seem to be activated after centrifugation and passing RTH filter. Later inconvenient mixing of the centrifuged blood (e.g., by vortex for 3 min as recommended by SOP-1) could stimulate PLT resulting in PLT hyperactivation leading to PLT aggregations and/or agglutinations. Thus, “*PLT particles*” with different sizes are formed. These particles seem to be mainly like WBC in size, “WBC-like particles” and/or “RBC-like particles” and less probably bigger than WBC “above-WBC particles”. We hypothesize that these “PLT particles” may be misread by the automatic blood cell counters (Sysmex; p.3-2-4) as WBC or RBC whenever they are within the corresponding size discriminators leading to: **a.)** False increase in PLT-RI related to the decrease in the a.F PLT count (after passing RTH filter) due to the consumption of PLT by PLT particles. **b.)** False decrease in WBC-RI and/or RBC-RI proportional to the false increase in the a.F count of WBC and/or RBC related to the increase in WBC- and RBC-like particles. The mode of the variations in RIs of PLT, WBC and RBC may differ according to the sizes and amounts of the resulted PLT particles.”

5-1-2-3. Validation of the “PLT particles phenomenon” and standardizing a convenient mixing method due to morphological control by phase-contrast microscopy

The validity of this hypothesis was inspected in the course of investigating the influence of mixing cWB on PLT count and RTH measurements (p.4-1-2-3). Two approaches were used:

- 1.)** The morphological pilot study of “blood mixing effect” (p.4-1-2-4). The observations and the conclusions of this study (explained in p.5-1-2-3-B and A) could together help in: **(a)** Establishing the rightness of the initial hypothesis of PLT particles phenomenon and providing further insights into this phenomenon and its implications. **(b)** Standardizing a convenient method for mixing the a.F blood that could deliver accurate and precise measurements of complete blood counts (CBC) and consequently of RIs measurements. **(c)** Providing indicators on the inconvenient blood mixing and the consequent errors in the accuracy and the precision of RTH measurements.
- 2.)** Inspecting the responsibility of “PLT-particles phenomenon” on the errors in the accuracy and the precision of PLT-RI and the other RIs measurements in stages 1-3 of the improving protocol by

investigating the possibility of avoiding such errors by applying the new standardized vortex in stage 4 (p.4-1-2-3; Tab.4-1-2-2-2).

A. Addressing the influence of vortex on mixing normal resting cWB

Several conclusions could be set from the investigations on blood mixing effect using normal resting blood in the morphological pilot study (p.4-1-2-4-2):

1. The application of higher vortex modes on the blood led to evident decreases in PLT count (down to 90.3%) inversely correlated ($p < 0.01$; $r = -0.78$) with significant increases in WBC count (up to 29.9%), as shown in Fig.4-1-2-4-2-a and b. Microscopically, the decreases of PLT count were related to PLT hyperactivation as demonstrated mainly by the formation of PLT aggregates (Aggr; Tab.4-1-2-4-2-a and b). The percentages of WBC-like Aggr were also proportional to the increases in WBC count (Tab.4-1-2-4-2-a and b).

2. This pattern of correlations were in some instances interrupted e.g., the 2nd experiment (p.4-1-2-4-2-b). The application of further high vortex on the Epp tube did not lead to a decrease but a slight increase in WBC count (7.7%) despite there was a sharp decrease in PLT count (83.0%: Fig.4-1-2-4-2-b). This controversy could be explained microscopically by the different size of Aggr. The percentage of aggregated PLT increased sharply but the percentage of WBC-like aggregates (particles) declined due to their partial degradation (Fig. and Tab.4-1-2-4-2-b). This observation underline that the PLT aggregations induced by inconvenient vortex are degradable.

3. In parallel with the formation of Aggr which represent the main reason (up to 80%) for PLT count decrease due to PLT hyperactivation induced by inconvenient vortex, the incidence of rosette forms objected microscopically (up to 20%) can be interpreted as a minor reason for such PLT decrease.

4. In addition to PLT, blood cells especially RBC are also activated by inconvenient mixing. This was evident by the co-existence of the rosette forms of WBC and the rouleaux forms (coin-rolls) of RBC (Tab.4-1-2-4-2-a and b).

5. The decrease of PLT count triggered by high vortex seems to be significantly more pronounced by using Epp tubes (1.5 mL: 90.3%) than the bigger SM tubes (5 mL: 36.0%). This effect is also associated with significantly higher increase of WBC count Epp tubes than in SM tubes (26.3% vs. 18.4%), as shown in Fig.4-1-2-4-2-b. The microscopic findings were quite compatible (Aggr: 75% vs. 25%; Tab.4-1-2-4-2-b). This phenomenon is attributed to the contact effect occurring during vortex between the blood and the inner wall of the tested tube which is much bigger in the case of Epp tube than in SM one.

B. Superiority and standardization of the slight vortex mode for mixing cWB after centrifugation and passing RTH filter

The morphological pilot study of the effect of mixing the a.F blood (cWB after centrifugation and passing RTH filter) on RTH measurements (p.4-1-2-4-3) revealed clear evidences on the convenience and the superiority of the standardized slight vortex mode (described in p.4-1-2-4-4) for mixing the a.F blood:

1.) Higher vortex modes led to substantial increases in PLT-RI (up to 49%; Fig.4-1-2-4-3-a1 and b) proportional to the decreases in the a.F PLT count (down to 63%). Similarly to the experiments on resting blood, the PLT decreases were related to a.F PLT hyperactivation as demonstrated microscopically by the increased formation of mainly (80%) Aggr and secondarily also rosette forms (Tab.4-1-2-4-3-a, and b).

2.) The increase in a.F Aggr upon PLT hyperactivation by high vortex was demonstrated in different sizes. The decreases of WBC-RI and RBC-RI were related with the false increases of the a.F count of WBC and RBC associated with the increases of WBC-like and RBC-like Aggr which were also inversely proportional with PLT-RI (Fig.4-1-2-4-3-a1 and b). The conversion of WBC-RI levels from positive to negative was attributed to the extensively false increase of the a.F WBC count and WBC-like Aggr. The decreases of RBC-RI were less pronounced than that of WBC-RI (down to 2.2% vs. 25.6%; Tab.4-1-2-4-3-a1 and b). This is due to the fact that the concentration of RBC is 10^3 times higher than that of WBC in blood ($\times 10^6/\mu\text{L}$ vs. $\times 10^3/\mu\text{L}$). Thus, the RBC count and consequently RBC-RI are much less affected with the increases of Aggr than WBC count. The inverse correlations of PLT-RI with WBC-RI and RBC-RI are considered, therefore, as a rapid indicator for probable PLT hyperactivation, especially if associated with elevated PLT-RI levels and reduced levels for WBC-RI and RBC-RI.

3.) The positive RBC-RI levels obtained by using pipette for mixing the a.F blood compared with the significantly lower levels associated with using SV in the 2nd experiment (mean= 6.0% vs. -2.1%; Tab.4-1-2-4-3-b) were microscopically attributed to the lower a.F RBC counts due to inadequate mixing. The levels of PLT-RI in both cases were comparable Fig.4-1-2-4-3-b. But in most experiments, the use of pipette delivered elevated PLT-RI levels with higher variation compared with SV (Fig.4-1-2-4-3-a2 and Fig.4-1-2-4-3-c) and it was occasionally associated with irrational levels of RBC-RI (Fig.4-1-2-4-3-c). These observations (1 to 3) confirm at least the superiority of using SV compared with the use of pipette since SV produce less errors and it is easier and faster to practice. The high RBC-RI value could be an indicator of inadequate mixing of the a.F blood.

4.) Of specific interest, the experiments done for optimizing the SV showed that the response of PLT count to inconvenient mixing or vortex varies inter-individually and that PLT may be in some instances not hyperactivated and consequently not aggregated, as illustrated in Fig.4-1-2-4-3-c. Compared with SV, higher vortex modes led in such case to no significant increases in PLT-RI. This is compatible with the microscopical objection of no remarkable increase of Aggr.

5.) PLCR-RI varied in parallel with PLT-RI by different mixing modes (Fig.4-1-2-4-3-a1, a2, b, and c).

5-1-2-4. Reliability of applying the new standardized vortex method to correct the errors of the main retention indices of RTH

The basic correction of the RIs errors in stages 1-3 of the improving protocol by applying the new standard vortex method (<3 sec; p.4-1-2-4-4) in stage 4 instead of 3 min (acc. to SOP-1; p.3-4-3-2-2-6) in stage 1-3 (Fig.4-1-2-3-2-1 to 12) support firmly its reliability for mixing the a.F blood and improving the significance of RTH assay.

A.) Substantial corrections of PLT-RI accuracy and precision

The main corrections of the precision and the accuracy are summarized as follows:

1. The big in-series as well as horizontal variations of PLT-RI in stages 1-3 were substantially reduced (>65%; $p < 0.0005$) to acceptable lower levels in stage 4 (SD= 4.7 vs. 13.8 and 8.2 vs. 2.9 respectively; Fig.4-1-2-3-2-1 and 3).
2. The falsely elevated PLT-RI levels in stages 1-3 declined significantly (55.4%; $p < 0.0005$) to rational levels in stage 4 (mean = 62.5% vs. 27.9%), as demonstrated in Fig.4-1-2-3-2-1.

3. As explained in p.5-1-2-1, the big systemic increases of PLT-RI by time (in-series increase: up to 61.4%; $r=0.717$) and horizontally (the increase in the 2nd in-double measurements compared with the 1st ones: mean=10.3%) found in stages 1-3 were actually eliminated in stage 4 ($p<0.0005$) and converted to insignificant negative ranges (mean= -2.7% and -0.6% respectively: $r>-0.046$; Tab.4-1-2-2-2), as illustrated in Fig.4-1-2-3-2-2 and 3.

These substantial corrections can be interpreted by the “PLT particles” phenomenon. PLT activated by shear forces (centrifugation) passing through the micro pores of RTH filter seemed to be hyperactivated by later inconvenient vortex (SOP-1; p.3-4-3-2) before the a.F measurements in stages 1-3. PLT hyperactivation led to the formation of PLT aggregates that increased with time resulting in relevant decreases of the a.F PLT count proportional to the false increases of PLT-RI. The evident corrections in stage 4 proved that the new standardized vortex could successfully avoid the occurrence of this phenomenon.

B.) Substantial correction of PLCR-RI accuracy and precision

PLCR-RI followed the same patterns of the variation and corrections demonstrated by PLT-RI (Tab.4-1-2-2-2) since they were strongly correlated throughout the whole stages of the improving protocol ($r>0.75$, $p<0.0005$; Fig.4-1-2-3-2-7). The unacceptable in-series increase of PLC-RI (increase by time) in stages 1-3 was eliminated converting to insignificant negative range in stage 4 (32.4 vs. 3.4%; Fig.4-1-2-3-2-8). The elevated levels of PLCR-RI values as well as their big in-series variations in stages 1-3 were substantially ($p=0.002$) reduced to rational levels in stage 4 (mean PLCR-RI= 41.0 % vs. 26.6%; SD= 18.5 vs. 5.8; Fig.4-1-2-3-2-8).

5-1-2-5. Correction and significance of the systemic difference between PLT-RI and PLCR-RI (RI-Diff)

Interestingly, PLCR-RI values were found to be systemically lower than PLT-RI in the improving protocol. This difference (RI-Diff) declined substantially (95.3%; $p<0.0005$) in stage 4 compared with stages 1-3 (RI-Diff=26.2% vs. 1.2%; Tab.4-1-2-2-2). Three conclusions could be set from these observations:

A. Shift of PLT size distribution to the right by hyperactivation of the blood after the passage of RTH filter

The significant decline of RI-Diff suggests that the big shift of PLT size distribution to the right in stages 1-3 shrank sharply in stage 4. This is interpreted by the hyperactivation of PLT in the a.F blood in stages 1-3 induced by inconvenient vortex which resulted in: **(1)** Changes in PLT shape disposing variable increases in PLT surface (up to 3-5 folds; White 1994) which could be misread by cell counters, at least partially, as large PLT. **(2)** Formation of Aggr related to the false increase in PLT-RI (as explained in p.5-1-2-4-A.); the small Aggr (large PLT-like Aggr= 2-3 PLT) seemed to be occasionally misread by cell counters as individual large PLT. These incidences (1 and 2) led to a relevant spurious shift of PLT size distribution to the right demonstrated by proportional false increase in a.F PLCR and consequently by significant false declines in PLCR-RI levels. Thus, avoiding the shift of PLT size distribution to the right in stage 4 by using the standardized vortex could minimize RI-Diff substantially. This effect of PLT hyperactivation is further supported by the findings of PDW-RI and MPV-RI (p.5-1-2-6-B) and it was therefore integrated in the implications of PLT particles phenomenon (p.5-2-7-B). We may at least suggest that a

trend of significantly lower levels of PLCR-RI compared with PLT-RI is an indicator for possible errors in RTH measurements due to PLT hyperactivation and/or inconvenient vortex.

B. Large PLT are of less active than the normal sized PLT

In addition to the main interpretation of the significant increase of RI-Diff in stages 1-3 compared with stage 4 by the false shift of PLT size to the right already explained in p.5-1-2-5-A, a minor explanation for this phenomenon could be that large PLT are less predisposed for hyperactivation compared with normal sized PLT. The consequent formations of a.F Aggr were therefore composed of normal sized PLT more than large PLT. This was demonstrated by the relative reduction of the elevated levels of PLCR-RI compared with PLT-RI. The slightly lower levels of PLCR-RI compared with PLT-RI in stage 4 support our suggestion that large PLT are less active than normal sized PLT. This conclusion is firmly supported by the significantly lower levels of PLCR-RI versus PLT-RI revealed by the reference ranges (n=70: mean=20.5% vs. 25.2%; Tab.4-3-2-3), as explained in p.5-1-1-7.

C. Basic role of the corrected formula of PLCR-RI for addressing the RI-Diff phenomenon

As illustrated in Fig.5-1-2-5-B, the old formula of PLCR-RI delivered unexplainable negative levels in stage 1-3 of the improving protocol (mean=-55.5%) which were inversely and insignificantly correlated with PLT-RI: $r=-0.150$; $p>0.01$). The PLCR-RI values turned to low positive levels in stage 4 (mean= -2.0%) reflecting no clinical significance with no meaningful positive correlation with PLT-RI ($r=0.033$; $p>0.01$). This conversion implicated no conclusion or sense for the correction of PLCR-RI measurements by applying the standard vortex in stage 4. In contrast, the corrected formula preserved a significant and positive correlation with PLT-RI in all stages ($r>0.75$; $p<0.0005$). The corrected formula delivered meaningful and positive levels of PLCR-RI in stage 1-3 which decreased significantly by applying the new standard vortex in stage 4, (mean=41.0% vs. 26.6%). This shift of PLCR-RI values to the left and the constant positive correlation between PLCR-RI and PLT-RI values have essential implications for both the correction of PLCR-RI measurements (p.5-1-2-4-B and p.5-1-2-5-A and B) and their clinical significance (p.5-4-4-3-A).

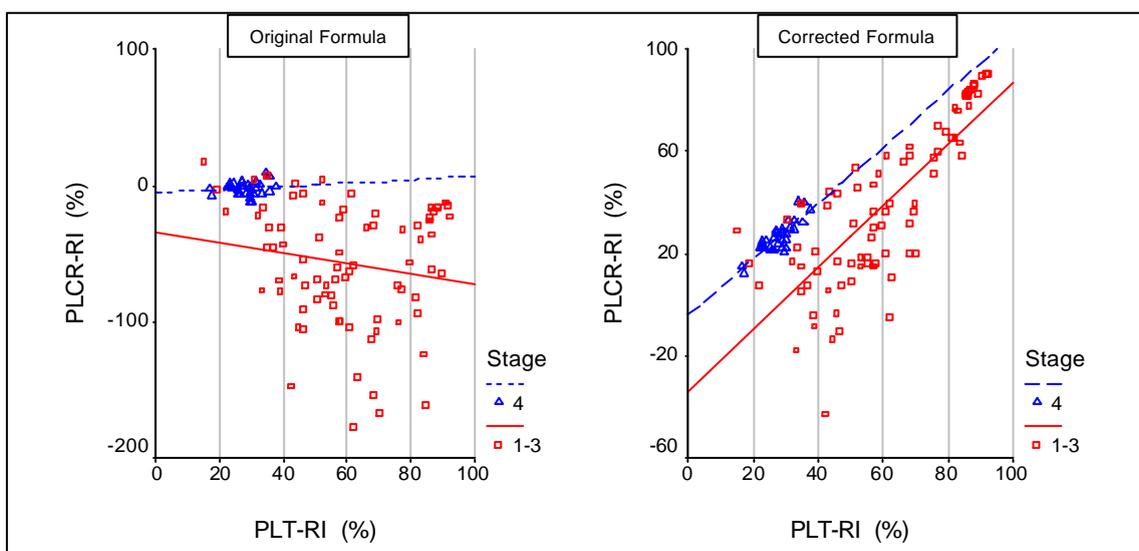


Fig. 5-1-2-5-C: Corrected formula of PLCR-RI demonstrating the difference between PLT-RI and PLCR-RI values in the improving protocol and their strong mutual correlation in contrast to the old formula.

5-1-2-6. Nobel identification of retention indices for CBC parameters and their interactions with PLT-RI: Crucial role in improving the reliability of RTH measurements

The retention indices (RIs) for the whole parameters detected by the complete blood counting (CBC: i.e., PLT size distribution, leukocytes and their main normal types as well as erythrocytes and RBC indices) were identified (p.4-1-2-3-1-A) in order to get more insight into RTH errors found in stage 1-3 of the practical protocol. The variations of these RIs and their correlations with PLT-RI played essential role in the development of RTH assay by: (1) Finding out the reasons of the errors of PLT-RI measurements in the improving protocol and the morphological study, as explained later in this section. (2) Addressing and validating the related modifications in RTH procedures. (3) Setting convenient protocol for the quality assurance of the measurements (p.5-4-6).

Actually, this is the *first time ever, in PLT retention assays; a retention index is identified for any of CBC parameters other than PLT-RI and PLCR-RI*. The RI measurements are basically more informative than CBC parameters in respect of data analysis because: a.) The unified unity of RIs (percentage: %) in contrast to the diverse unities of CBC parameters. b.) RIs reflect PLT function providing significant linkage between the b.F and a.F counts of CBC parameters. This was especially evident by analyzing the data of the morphological pilot study on the effect of mixing the “resting blood” (i.e., by using CBC findings; p.5-1-2-3-A) compared with the “a.F blood” (after passing RTH filter i.e., by using RIs findings p.5-1-2-3-B). The conclusions were easier to be set by using RIs measurements than CBC ones. The insight provided by new RIs was an essential trigger of hypothesizing the “PLT particles” phenomenon (p.5-1-2-2-C) which revealed to be the prime error in previous RTH measurements. The measurements of these RIs provided crucial evidences on this hypothesis in due of morphological control (p.5-1-2-3) and added further implications of this hypothesis (p.5-1-2-7-A). The measurements of the new RIs were also essential to address the standardized vortex method for mixing the a.F blood and to validate the resulted improvement in the reliability of RTH in stage 4 of the improving protocol, as described as follows:

5-1-2-6-A. Significance of the RI of leukocytes (WBC-RI) and erythrocytes (RBC-RI)

1.) In the morphological pilot study of the “blood mixing” effect

As described in p.5-1-2-3-B-2 and 3, the levels and variations of WBC-RI and RBC-RI and their mutual correlations with PLT-RI were compatible with the morphological findings and also essential for:

- (a) Proving the initial hypothesis of “PLT particles” phenomenon as well as providing further insights into their implications.
- (b) Indicating the errors and improvements in the accuracy and the precision of RTH measurements by using the different modes of blood mixing e.g., those attributed to unacceptably strong vortex, convenient slight vortex, or inadequate mixing by pipette
- (c) Addressing the new standardized vortex method that could deliver accurate and precise RTH measurements.

2.) In the improving protocol

The significant inverse correlation of the falsely elevated PLT-RI levels with the remarkably negative levels of WBC-RI and RBC-RI ($r < -0.33$; $p < 0.002$) in stages 1-3 of the improving protocol (Fig.4-1-2-3-2-4 and 6) were prim triggers of our investigations on the effect of blood mixing (PLT particles phenomenon; p.5-1-2-2-C). In addition and in concordance with the morphological pilot study of the blood mixing effect, the

patterns of the levels and variations of WBC-RI and RBC-RI as well as their correlations with PLT-RI could play a beneficial role as indicators for validating the accuracy and the precision of RTH assay in the improving protocol. This was evident by the substantial changes in the characteristics of these patterns in stage 4 compared with stages 1-3 i.e. the changes associated with the basic correction of the PLT-RI accuracy and precision by using the standard vortex (p.4-1-2-3-2), as summarized as follows:

- a.) Significant conversion ($p < 0.0005$) of the correlation between WBC-RI and PLT-RI from strongly negative to a positive one ($r = -0.82$ vs. 0.30 ; Fig.4-1-2-3-2-4).
- b.) Similar significant effect ($p < 0.0005$) was registered for RBC-RI ($r = -0.33$ vs. 0.49 ; Fig.4-1-2-3-2-6).
- c.) Sharp rise up of WBC-RI from strongly negative to positive levels (-19.5% vs. 5.9% ; $p < 0.0005$; Fig.4-1-2-3-2-5).
- d.) Significant reduction of the variation of WBC-RI and RBC-RI values ($p < 0.01$; Fig.4-1-2-3-2-5 & 6).

These observations could be interpreted by the PLT particles phenomenon taking into consideration the conclusions of the morphological pilot study of "blood mixing effect" (p.5-1-2-3-B-1 and 2). The increased and variant formations of WBC-like and RBC-like Aggr in the a.F blood due to unacceptable hyperactivation by inconvenient vortex in the stages 1-3 resulted in: a.) Relevant false decrease in a.F PLT counts negatively proportional to PLT-RI, and b.) Relevant false increases in a.F WBC and RBC counts that were negatively related to WBC-RI and RBC-RI. Thus, the falsely reduced levels of WBC-RI and RBC-RI were negatively correlated with the falsely elevated levels of PLT-RI. The lower impact of Aggr increases on the retention of RBC compared with WBC is due to the much higher (10^3) concentration of RBC than WBC in the blood, as explained in the morphological study (p.5-1-2-3-B-2). The application of the standardized vortex method in stage 4 could successfully avoid the unacceptable PLT hyperactivation and the consequent false variations and lead to accurate and precise measurements reflected by acceptable variation and rational levels of WBC-RI and RBC-RI that were interpreted as mainly "unspecific" retention due to the blood cell sizes, as explained in the chapter of the specificity of RTH (p.5-2-9-A). Thus, the measurements of WBC-RI and RBC-RI were critical for the validation of the improvement in the reliability of RTH assay by using the new standard vortex in stage 4.

5-1-2-6-B. Significance of the RIs of PLT size distribution: PDW-RI and MPV-RI

The extremely negative and variable levels of PDW-RI and MPV-RI in stages 1-3 compared with stage 4 of the improving protocol (Fig.4-1-2-3-2-9) support strongly our conclusion that the PLT hyperactivation by inconvenient vortex of the a.F blood in stages 1-3 led to relevant spurious shift of the PLT size distribution to the right, as explained in p.5-1-2-5-A. This shift was reflected by false and variant declines in PDW-RI and MPV-RI levels that differed proportionally from the variety of PLT shape changes and the large PLT-like Aggr induced by inconvenient vortex. The basic reduction of PLT hyperactivation in stage 4 by using the standardized vortex for mixing the a.F blood resulted therefore in a significant ($p < 0.0005$) rise up for both PDW-RI and MPV-RI levels (Tab.4-1-2-2-2). This linkage with the applied vortex mode explains the significant conversion of the correlation of PDW-RI and MPV-RI with PLT-RI values from negative in stages 1-3 to positive in stage 4 (Fig.4-1-2-3-2-10). The extreme negative and variable values of PDW-RI and MPV-RI that are inversely correlated with elevated PLT RI levels may be therefore considered as a sign of hyperactivation and/or inconvenient vortex.

5-1-2-6-C. RIs of the main normal types of leukocytes (NEUT-RI, LYMP-RI, MXD-RI)

The retention indices (RIs) of the main normal types of Leukocytes (p.4-1-2-3-1-A) delivered the same pattern of intimate and proportional correlation with WBC-RI throughout the whole stages (1-4) of the improving protocol ($p < 0.001$; Fig.4-1-2-3-2-11). The absence of a significant correlation between the variations of WBC-RI with the types of leukocytes suggest that the misreading of WBC-like particles as WBC by the cell counters is not on the account of either of these types. In addition, the main normal types of WBC seem to be comparably retained by RTH filter by applying the standard vortex in stage 4. The RIs of the main normal types of leukocytes present therefore no further advantage upon WBC-RI in regard of their correlation with PLT-RI errors and variances.

5-1-2-6-D. RIs of the erythrocyte indices (HGB-RI, HCT-RI, MCV-RI, MCHC-RI and RDW-RI)

The retention values (RIs) of the erythrocyte indices (p.4-1-2-3-1-A), demonstrated either positive or negative patterns of close correlation with RBC-RI throughout the whole stages (1-4) of the improving protocol, as illustrated in Fig.4-1-2-3-2-12. These patterns of correlation were more evident in stage 4 than in stages 1-3 which could be attributed to the formation of RBC-like Aggr in stages 1-3 (p.5-1-2-6-A). This trend was mostly evident for HGB-RI and HCT-RI ($r = 0.87$ and 0.96 vs. 0.43 and 0.46 ; $p < 0.0005$), and therefore, their measurements could be of advantage for further control of the reliability of RTH. Otherwise, the differences of the other RIs were not significant and actually did not provide further significant insight into the variation errors of RBC-RI and PLT-RI.

5-1-2-7. Hypothesis “PLT particles phenomenon”**5-1-2-7-A. Accumulative clues and implications**

The morphological pilot study on the “blood mixing” effect could establish the rightness of the initial hypothesis of “PLT particles” phenomenon and provide further significant insight and implications, as described in p.5-1-2-3-A and B. The success of applying the new standardized vortex method (stage 4 vs. stages 1-3 in the improving protocol; p.5-1-2-4 to 6) is a further clear proof on the reliability of our hypothesis. The variations and the levels of PLT-RI and the other RIs and their mutual correlations before and after this correction provided basic insights into the interpretation and implications of PLT particles phenomenon in RTH measurements, as described in p.5-1-2-4, 5-A, and 6. In addition, further significant evidences on this phenomenon as well as insights on its implications and interactions with RTH measurements were accumulated from different investigations of our study, as summarized in the followings:

1.) The agglutinates of iPLT (PLT isolated by gel-filtration; p.4-2-21), objected after the passage of RTH filter (a.F), were misread by the cell counter as blood cells. Both, the CBC (complete blood counting) and the morphological control by PLT spreading proved the absence of blood cells in the suspension of iPLT (p.4-2-2-1-2). The remarkable a.F counts of WBC (up to $0.8 \times 10^3/\mu\text{L}$) were proportional to WBC-like particles, as revealed by the microscopic evaluation (Tab.4-2-2-3). This could be explained only by the occurrence of PLT particles phenomenon. Less pronounced effect was demonstrated by RBC-like particles. Due to the absence of plasma fibrinogen in iPLT, PLT particles are formed mainly of PLT agglutinates (Aggl) instead of aggregates (Aggr) due to the lack of plasmatic fibrinogen. Nevertheless,

the hyperactivated iPLT may occasionally form Aggr by the help of their internal content of fibrinogen [Jurk 2005, Levi 2005].

2.) PLT are predisposed to hyperactivation by partial absence of the normal blood medium e.g., in iPLT suspension and in the PLT standards prepared from fixed plasma, as explained in p.5-2-3.

3.) Day-by-day control on the PLT standards prepared from fixed cPRP (p.4-2-5-3 to 5). The significant decreases of PLT count of the different PLT standards by time (mean=38.4%; $p < 0.0005$; Fig.4-2-5-3-1, 4-2-5-4-1 and 4-2-5-5-1) were proportional to the increases in PLT particles observed by phase-contrast microscopy (Tab.4-2-5-3-2, 4-2-5-4-2 and 4-2-5-5-2). In parallel, the initial low counts of WBC of the PLT standards increased dramatically (e.g., from $0.2 \times 10^3/\mu\text{L}$ up to $12.4 \times 10^3/\mu\text{L}$; $p < 0.0005$) and proportionally to the increases of WBC-like and RBC-like particles found microscopically. The formation of PLT particles was attributed to PLT agglutination and not to aggregation since the PLT standards were prepared from fixed plasma. Performing RTH for the day-by-day control provided specific insights into PLT particles phenomenon. Three effects of PLT particles have to be taken into consideration:

- (a) The *b.F Aggl* were trapped by RTH filter. The retention of *b.F WBC-like Aggl*, falsely interpreted as WBC, resulted in falsely elevated levels of WBC-RI (mean=35.9%; Tab.4-2-5-5-1). Much lower effect is expressed by RBC-RI as RBC count is 10^3 times higher than that of WBC.
- (b) The increases of *a.F Aggl* resulted in relevant false decreases in *a.F PLT* count that led to proportional increases of PLT-RI. In parallel, the increases of the *a.F WBC-like Aggl*, led to proportionally false decreases in WBC-RI. The final values of WBC-RI depended therefore on the false variations of their *b.F* and *a.F* counts and the percentage of the trapped Aggl.
- (c) The negative PLT-RI values were attributed to the degradation of the excessive *b.F Aggl* after passing RTH filter leading to decrease in *a.F PLT* count relevant to the degree of degradation; e.g., PLT-RI on the 3^d day of controlling the 3^d PLT standard declined to negative levels (Fig.4-2-5-5-4). This observation underline that physical effects may also degrade Aggl, as well as Aggr (p.5-1-2-3-A-2) e.g., by passing through RTH filter or by vortex.

4.) The electron microscopy of RTH filter proved the activation of PLT by shear stress (centrifugation) during the passage of the inter-connected micro pores of RTH filter (p.5-2-9-A). In parallel, the activation of the blood cells was also demonstrated electron microscopically mainly by granulocytes degranulation (Fig.4-2-6-3-c1 and 2) and by the rouleaux (coin roll) forms of erythrocytes (Fig.4-6-2-3-b). Such activation process may lead to the formation of rosette forms by further hyperactivation e.g., by vortex.

5-1-2-7-B. Integrated identification/description of “PLT particles phenomenon”

Taking into consideration the initial formula of the “PLT particles” phenomenon (p.5-1-2-2) as well as its accumulative evidences and implications already explained in p.5-1-2-7-A, the integrated formula of this hypothesis may be described as follows:

(1) Hyperactivated PLT may aggregate and/or agglutinate and form *PLT particles* with different sizes: “large PLT-like” particles, “RBC-like” particles, “WBC-like” particles, and particles above the size of WBC i.e., “above-WBC” particles. These particles may be misread by the automatic cell counter (Sysmex; p.3-2-4) as large PLT, RBC, and/or WBC respectively whenever they are within the corresponding size discriminators of these “blood cells” leading to relevant *false increases* in the counts

of these cells. The false increases of RBC count attributed to the formation of PLT particles are less significant than that of WBC and occasionally not remarkable because the concentration of RBC is 10^3 times higher than that of WBC in the blood. The false increase in WBC count, proportional to the formation of WBC-like particles, is not related to a specific type of leukocytes. The “above-WBC” particles are not read as blood cells but they lead to a false decline of PLT count proportional to the sizes and the amounts of these particles. The main consequence of the formation of PLT particles, however, is the false decrease in PLT count which is proportional to the amount of the consumed PLT.

(2) Inconvenient mixing of the blood (e.g., by aggressive vortex) may induce PLT hyperactivation especially if the PLT are activated after centrifugation (shear forces) while passing the inter-connected micro pores of RTH filter. PLT are more exposed to hyperactivation by inconvenient mixing when they are in contact with a relatively larger inner surface than with a smaller one (blood in RTH tubes vs. SM tubes; p.5-1-2-3-A). PLT are more opposed to hyperactivation by the partial absence of the other blood components e.g., in PLT isolated by gel-filtration (iPLT) compared with cWB, and PLT in prefixed washed cPRP compared with the unwashed one (p.5-2-3).

(3) The PLT particles formed in blood or in cPRP compose mainly of aggregates (Aggr) and secondarily of agglutinates (Aggl). PLT agglutinates are the main component of PLT particles in iPLT; iPLT may aggregate only by their intern content of fibrinogen due to the absence of the plasma fibrinogen. Fixed PLT can also form particles which are not composed of Aggr but of Aggl attributed to increased stickiness (p.5-2-3).

(4) Large PLT are less predisposed for hyperactivation and aggregation than normal sized PLT. Consequently and due to PLT shape changes and the probable formation of large PLT-like Aggr triggered by PLT hyperactivation, the PLT size distribution is mostly shifted to the right (p.5-1-2-5-A).

(5) Both Aggr and Aggl may degrade upon further physical effects e.g., vortex and/or passing RTH filter. The degradation of PLT particles results in changes in the blood cells relevant to the new configurations of these PLT particles as well as in an increase of PLT count proportional to the non aggregated and/or agglutinated PLT released from the degraded PLT particles. The sizes of PLT particles and their amounts may also change by time and the “Above-WBC” particles are more disposed to be disrupted due to their big size.

Note: Rosette forms In addition to PLT, blood cells can also be hyperactivated by inconveniently strong mixing, especially if they are preactivated by shear stress passing RTH filter (p.5-1-2-3). This is demonstrated mainly by rosette forms (satellitosis phenomenon) and the coin-roll (rouleaux) configurations associated with PLT particles in both, the resting and the a.F blood (Tab.4-1-2-4-2a and b, Tab.4-1-24-4-3-a, b and c). Despite the incidence of the rosette forms is much lower than that of PLT particles (1:5), it contributes constantly in the false decrease of PLT count. We integrate the formation of rosette in the PLT particles phenomenon in order to have more comprehensive insight into the interpretation of the incidences induced by inconvenient mixing of the blood and especially for the right interpretation of RTH measurements.

5-1-2-7-C. Implication/Significance of PLT particles phenomenon in RTH assay

The PLT particles phenomenon may occur or be triggered mostly if PLT are pre-activated by shear stress (centrifugation) while passing through RTH filter (a.F particles). The b.F particles are less frequent and are mainly encountered in the relative absence of the other blood components especially for long time e.g., in the day-by-day control of the PLT standard (Tab.4-2-5-3-2, 4-2-5-4-2 and 4-2-5-5-2; also p.5-1-2-7-

A-3). The relative absence of blood medium increases the predisposition of PLT to hyperactivation, especially by stimulation after passing RTH filter (p.5-1-2-7-A-2 and p.5-2-3). The occurrence of b.F and/or a.F PLT particles has serious implications on the reliability of RTH measurements since it leads to false values RIs and increases their variability. The response of PLT and blood cells to inconvenient vortex varies inter-individually and may disappear occasionally (Fig.4-1-2-4-3-c). Understanding the integrated formula of PLT particles phenomenon (p.5-1-2-7-B) and its implication is, therefore, essential for the right interpretation of RIs as well as for suspecting false results and assuring reliable quality for RTH measurements, as summarized as follows:

a. Implications on the measurements before the passage of RTH filter

The b.F PLT particles are associated with variable decrease of b.F PLT count (p.5-1-2-3-A), and they are mainly trapped by RTH filter. The retention of WBC-like particles leads to falsely proportional elevated levels of WBC-RI (p.5-1-2-7-A-3-a). Much lower effect may be encountered for RBC-RI. The b.F particles may occasionally degrade after passing RTH filter leading to relevant decrease in PLT-RI values that can even step to negative levels (p.5-1-2-7-A-3-c).

b. Implications on the measurements after the passage of RTH filter

1.) The decline of the a.F PLT count due to the formation of PLT particles leads to proportional relevant false increase of PLT-RI and less significant increase of PLCR-RI (p.5-1-2-4). **2.)** In parallel, the changes of the PLT shapes associated with PLT hyperactivation and the resulted increase of "large PLT-like" particles lead to a proportional shift in PLT size distribution to the right (p.5-1-2-5-A). This shift is also reflected by a proportional decrease of PDW-RI and MPV-RI (p.5-1-2-6-B) as well as by a relevant relative decline of PLCR-RI (p.5-1-2-5-A). **3.)** The increases in the amount of the a.F WBC-like and RBC-like particles result in false decreases in WBC-RI and RBC-RI; the increase of RBC-RI is much less pronounced than that of WBC (p.5-1-2-6-A). **4.)** The variations in WBC-RI and RBC-RI resulting from the formation of PLT particles lead to proportional relevant variations in the RIs of the normal types of leukocytes as well as the RIs of the erythrocytes indices. **5.)** Occurrence of rosettes forms upon inconvenient vortex contributes significantly (up to 20%) to the decrease and the variation of a.F PLT and consequently to the false increase of PLT-RI

Since the formation PLT particles is time dependant (Fig.4-1-2-3-2-2), the a.F measurements should be performed shortly (within 1-2 min) after mixing the centrifuged blood by vortex in order to minimize the probable errors related to the PLT particles phenomenon, as we recommended in SOP-3 (p.4-1-2-5).

5-2. Specificity and accuracy of RTH assay

The extensive improvements on the accuracy of RTH procedures by excluding the sources of the variability in SOP-1 (p.5-1-1) and SOP-2 (p.5-1-2) were concluded by the basic correction of the accuracy of RIs in SOP-3, as explained in p.5-1-2-4 to 6. We could afterwards step further to validate and evaluate the specificity and the accuracy of RTH assay for screening PLT function. Diverse approaches were investigated, and the accumulative conclusions and evidences are described as follows:

5-2-1. Measuring RI with and without RTH filter

This study (p.4-2-1) provided clear clues that the acceptable precision of RTH using SOP-3 was attributed to a further specific effect of the filter on PLT in cWB. By excluding RTH filter (Tab.4-2-1-2), the values of PLT-RI (mean= 3.2%) as well as PLCR-RI (mean PLCR-RI=-1.2%) had no significance. Getting substantially lower PLT-RI levels upon excluding the filter (mean: Epp tubes 3.2% vs. RTH tubes 23.8%: relative decrease= 86.6%: $p < 0.0005$; Fig.4-2-1-2-1) points out the specific retention of PLT by RTH filter. In parallel, PLCR-RI values demonstrated even sharper decline (106.4%: $p < 0.0005$; Fig.4-2-1-2-2). Actually the mean of PLCR-RI converted from a significant positive level by using RTH tubes (18.4%) to a negative one (-1.2 %) by using Epp tubes. The low levels of WBC-RI and RBC-RI (Fig.4-2-1-2-5 and 6) in both RTH and Epp tubes suggest that RTH filter has no significant specific effect on WBC and RBC. Nevertheless, the remarkable decreases of WBC-RI and RBC-RI levels in RTH tubes compared with Epp ones suggest that the concentration of these cells increase slightly after passing RTH filter. This could be interpreted by the plasma portions absorbed by RTH filter, as explained in p.5-2-9-B.

5-2-2. Compatibility between the findings of RTH and PLT spreading for both cWB and platelets isolated by gel-filtration (iPLT)

The investigations using cWB and iPLT (PLT isolated by gel-filtration; p.4-2-2) established the reliability of applying RTH assay on iPLT as well as on cWB. The morphologic validation of PLT in both cases before passing RTH filter showed that they were intact, unstimulated, active, and demonstrated well spread preparations (Tab.4-2-2-3). The RTH assay was able also to reflect the integrity and the function of PLT by delivering significantly high positive PLT-RI values for cWB as well as iPLT (Tab.4-2-2-2-1 and 2). In comparison with the PLT spreading before the passage of RTH filter (b.F), the PLT were significantly inhibited or at least less spread after passing RTH filter (a.F). This observation suggests that PLT with normal function are mainly trapped by RTH filter, whereas those of lower function are mainly allowed to pass the filter. The compatibility between RTH findings and PLT spreading in both cWB and iPLT (Tab.4-2-2-2-1 and 2 compared with Tab.4-2-2-3) proved the specificity and the accuracy of RTH assay for screening PLT function.

5-2-3. Insight into platelet predisposition to hyperactivation by partial absence of normal blood medium

The levels of PLT-RI increased substantially in iPLT (PLT isolated by gel-filtration; p.4-2-2) compared with cWB in the first 7 samples (mean increase=92.8%; $p=0.018$: Fig.4-2-2-2-2) despite the PLT in the iPLT

suspension were deactivated by resting them in a bath chamber (37 °C/ 30-120 min). Beyond it, the integrity and normal activity of PLT were controlled morphologically before passing RTH filter. The increase of PLT-RI demonstrated no significant correlation with the longer resting periods used in samples 8-12 (30-120 min applied to assure iPLT deactivation), and it preserved similar high rates as in samples 1-7 (mean increase= 96.1%; Fig.4-2-2-2-2). This suggests that iPLT have lower activation threshold than PLT in cWB. The phenomenon of “PLT-RI increase” varied inter-individually (40.6-181.4%; $p=0.002$) and was proved morphologically by PLT spreading (Tab.4-2-2-3) which showed higher PLT activity in iPLT suspension compared with cWB especially after passing RTH filter as described as follows:

- a.) The iPLT suspensions before the passage of RTH filter were better spread than of PLT in cWB.
- b.) The preparations of iPLT after the passage of RTH filter were relevantly thinner and less concentrated than that of PLT in cWB.
- c.) After the passage of RTH filter, the PLT in cWB were not spread and demonstrated few small Aggr and/or Aggl, if any. Whilst, the iPLT were partly spread with frequent intermediate forms and had a lot of Aggl. This reflects significantly higher PLT activity for iPLT than the PLT in cWB after the passage of RTH filter.

The similar sharp increases of PLCR-RI values in iPLT compared with cWB (mean increase= 88.8%; Fig.4-2-2-2-2) enhance our conclusions and provide further insight on the impact of PLT size. We may thus suggest that the significant increase of the retention of the platelets associated with proportional reduction of their activation threshold is related to the partial absence of the normal blood medium of PLT. Such effect was significantly less compensated by prefixed PLT which dispose and substantial increase in stickiness and agglutination, as demonstrated by the investigations on our PLT standard (prepared from fixed cPRP). The PLT-RI of the standard of washed PLT (Sw) was 536% higher than that of the unwashed PLT (Suw) (35.6% vs. 5.6%: the measurements after preparation; Tab.4-2-5-5-1). The phase-contrast microscopy proved this effect and reflected increased PLT stickiness and consequent agglutination in Sw compared with Suw Tab.4-2-5-5-1). Further investigations on the standards revealed similar high increases of PLT-RI (mean increase=483%; $p=0.003$) in Sw compared with Suw (5.7 vs. 33.5%), as previously described in [Nemeh 2005 and 2006]. We may suggest, therefore, that the mutual interactions between PLT and their normal blood medium preserve the dynamic balance of PLT function in normal hemostasis and hypothesize that PLT are more predisposed to hyperactivation by partial absence of the other blood components. Despite further studies are required to address this effect, triggering this phenomenon also by RTH – compatibly with the morphologic control – provides at least further significant support to the reliability of RTH assay for evaluating PLT function specifically. The significance of RTH assay exceeded, in this case, that of the morphological tests (PLT spreading and phase-contrast microscopy) which had just a confirmatory role.

5-2-4. Indication of the variable dose-dependent effect of Ca^{+2} with hirudin on the retention of platelets

The significant positive correlation of PLT-RI and PLCR-RI with the reactivating reagent ($CaCl_2$ with r-hirudin) could demonstrate the dose-dependent response of PLT to Ca^{+2} (Tab.4-1-1-2-B). The

conversion of this correlation from a positive to a negative one is attributed to the presence of hirudin (Fig.4-1-1-2-B-1 and 2). This correlation reflected also the inter-individually variable response of PLT to the reactivating reagent, as illustrated in Fig.4-1-1-2-B-2. Despite further investigations are required to address this effect; indicating these interactions between PLT and the reactivating reagent by RTH assay at least supports its specificity and accuracy for analyzing PLT function.

5-2-5. Triggering and proving the hypothesis of PLT particles phenomenon

As explained in the investigations to improve the reliability of RTH, the observations delivered by RTH measurements triggered the hypothesis of PLT particles phenomenon (p.5-1-2-2). The investigations based on RTH measurements could also provide evident clues on the rightness and the implications of the PLT particles phenomenon (p.5-1-2-3, 4, 5-A, 6 and 7-A). The demonstration of these evidences by RTH assay and in concordance with multiple morphological controls (p.5-1-2-3 and p.5-1-2-7-A) promotes strongly the reliable specificity, accuracy and precision of RTH assay as well as its significance for screening PLT function.

5-2-6. Reliable assessment of the PLT standard in due of morphological control

As shown by the day-by-day control of the PLT standard prepared from fixed cPRP (p.4-2-5-3 to 5), the findings of RTH assay were more informative than CBC and demonstrated evident compatibility with the morphological control by phase-contrast microscopy. This compatibility prove evidently the accuracy and the specificity of RTH assay for screening the function of fixed PLT as well as sustained PLT in cWB.

5-2-7. “Athrombogenicity” of RTH filter and the specific responses of PLT to activation characterized by electron microscopy

The response of PLT in cWB to activation by shear stress forcing the blood to pass through the inter-connected micro pores of the polyurethane (PU) filter is demonstrated extensively by the PLT adherence and spreading on the PU surfaces with extremely prolonged pseudopodia (Fig.4-2-6-3-a-1, 2, and 4). The changes of PLT shape are associated with moderate degranulation (Fig.4-2-6-3-a-4 and 8). PLT activation is primarily demonstrated by wall-bound PLT aggregates that vary in size. In contrast to the in-vitro aggregates without the influence of shear forces [Harrison 2000, Rand 2003], the PLT aggregates in RTH filter built a network characterized by extremely prolonged pseudopodia mostly bridging between the opposite PU surfaces (Fig.4-2-6-3-a-1 and 2). These specific interactions of PLT with RTH filter in response to activation by shear stress besides the characteristic PLT contacts within aggregates, (Fbr-mediated and direct contacts; Fig.4-2-6-3-a-5 and 6) are comparable to the physiologic reaction of the circulating PLT at a disturbed endothelium and/or subendothelium [Miller 2001, Huntington 2005, Jurk 2005]. This comparability underlines evidently the athrombogenicity of RTH filter. The occasional free PLT aggregates in the lumina of the pores of the filter (no contact to the filter surfaces; Fig.4-2-6-3-a-4) could be attributed to the availability of activating substances by PLT degranulation during the passage of the filter.

On the other hand, the occasional increase in the disrupted PLT forms objected by PLT spreading after the passage of RTH filter by using cWB as well as PLT isolated by gel-filtration (Tab.4-2-2-3) suggests that RTH filter may disrupt PLT and put a question on its athrombogeneity.

We may conclude that RTH filter is rather athrombogenic especially if compared with the filter systems used in previous PLT retention assays i.e., Salzman [1963], Hellem-II [1970], O'Brien [1987]. Thus, the PLT activation during the passage of RTH forced by centrifugation is attributed mainly to the shear stress of centrifugal force and not to a thrombogenic effect related with the filter.

5-2-8. Reliability and specificity of the reference ranges for the RIs and the weight parameters of RTH by using Eightcheck control

The different batches of the normal level Eightcheck control (EC) used in the study of the in-series (Tab.4-2-4-2-2) and the day-by-day (Tab.4-2-4-4) precision of RTH assay revealed no significant differences ($p>0.1$; t test) in the mean values of the retention indices (RIs) as well as the weight parameters of the quantity assessment (p.4-2-3-1) and demonstrated rather homogeneous low variances ($p>0.05$ Levene test), as shown in Fig.4-2-4-4-3 to 8. The accuracy of the RIs of PLT and blood cells was proved by phase-contrast microscopy. Taking into consideration that EC consists of prefixed medium, the low and homogeneous variations of the resulting RIs and weight parameters underline the ability of RTH assay to reflect accurately and precisely the loss of the physiologic activity of the prefixed PLT in EC. The total 32 measurements performed in the study of the in-series and the day-by-day precision of RTH assay using EC could be, therefore, statistically considered as one integrated sample addressing to the reference ranges of RTH by using EC. Since the values of EC measurements demonstrated mainly normal distribution ($p>0.05$), their reference ranges are based on the theory of normal assumption defined as $\text{mean} \pm 2\text{SD}$, as described in Tab.5-2-7-A.

Table 5-2-8: Descriptive Statistics of the reference ranges of the RIs and the weight parameters of RTH by using Eightcheck control (EC).

	N	Range	Minimum	Maximum	Mean	SD (CV %)	Mean -2SD	Mean +2SD	KS ^a (p)
PLT-RI (%)	32	8.9	-2.7	6.2	2.6	2.1	-1.6	6.8	0.200 ^b
PLCR-RI (%)	32	26.2	-7.5	18.7	7.3	7.1	-6.9	21.5	0.200 ^b
PDW-RI (%) ^c	32	16.4	-8.1	8.3	1.0	3.5	-5.9	8.0	0.176
MPV-RI (%)	32	7.1	-2.8	4.3	1.1	1.8	-2.5	4.7	0.200 ^b
WBC-RI (%)	32	12.9	4.1	17.0	11.3	2.8	5.7	17.0	0.096
RBC-RI (%)	32	22.6	-24.0	-1.4	-4.2	3.9	-12.0	3.7	0.000 ^c
HGB-RI (%)	32	21.6	-21.6	0.0	-5.2	5.3	-15.8	5.4	0.000 ^c
HCT-RI (%)	32	19.9	-22.4	-2.5	-4.4	3.6	-11.6	2.8	0.000 ^c
EC Weight (mg)	27	11.5	518.4	529.9	523.6	1.9 (0.4)	519.7	527.5	0.110
RTH filter (mg)	27	3.6	41.2	44.8	42.4	0.8 (1.9)	40.8	44.0	0.200 ^b
Trapped EC (mg)	27	3.7	23.0	26.7	25.2	0.9 (3.7)	23.4	27.1	0.200 ^b
WRI (%)	27	0.7	4.4	5.1	4.8	0.2	4.5	5.2	0.200 ^b

^aKolmogorov-Smirnov test of normality; Lilliefors significance correction. ^bThis is a lower bound of the true significance. ^cSkewness for these secondary RIs attributed to an extreme outlier within one of the measured series (Fig.4-2-4-2-6).

5-2-9. Integrated interpretation of the specificity and the accuracy of RTH assay

The clues of our multiple investigations, described in p.5-2-1 to 8, provided firm evidences and insights on the specificity and the accuracy of RTH assay (using the improved SOP: "SOP-3") for the evaluation of PLT function and activity. Taking into consideration the reference ranges of RTH in cWB (Tab.4-3-2-2) and in EC (Tab.5-2-8) and the findings characterizing PLT and blood cell interaction with RTH filter (p.4-2-6-2 to 4) besides the findings of the quantity assessment of RTH by using cWB (Tab.4-2-3-2) and EC (Tab.5-2-8), we may suggest the integrated interpretation of the specificity and accuracy of RTH assay as described in the next sections (A and B) respectively. The mean values are considered for comparative analyses.

A.) Nobel significance of the retention of PLT in view of the RIs of blood cells in cWB and EC and in due of phase-contrast and electron microscopic control

The significantly lower retention level of erythrocytes (RBC-RI=-3.25%) compared with leukocytes (WBC-RI=4.21%) by using cWB (Fig.5-2-9-A) could be attributed to their significantly smaller size allowing easier passage through the pores of RTH filter (16-22 μm). Despite PLT are basically smaller than erythrocytes (6.5-12 fl vs. 80-96 fl), they demonstrated substantially higher retention level (PLT-RI= 25.2%; $p < 0.0005$: Fig.5-2-9-A) which reflects a specific effect of the RTH filter. This effect was proved and characterized electron microscopically mainly by: a.) the extensive adherence of PLT aggregates on the polyurethane (PU) surfaces of RTH filter bridging its inter-connected micro pores, and b.) the substantially less adherence of leucocytes and erythrocytes (p.4-2-6-3-A) despite the blood cells as well as PLT were activated by shear stress during the passage of the inter-connected micro pores of RTH filter (Fig.4-2-6-3-a 1 to 8, b, and c1 & c2; p.4-2-6-3-a, b and c). In addition, the incidences of leukocyte and erythrocyte agglutinates in the filter by using cWB compared with EC were rather comparable (p.4-2-6-4) and proportional to the levels of WBC-RI (4.2% vs. 11.3%) and RBC-RI (-3.6% vs. -4.2%) in EC and cWB (Fig.5-2-9-A). This supports strongly our initial suggestion that platelets are specifically retained by the filter due to their activation by shear stress in contrast to the other blood cells which are retained *mainly* unspecifically due to their size (p.5-2-1). On the other hand, the sharp reduction in the levels and variability of PLT-RI in EC compared with that in cWB (mean= 2.6% vs. 25.2%, SD= 2.1 vs. 6.3; $p < 0.0005$: Fig.5-2-9-A) proves the ability of RTH to reflect PLT activity proportionally by demonstrating the low and invariable activity of the prefixed PLT in EC as well as the physiologic activity and biovariability of the sustained PLT in cWB. The electron microscopy provided further evidences on this conclusion by the characterization of PLT interactions with RTH filter

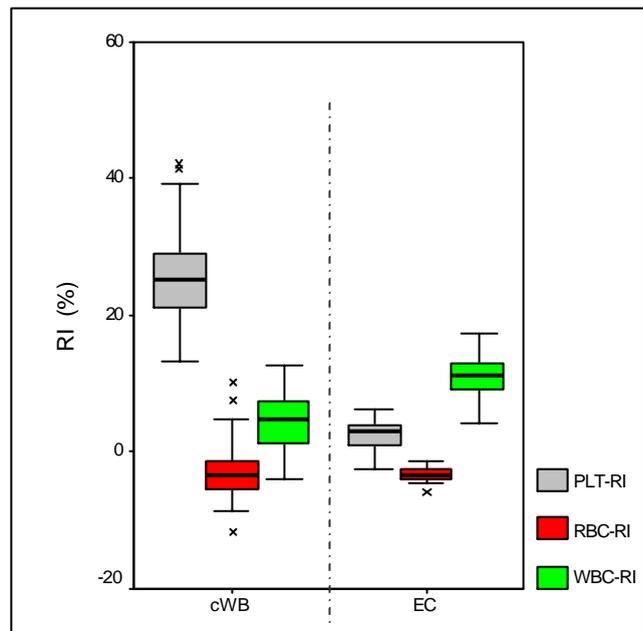


Fig. 5-2-9-A: Comparison between the reference ranges of the retention indices (RIs) of the blood cells by using citrated whole blood (cWB: n=70) and Eightcheck control (EC: n=32).

(p.5-2-7): **(A)** The extensive PLT adherence, wide spreading and aggregation on the PU surfaces of RTH filter by using cWB as well as the associated PLT shape change and mild PLT degranulation prove: (1) PLT-RI values reflects directly the multiple response of sustained PLT to activation by shear stress during the passage of the pores of RTH filter (Fig.4-2-6-3-a-1 to 8). (2) This PLT response is, thanks to the athrombogeneity of the RTH filter, comparable with that found by disturbed endothelium/sub-endothelium. **(B)** Prefixed PLT in EC could form mild PLT agglutinates and demonstrated no real adherence but only selective focal contacts on the PU surfaces of the filter (Fig.4-2-6-4-1 to 3). The accuracy of the variant retention indices were additionally proved for PLT, RBC, and WBC in cWB and EC by using phase-contrast microscopy (p.4-3-1 and p.4-2-4-1). In parallel with PLT-RI, the levels of PLCR-RI showed also significant decline in EC compared with cWB (mean= 7.3% vs. 20.5%: $p < 0.0005$; Tab.5-2-7-A and Tab.4-3-2-2).

B.) Nobel significance of the weight retention index (wRI): “Absorbance effect” of RTH filter

By the centrifugation of cWB in RTH tube, a significant amount of the blood equivalent to wRI (mean=4.6%; Fig.4-2-3-2-4) is absorbed by filter. This portion comprises mainly of the plasma because of its liquid phase and its lower specific gravity compared with the blood cells [Moris 2001, Sugai 2003]. This “*absorbance effect*” is supposed therefore to lead to relevant increases in the concentrations of the blood cells after the passage of RTH filter and consequently to relevant *negative RI values*. The RI value of blood cells is determined therefore by the total of: **(1)** the positive RI attributed to the “unspecific” retention by RTH filter mainly proportional with the size of the cell (p.5-2-9-A); and **(2)** the negative RI related to the absorbance effect (the virtual level = -mean wRI= -4.6%). Thus, the low negative level of RBC-RI (-3.25%) reflects mainly the “*absorbance effect*”, whilst the WBC-RI level which stepped significantly over it (4.2%) implicates a retention effect on leukocytes significantly higher than that on erythrocytes since the latter are 3 times smaller in size [Moris 2001]. In addition, the value of WBC-RI is significantly more affected by such retention effect than RBC-RI since the concentration of leucocytes in blood is 10^3 times lower than that of erythrocytes. Comparably, the substantial increase of PLT-RI levels (25.2%) over the virtual negative level related to the absorbance effect (-4.6%), in spite PLT are 7-12 times smaller than RBC [Diamant 2004], confirms firmly the specific and accurate effect of PLT retention under the conditions of SOP-3. The absorbance effect is also evident by using EC as tested material. But the gap between PLT-RI levels and the virtual negative level related to the absorbance effect of EC (-4.8%) was significantly smaller by using EC than by using cWB (7.4% vs. 29.8%). This is attributed to loss of PLT function in EC, as explained in p.5-2-9-A.

5-2-10. Reliability and specificity of RTH assay pronounced by the quantity assessment using cWB and EC

Taking into consideration the results of the reference ranges of the weight parameters by using cWB (Tab.4-2-3-2) and EC (Tab.5-2-8), we may conclude the followings:

1. Accuracy of the amount added to RTH tubes (b.F amount)

The low variations of the reference ranges of the b.F amount added to RTH tubes i.e., passing RTH filter by using cWB and EC ($CV < 0.4\%$) enhance the accuracy of performing RTH assay. Despite the trend of positive correlation between PLT-RI levels and the b.F amounts of cWB was not statistically significant

($r=0.06$; $p>0.01$; $p.4-2-3-2$), but it underlines the necessity of standardizing and controlling the b.F amount to preserve the reliability of RTH measurements. The higher weight of the 500 μL of EC compared with cWB (523.6 mg vs. 502.6 mg) is interpreted by the higher specific gravity of EC [Moris 2001, Sugai 2003].

2. Accuracy of the weight of RTH filter: A discriminator of the filter integrity

The low variation of the reference range of the weight of RTH filter by using cWB and EC ($CV=2.6\%$) promotes further the reliability of RTH filter and consequently RTH assay. Despite the correlation between PLT-RI and the weight of RTH filter was not statistically significant ($p>0.01$), it was quite remarkable ($r=-0.296$). Thus, the reference range of the weight of RTH filter represents a helpful discriminator for controlling the integrity of RTH filter besides the quality assurance of the reliability of RTH measurements.

3. Accuracy of the weight retention index (wRI): Indicator of the reliability of RTH filter

The wRI proved to demonstrate proportionally the amounts of the blood trapped/absorbed in RTH filter ($p<0.0005$; $p.4-2-3-2$). The significant increase of wRI by using EC compared with cWB (4.82% vs. 4.57%) is attributed therefore to the higher specific gravity of EC [Moris 2001, Sugai 2003]. The variability of the wRI was very low ($SD=0.21$) with no significant correlation with PLT-RI. But the levels of wRI demonstrated marked negative correlation with the weight of RTH filter in both cWB ($r= -0.23$) and EC ($r= -0.12$), as shown in Fig.5-2-10-3. The reproducible levels of wRI are considered, therefore, as an essential indicator for the integrity as well as the reliability of RTH filter and RTH measurements under the centrifugation conditions of 110 g/ 5 min. Also the reference ranges of wRI for both cWB and EC (Tab.5-4-6-3) are consequently essential discriminators for the quality assurance of the integrity of the filter and the reliability of RTH assay.

The negative correlation between wRI and the weight of RTH filter in both cWB ($r= -0.233$) and EC ($r= -0.12$) as well as the marked negative correlation between PLT-RI and the weight of RTH filter ($r=-0.269$) may together suggest the perspective for setting even further improvement in the reliability of RTH assay. This might be achieved by developing more standardized construction of the filter system.

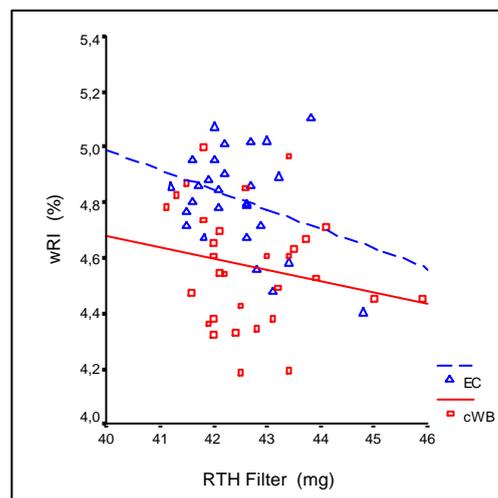


Fig. 5-2-10-3: wRI against the weight of RTH filter by using cWB from blood donors (n=31) and Eightchek control (EC: n= 27).

5-3. Precision of RTH assay

The essential improvements on the precision of RTH were accomplished in the course of the stepwise development of the methodology and the reliability of RTH assay addressing SOP-2 and then SOP-3 which implicated acceptably precise measurements for the retention indices (RIs). The accumulated experience in the application of RTH assay using SOP-3 emerged further investigation on its precision in cWB in order to inspect further probable improvement as well as to study the in-series and the day-by-day precision by using normal level Eightcheck control (EC). The precision study set further evident clues on the reliability of the precision of RTH measurements, as summarized as follows:

5-3-1. Reliability of the in-series precision of RTH assay using cWB

The modifications addressed to SOP-2 excluded major sources of the variability and errors in the SOP-1 of RTH, as described in p.5-1-1. Nevertheless, the resulted accuracy and precision of RTH could not reach acceptable levels, as revealed by the measurements in stages 1-3 of the improving protocol (high SD values; Tab.4-1-2-2-2). The modifications addressed to SOP-3 in stage 4 of the improving protocol, delivered further basic corrections and resulted in acceptable precision (substantially lower SD values; Tab.4-1-2-2-2) and accuracy especially by avoiding the errors related to the PLT particles phenomenon, as described in p.5-1-2-4-A & B and p.5-1-2-6-A-1 & p.5-1-2-6-B. The high systemic increases of PLT-RI by time (in-series increase: mean=38.5%) and horizontally (the increase in the 2nd in-double measurements compared with the 1st ones: mean=10.3%) found in stages 1-3 (SOP-2) were actually eliminated ($p < 0.0005$) in stage 4 (SOP-3) and even converted occasionally to insignificant negative ranges (mean= -2.7% and -0.6% respectively: $r > -0.046$), as described in Tab.4-1-2-2-2 and illustrated in Fig.4-1-2-3-2-2 and 3. The high in-series and horizontal variations of PLT-RI in SOP-2 (stages 1-3) were also sharply reduced to acceptable limits in SOP-3 (stage 4) (SD= 13.8 vs. 4.7 and 8.2 vs. 2.9 respectively; Fig.4-1-2-3-2-1 and 3). In parallel, the serious errors of the other RI measurements in SOP-2 were basically corrected in SOP-3 leading to acceptable precision and accuracy, as described in Tab.4-1-2-2-2 and demonstrated in Fig.4-1-2-3-2-5, 6, 8 and 9.

The latest precision study performed on 3 series of cWB from different healthy blood donors (Tab.4-2-4-2-1) enhanced the acceptable precision of SOP-3. Similarly to stage 4 of the improving protocol, the precision study demonstrated also no significant horizontal variation and it resulted in even further reduction of the in-series variation of PLT-RI (SD=4.8 vs. 2.9) associated with conveniently small ranges of the 95% confidence intervals of the mean values (Fig.4-2-4-2-1). The resulting *low variation* of the PLT-RI by using SOP-3 is of little practical consequence because of the physiologic variation of PLT activity [Kunicki 2002, Jurk 2005]. In parallel with PLT-RI, the precision of the other RIs was similarly further improved, as described in Tab.4-2-4-2-1 and illustrated in Fig.4-2-4-2-2 to 6. The reliability of the acceptable in-series precision was further enhanced by the constant demonstration of normal distribution by the PLT-RI and other RI values within each series of measurements ($p > 0.05$). This significant improvement in the precision is attributed to the accumulated experience of improving the performance of RTH. Having acceptable precision on 3 series of cWB in stage 4 of the improving protocol besides further lower variation in the other 3 series cWB from different blood donors (in the precision study) confirm evidently the reliability of RTH precision.

In parallel with PLT-RI, the precision of the RIs of PLT size (PLCR-RI, PDW-RI and MPV-RI) and blood cells (WBC-RI, RBC-RI) revealed basic improvement (Fig.4-1-2-3-2-5, 6, 8 & 9 and Fig.4-2-4-2-2 to 6). The reliable precision of these RIs (Tab.4-2-4-2-1), enhances the enlarged significance of RTH assay (p.5-4-4-3 and p.5-4-5) and support strongly the reliability of RTH measurements in view of the significance of the models of the mutual correlations between PLT-RI and the other RIs (Fig.4-2-4-2-7) for the interpretation and the quality assurance of RTH assay (p.5-4-6-1 & 2). The variation of the RIs of PLT is dependant on the reliability of CBC measurements and the biological variability.

5-3-2. Reliability of the in-series and the day-by-day precision of RTH assay using EC

The day-by-day precision of RTH assay was studied by using EC. The cWB from blood donors was not used since it is not acceptable to perform PLT function analyses after 180 min from blood collection [Harrison 2000, Miller 2001]. The in-series as well as the day-by-day precision of RTH using EC revealed rather homogenous of PLT-RI (SD 2.3 vs. 1.7; $p>0.01$) which was significantly ($p<0.05$) lower than that of the in-series precision performed on cWB (SD=2.9). This is interpreted by the loss of PLT activity in the prefixed medium of EC and underlines the reliability of RTH to reflect this fact precisely and accurately. The acceptable reproducibility of PLT-RI and the other RI levels as well as the high homogeneity of their variances (Levene test) by using 5 different EC series in the precision study (3 for the in-series and 2 for the day-by-day studies: Fig.4-2-4-4-3 to 8; Tab.4-2-4-2-2 and 2) underlines the reliable in-series as well as day-by-day precision of RTH assay. The rather normal distribution of the measurements of RIs in EC series enhances the reliability of RTH precision using EC. The occasional presence of outliers in the in-series measurements was limited to a minority of series of the secondary RIs (e.g., RBC-RI) causing no disruption in the significance of their normal assumption neither their small ranges of the 95% confidence intervals of the mean values, as illustrated in Fig.4-2-4-4-3 to 8.

5-3-3. Reliability of the precision of the weight parameters of RTH assay

The weight parameters demonstrated quite acceptable in-series precision by using cWB and EC as well as day-by-day precision by using EC. This was evident by the low variation of these measurements (CV=4.1%; (Tab.4-2-4-2-2-1 & 2 and Tab.4-2-4-4) besides their normal distribution ($p>0.05$) and the small range of the 95% confidence intervals of the mean values, as illustrated in Fig.4-2-4-3-1 to 4 and 4-2-4-5-1 to 4. The variations of wRI were in intimate concordance with that of the weight of the material trapped by RTH filter (cWB as well as EC; $p<0.0005$). The significant increases in the mean values of EC compared with cWB are attributed to its lower specific gravity [Moris 2001, Sugai 2003].

5-3-4. Compatibility of the serial and day-by-day measurements of RTH with the morphological control by phase-contrast microscopy

As described in p.4-2-4-1, the accuracy of the retention indices of PLT and blood cell in the the serial as well as the day-by-day measurements of the precision study using cWB EC was proved by phase-contrast microscopy. This morphological control enhances the credibility of the precision study of RTH assay. The compatibility between the findings of RTH and the phase-contrast microscopy supports

the reliability of the resulted low variances of RIs and acceptable precision of RTH. Similar evident compatibility between the findings of RTH and the morphological control by phase-contrast microscopy was objected in the day-by day precision of the 3rd lot of our PLT standard, as described in Tab.4-2-5-5-1 and 2.

5-4. Introduction of RTH-II: A developed, standardized, reliable, and simple version of RTH

5-4-1. Reliability and evidence based specificity of the developed standard procedure compared with the variability and errors of previous RTH procedures and studies

The data obtained from the last multicenter investigations on RTH underlined that the reliability of RTH assay is at least unacceptable [König 2002]. Since its development in 1993, the RTH assay has been suffering from serious problems in the reliability preventing its application in the clinical practice. The studies investigating the precision of RTH delivered variant results; some of them are not interpretable [Koscielny 2002]. The accuracy of RTH assay in healthy individuals has never been investigated, and the clues on its specificity have been insufficient and presented no comprehensive interpretation. Nevertheless, some works reflected considerable response of PLT-RI to PLT agonists and PLT inhibitors and promised vital clinical significance for RTH as PLT function screening test [Wieding 2001 and 2003, Schenk 2001]. This has meanwhile to be interpreted as rather hypothetic and throws doubts on the reproducibility. Few studies concluded the “athrombogeneity” of the RTH filter system [Wieding 2001, Schenk 2002], despite the evidences were not adequate and the filter is proved actually to be not completely athrombogenic, as explained in our study (p.5-2-7). The main weakness point of RTH studies was the use of variant and unreliable procedures stepping unacceptably over defining reliable and evidence based SOP (standard operation procedures). This was evident in the improvements and modifications addressing our developed SOP of RTH (SOP-3), as explained in p.5-1 and *summarized in Tab.5-4-1*. In addition, previous studies on RTH had frequently serious errors in their methodology. As examples we may briefly mention the followings:

- 1) The centrifugation conditions were not specified and varied extensively in the guideline of RTH and related studies (79-160 g for 5-16 min) [Wenzel 2000, Wienefoet 2003, Wieding 2004, and Krischek 2005]. A main investigation on RTH described the used “g” value but it neglected defining the time of centrifugation [Wieding 2002 and 2003]. Our investigations were, however, based on well-defined centrifugation conditions (p.4-1-1-1 and p.5-1-1-2).
- 2) PLCR-RI values were calculated according to the old formula and their levels were significantly higher than PLT-RI ones; mean= 26% vs. 21.0% as mentioned by Koscielny [2002], Schenk [2002], and Krischek [2005] (the number of the studied group was mentioned only by Krischek). But the diverse investigations in our study proved firmly that the old formula can deliver only very low or negative levels of PLCR-RI e.g., the reference range (n=70) (mean = -6.4%; Fig.5-1-1-7). Even the PLCR-RI levels based on our corrected formula (p.4-1-1-6) were lower and not higher than PLT-RI levels (mean=20.5% vs. 25.2%; Tab.4-3-2-3). The PLCR-RI values preserved significant correlation with PLT-RI thought the diverse investigations of our study which was essential in the interpretation of the assay and improving the reliability of the measurements in contrast to the meaningless results by using the old formula, as explained in p.5-1-2-5-C (Fig.5-1-2-5-C). These controversies put a serious question on the accuracy and the credibility of the related previous studies.
- 3) The PLT-RI values by using fixed PLT medium (Eightcheck control) revealed low levels and variation in our precision study (mean=2.6%, SD=2.1; Tab.5-2-8) which demonstrated proportionally

the low and invariable activity of the prefixed PLT. Whilst the study of Krischek [2000 and 2005] stated irrationally high levels for PLT-RI (mean= 21.8%, SD=4.4) which could be attributed to the errors of PLT particles phenomenon. The day-by-day variations were substantially higher than the in-series ones by using the Eightcheck control, as described by Wienefoet [2003]. This is also irrational since the prefixed medium of Eightcheck control delivers rather stable retention values regardless of the time, as also revealed by our study (no significant differences between the serial variations and the day-by-day ones: $p>0.01$; Fig.4-2-4-4-3 to 8).

4) The amount of the tested material was variable (450-500 μ L) and the period of its incubation in RTH tube (3-10 min) differed significantly [Wenzel 2000, Nickels 2003, and Wienefoet 2003].

5) The time window of performing the test varied up to 180 min after blood withdrawal [Schenk 2001, Koscielny 2002], despite the limit 180 is not recommended for PLT function assays [Miller 2001] and our study revealed also unacceptable variability associated with intervals above 120 min (Fig.4-1-2-3-2-2; p.5-1-2-1).

6) Surprisingly and apart from these variability and errors of RTH procedures, some of RTH related studies did frequently not define main parts of the procedures applied for performing RTH e.g., (a) The amount of the tested material [Wieding 2002], (b) the period of the incubation step [Schenk 2002], (c) the conditions of centrifugation (the “g” value and the time: Wieding 2001), (d) the way of mixing the blood after centrifugation [Schenk 2001, König 2002, Wieding 2004] which is quite essential for reliable measurements (Fig.4-1-2-3-2-1 and 3), as revealed in our study (p.5-1-2-7), (e) the time limit of performing the measurements after centrifugation [e.g., Koscielny 2002, Schenk 2002, Wieding 2004, König 2002] which is quite critical for their reliability (as revealed in our study: p.5-1-2-7-C-b), (f) the time window of performing the test which [König 2002, Wieding 2003 and 2004], (g) the formula of calculating PLT-RI e.g., [Schenk 2001, Wieding 2002].

7) Some studies performed by using cPRP, but they unacceptably generalize their conclusions on the RTH assay using cWB [Schenk 2001].

These errors in the methodology of the previous studies negate their credibility and put a question on the reliability of their findings e.g., the specificity and the reference ranges.

In contrast, our study addressed the reference range of RTH assay and its specificity after developing a reliable and standardized procedure for RTH (SOP-3). A stepwise approach was followed for the radical validation and development of RTH procedures. Each step was evaluated and then new modifications, procedures and/or identifications were set/addressed and incorporated in RTH assay primarily to exclude or minimize the sources of the variability and errors and to assure the reliability and the and the specificity of the assay. As a further step, the convenience of the RTH as simple and cost effective assay should be improved. Our basic improvements on SOP-1 were incorporated in SOP-2 which was in turn further evaluated and developed to SOP-3. Multiple approaches were applied for the investigations validating our modifications as well as the resulting SOPs by using cWB, EC, iPLT, cPRP, and our developed PLT standard (p.4-1 & 42). The novel identification of the secondary RIs (p.5-1-2-6), the correction of PLCR-RI formula (p.5-1-2-4-B & 5), and wRI (p.5-2-9-B, p.5-2-10 and p.5-3-3) along with the use of multiple morphologic control (p.3-5) were quite essential for: (1) assuring the reliability of the measurements, (2) getting comprehensive insights into the interpretation of RTH measurements,

(3) defining the sources of errors and variability and setting the related modifications, and (4) validating the reliability of the developed SOP. This methodology was crucial for addressing a comprehensive and evidence based interpretation for the specificity and the accuracy of RTH assay, for the first time, under the standard conditions of SOP-3 (p.5-2); and *b.*) defining adequate clues characterizing the rather “athrombogeneity” of RTH filter (p.5-2-7).

Table 5-4-1: Summary* of the modifications on RTH and identifications addressing the standard procedures of RTH-II.

Procedures and Identifications	RTH	RTH-II	Advantages of RTH-II
Tested material in clinical practice	Mainly ^A cPRP, occasionally ^B cWB	cWB	<ul style="list-style-type: none"> • Avoid variability of PLT & cell gradients and their diverse interactions with PLT. • Reflects the actual physiological situation. • Minimize the required blood amount to 0.5 vs. >1.5 mL. • More convenient: Easier and save the preparation time of cPRP (>15 min).
Tested amount	450-500 μ L ^C	Standard 500 μ L	Standardize the amount passing RTH filter
Additional Re-agents	50 μ L mainly reactivating reagents ^D	Non	<ul style="list-style-type: none"> ○ Unjustified addition: PLT response to such additives was not defined or standardized. ○ Avoid unstandardized convertible PLT response that varies inter-individually. ○ Save preparation time (3-6 min) with greater ease.
Incubation step	3-10 min ^C , ND	Non	<ul style="list-style-type: none"> ❖ Exclude errors attributed to PLT activation by long contact with the filter. ❖ Standard contact time with RTH filter: Avoid variability of PLT activation due to different incubation periods and the inter-individual variability PLT response to such activation.
b.F measurements	On the materials added to RTH tube: After the incubation step	On the materials in the collection tube: No incubation required	<ul style="list-style-type: none"> ✓ Standardize the amount passing RTH filter: Eliminate variability due to variances in the amounts aspirated from the different cell counters (100-400 μL). ✓ Sufficient quantity for performing the in-double b.F and a.F measurements and additional control. ✓ Assure blood homogeneity on mechanical rotator, contrast to the variability of the measurements on RTH tube attributed to variant rates of sedimentation and blood heterogeneity during its stand. ✓ Blood in big tubes is less opposed to hyperactivation by inconvenient mixing than in the smaller Epp tubes. ✓ Save time: Allow performing the b.F measurements during the centrifugation of RTH.
Centrifugation of RTH tube	79-160 g for 4-16 min ^E , ND	110 g/ 5 min	<ul style="list-style-type: none"> ▪ Deliver reliable procedure and measurements of PLT-RI and other RIs. ▪ Acceptable distribution of PLT in the filter: from its upper until middle-lower parts ▪ High precision of the weight retention (wRI: CV<2.6%) and significant reference range. ▪ Reliable and meaningful reference range of PLT-RI and the other RIs with acceptable bio-variability and adequate margins for assessing pathological variations.
Mixing RTH tube before a.F measurements	Vortex 3 min	Slight vortex: standard 1-3 sec.	<ul style="list-style-type: none"> ○ Avoid PLT hyper-activation triggering the occurrence of PLT particles phenomenon that is associated with serious errors destroying the reliability of a.F measurements. ○ Save time and greater convenience.
a.F Measurements	On RTH tube	On RTH tube <2 min after mix	<ul style="list-style-type: none"> ❖ Preserve blood homogeneity. ❖ Minimize errors attributed to probable occurrence of PLT particles phenomenon.
Measurements Type	Not standardized	In-Double	Assure the measurements' quality: the patterns of the variation of the 2 nd in-double a.F measurements compared with the 1 st one indicate probable errors e.g., inadequate or aggressive mixing.
Time of performing RTH	<180 min., ND	Standardized <120 min	<ul style="list-style-type: none"> • Deliver reliable accuracy and precision for RIs measurements. • PLT-RI decrease remarkably after 120 min.
Tips of the micropipette	ND	New	Avoid unstandardized variation by using one tip for successive pipetting (e.g., in-double).
Correction of PLCR-RI formula	PLCR-RI (%) = [(b.F PLCR) – a.F PLCR] / (b.F PLCR) x 100	PLCR-RI (%) = [(b.F PLCR x b.F PLT) – (a.F PLCR x a.F PLT)] x 100 / (b.F PLCR x b.F PLT)	<ul style="list-style-type: none"> ✓ Reliable and significant reference range contrast to the meaningless one using original formula. ✓ Correction of PLCR-RI correlation with PLT-RI and the other RIs. ✓ Essential role in identifying RTH errors and validating the due modifications approaching RTH-II ✓ Enlarging the significance of RTH-II assay: Significant insights into the characteristics of large PLT function and their interaction with RTH filter. ✓ Approaching key indicators for the quality assurance of PLT-RI measurements by the variation their levels and the pattern of their correlation with PLT-RI.
Identification of new RIs for PDW and MPV	Non	PDW-RI, MPV-RI	<ul style="list-style-type: none"> ➢ Reliable and significant reference range: RI (%)=(b.F measurement-a.F measurement) x100 / b.F measurement ➢ Beneficial role in validating the errors and due modifications approaching RTH-II ➢ Enlarging the significance of RTH-II assay: Significant insights into the interaction of PLT size distribution and RTH filter e.g., the mild shift of PLT size distribution to the right after passing the filter and its significant increase by PLT particles phenomenon ➢ Setting significant indicators for the quality assurance of RTH-II measurements by the pattern of the variation of their levels and correlation with PLT-RI.

Identification of new RIs for the blood cells	Non	WBC-RI, RBC-RI, HGB-RI, HCT-RI	<ul style="list-style-type: none"> ▪ Reliable and significant reference range: $RI (\%) = (b.F \text{ measurement} - a.F \text{ measurement}) \times 100 / b.F \text{ measurement}$. ▪ Crucial role in identifying RTH errors and validating the due modifications approaching RTH-II ▪ Provide essential indicators for the quality assurance of RTH measurements: Enhance the reliability of RTH-II. ▪ Expand the significance of RTH-II to other blood cells.
Identification of wRI (the weight retention index)	Non	wRI: weight percentage of blood trapped in the filter	<ul style="list-style-type: none"> ○ $wRI (\%) = (b.F \text{ filter weight} - a.F \text{ filter weight}) \times 100 / b.F \text{ filter weight}$. ○ Essential for standardizing the conditions of centrifugation and validating the reliability of the developed procedures of RTH-II. ○ Essential for the quality assurance of the integrity and the reliability of RTH filter.
Identification of PLT particles phenomenon	Non	Addressed experimentally: on cWB, cPRP, isolated PLT and prefixed PLT mediums (EC, PLT standard)	<ul style="list-style-type: none"> • PLT particles phenomenon: "PLT activated by centrifugation passing through the filter are opposed to hyperactivation by inconvenient vortex (RTH) leading to PLT degradable aggregates/agglutinates (particles) and rosette forms resulting in: a) False PLT count decrease and b) PLT particles misread by the automatic cell counter as large PLT, RBC, and/or WBC whenever they are within their corresponding size discriminators leading to relevant <i>false increases</i> in their counts and shift of PLT size to the right. A b.F PLT hyperactivation can lead to the same consequences. The total values of RIs vary according to the false b.F and a.F variations." • Mandatory for identifying RTH errors and setting the due modifications of RTH-II: Associated with defined patterns of RIs levels and their inter-correlation with PLT-RI. • Represent the main probable errors for RIs measurements. • Understanding their implications is necessary for interpreting and assuring the quality of the measurements.
Applicability on other materials	Not studied	Established for isolated* and fixed* PLT	<ul style="list-style-type: none"> ✓ Further comprehensive insight on RTH precision, accuracy and specificity. ✓ Characterization of PLT interaction with the filter and their different medium. ✓ Enhance RTH-II reliability for research purposes.
Automation	Non	Addressed	<ul style="list-style-type: none"> ○ On-line interpretation and quality assurance based on RIs levels and correlation with PLT-R. ○ Enhance reliability and satisfaction with higher cost effectiveness.

* Details are described in p.5-1: The procedures of RTH (SOP-1) were based mainly on the newest guideline of RTH assay at the time we start our study [Wenzel 2000] and considering the procedures used in the main RTH related studies at that time. ^A Krschek 2000, Schenk 2001, Koscielny 2002, Nickels 2003. ^B König 2002. ^C Wenzel 2000, Nickels 2003, Wienefoet 2003. ^D (r-Hirudin with CaCl₂) or occasionally Na-Cit 0.3% [Nickels 2003]. ^E Wenzel 2000, Wienefoet 2003, Wieding 2004, Krschek 2005. cWB= citrated whole blood (p.3-4-1); cPRP=citrated platelet rich plasma (p.3-4-2); ND= frequently not defined partly or totally (see the text); EC= normal level Eightcheck control.

Despite the essential improvements in the precision and the accuracy of RTH using SOP-2 due to excluding major sources of errors and variability in SOP-1 (p.5-1-1), the SOP-3 implicated further basic correction in the accuracy and the precision of RTH measurements (p.5-1). The phenomena of false elevations of PLT-RI levels (mean: 2.2 fold) and their increase by time (up to 61%) observed in SOP-2 (Tab.4-1-2-2-2), as well as the errors of PLT-RI correlation with the other RIs (Fig.4-1-2-3-2-4, 6, 7 and 10) were basically corrected in SOP-3, as described in p.5-1-2-4 to 6. The errors of SOP-2 were attributed mainly to PLT particles phenomenon which was defined under morphologic control. Approaching the PLT particles phenomenon was actually essential (p.5-1-2-2, 3 and 7). The correction of the accuracy and the precision of PLT-RI levels were associated with the correction of the other RIs (p.5-1-2-4 to 6). Compared with SOP-3, the variation of PLT-RI in SOP-2 was 4.7 times higher (SD= 2.9 vs. 13.8; Tab.4-2-4-2-1 and Tab.4-1-2-2-2 respectively). The in-series (38.5%) and horizontal increases (10.3%) in SOP-2 (Tab.4-1-2-2-2) were eliminated in SOP-3, as further explained in p.5-1-3. Investigating the precision by using Eightcheck control; in-series and day-by-day (for periods longer than ever before), revealed low and homogenous variations for RIs (e.g., PLT-RI: SD<2.9) that support further the reliability of SOP-3. In association with PLT-RI, similar basic improvements (p<0.0005) on the precision of other RIs was achieved in SOP-3, as described in p.5-3-1. The compatibility between the measurements of the precision and the phase-contrast microscopy control enhances strongly reliability of SOP-3 (p.5-3-4). The reliability of RTH assay using SOP-3 is dependent on precise and accurate CBC measurements (before and after the filter passage) and the biological variability, whilst the errors induced by additional sources of variability in previous procedures (e.g., PLT particles) increase the variability and false results unacceptably.

The significance of the improvements in the accuracy and the precision of RTH using SOP-3 (above mentioned and also described in details in p.5-1-2 and p.5-3) is further enhanced by taking into consideration that these improvements are based on the comparison with SOP-2 which actually implicates also essential improvement compared with SOP-1 by excluding different sources of variability. *Therefore, the improvements in the reliability of RTH measurements using SOP-3 compared with SOP-1 exceed significantly that described in the comparison of SOP-2 with SOP-3.* The advantages of the identifications and improvements addressed/set to RTH assay in our study (p.5-1) did not only represent basic development in its reliability and specificity (p.5-1 and 3), but also underlined the inconvenience and unacceptable reliability of SOP-1. The significance of SOP-3 is further enhanced by taking into consideration that SOP-1 was the newest “improved” guideline of RTH [Wenzel 2000] when we started our study.

5-4-2. Identification of RTH-II: Obligations and adaptation of the developed SOP

With regard to the frequent serious errors in previous RTH studies and the extensive and unacceptable errors and variability of previous RTH procedures compared with the basic developments of the standardized and improved SOP (SOP-3) of RTH implicating acceptable reliability (Tab.5-4-1; p.5-4-1), we identified “RTH-II” as the developed and reliable version of RTH which comprises mainly our improved SOP (SOP-3) holding consequently its advantages. The SOP of RTH-II exceeds further the excellency of SOP-3 by taking into consideration the advantages concluded by its validation and establishing the reference range, as summarized as follows: **A.)** The determination of the secondary RIs (WBC-RI, RBC-RI, MPV-RI and PDW-RI; p.4-1-2-5) is incorporated in RTH-II assay due to their substantial significance in: (1) its interpretation and specificity (p.5-1-2-6 and p.5-2-9), and (2) the quality assurance of PLT-RI measurements (p.5-4-6). In addition these RIs enhance and enlarge the clinical significance of RTH-II evidently (p.5-4-4 and 5). **B.)** The HGB-RI and/or HCT-RI could be optionally calculated instead of RBC-RI according to the menu of the cell counter. **C.)** The RIs of the main normal types of leukocytes (NEUT-RI, LYMP-RI and MXD-RI) and of the indices of erythrocytes (MCV-RI, MCHC-RI and RDW-RI) were excluded from RTH-II because they provided no additional significant insights into the specificity or the interpretation of RTH-II. This became evident in the improving protocol (p.5-1-2-6-C and D) and was further confirmed in our subsequent investigations e.g., on the reference range (Fig.4-3-3-1). **D.)** For further minimizing the turnaround time, the b.F measurements on the collection tube could be performed during the centrifugation of RTH tube. This was actually applied in the investigations for validating RTH and establishing the reference range. **E.)** The room temperature was defined as a standard condition for performing RTH-II since it delivered reliable results. **F.)** Specific care should be given to the interpretation of the variation of the 2nd measurements compared with the 1st ones for the quality assurance of the results. The patterns of these variations can indicate probable errors related to e.g., inadequate or aggressive (PLT particles phenomenon) mixing, as explained in p.5-4-6-1-a. The SOP of RTH-II is illustrated in Fig.5-4-2 and can be summarized as described as follows:

5-4-2-1. 500 µL of cWB (or the tested material) is pipetted carefully into the upper part of the RTH tube (p.3-1-2) and then centrifuged by 110 g/ 5 min at room temperature.

- 5-4-2-2. CBC before the passage of RTH filter (*b.F measurement*) is performed on the blood in the collection tube (p.3-4-1) during the centrifugation of RTH tube.
- 5-4-2-3. CBC after the passage of RTH filter (*a.F measurement*). After centrifugation, the upper part of RTH tube is removed and the blood in the lower part (p.3-2-1) is mixed thoroughly and carefully by vortex (standard vortex; p.4-1-2-4-4). The a.F measurement is performed on the blood in the lower part of RTH tube shortly (within 1-2 min) after mixing.
- 5-4-2-4. Calculation of the retention indices (RIs). PLT-RI and the secondary RIs (e.g., MPV-RI, PDW-RI, WBC-RI, RBC-RI) are calculated as follows:

$$\text{RI (retention index \%)} = \frac{(\text{b.F measurement} - \text{a.F measurement}) \times 100}{\text{b.F measurement}}$$

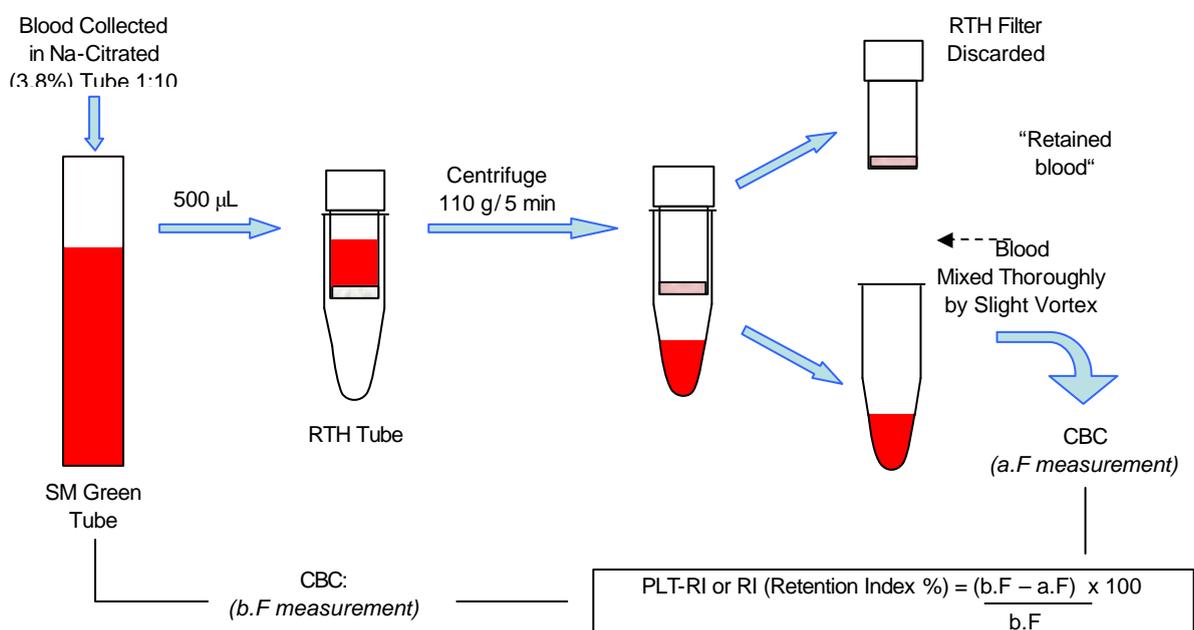
PLCR-RI is calculated according to the corrected formula (p.4-1-1-6).

Note 1: RTH should be performed within 120 min after blood withdrawal and at room temperature. Inter-laboratory investigations should better prospectively define the time limits of RTH performance in order to minimize the variation (p.5-1-2-1).

Note 2: The measurements are performed preferably *in-double* and the means are considered for the calculation of RIs. For expertise personnel, performing the a.F measurements one time is recommended to reduce the critical time required for delivering the results. Interpretation of the results should consider the key notes summarized in the protocol of quality control (p.5-4-6).

In contrast to RTH, the modifications incorporated in RTH-II implicate a.) basic improvement in the standardization and reliability, b.) the correction of PLCR-RI formula and the novel identification of the RIs for PLT size distribution and blood cells, c.) evidence based specificity. These modifications have consequently led to fundamental improvements reflected on: (1) identifying reliable reference ranges of RIs (p.5-4-3), (2) enhancing and enlarging the specificity on PLT function (p.5-2-9 and 5-4-4), (3) expanding the clinical significance (p.5-4-5), (4) improving the excellency (p.5-4-7 and 8) and the quality assurance of the assay (p.5-4-6) which in turn enhance further the reliability of RTH-II. This

Fig. 5-4-2: Standard Operation Procedures (SOP) of RTH-II.



enlarged furthermore the gap between the reliability and the significance of RTH-II and RTH. The introduction of RTH-II assay is therefore mandatory in order to prevent significant and basic “bias by reporting”.

Since the introduction of RTH-II in 2004, only one study has been created using the SOP of RTH-II [Klinkhard, 2006]. The newly published works of Krschek and Wieding were still attributed to RTH despite the procedures described are significantly adapted from RTH-II [Krschek 2005, Wieding 2005]. These findings should be therefore critically revealed.

5-4-3. Reliability of the reference range of RTH-II

5-4-3-1. Well established reference ranges for RIs and their significance

After addressing RTH-II which implicates acceptable accuracy and precision as well as evidence based specificity on PLT (p.5-4-1 and p.5-2 and 3), it became possible for the first time to identify a reliable reference range for PLT-RI by using cWB. The variability of RTH procedures used for addressing the reference ranges of PLT-RI in previous studies [Krschek 2000, Schenk 2002] disrupted the probable usefulness in clinical practice. In addition, RTH procedures implicated serious errors that negate the reliability of the measurements of such reference ranges, as summarized in Tab.5-4-1 and explained in p.5-4-1. Moreover, the previous studies used no conventional control on RTH measurements. In contrast for the reference range of RTH-II, the accuracy of PLT and blood cell retention indices besides the integrity of PLT were controlled by phase-contrast microscopy (p.4-3-1). However, the available data on the previous reference ranges shows that they were addressed on small samples of blood donors (e.g., n=14; Krschek 2000) and were based on the theory of normal distribution (mean \pm 2SD) despite the normality of the PLT-RI values was not statistically defined. Whereas the reference range of RTH-II was based on a statistically significant number of healthy individuals (n=70; Tab.4-3-2-1). The normality of PLT-RI and the other RIs was inspected in triple by: a.) the formal test of normal distribution (KS: p<0.05; Tab.4-3-2-1), b.) histograms of RIs distribution (Fig.4-3-2-1 to 8) and c.) Q-Q Plot test (Fig.4-3-2-9 to 16). The PLT-RI values were mainly in line of normal assumption with slight skewness ranging within acceptable limits (<2 Std error; p.4-3-2). These tests revealed that PLT-RI and the other RIs are acceptably distributed in line of normal assumption, and could be therefore reliably expressed by using the 95% reference ranges based on normal theory defined as mean \pm 2SD, as described in Tab.4-3-2-2. Regarding PLCR-RI, a previous study defined a reference range by using its old formula which should be therefore critically revealed, as explained in p.5-4-1-7. Nevertheless, the use of the old formula would have delivered no meaningful reference range (mean= -6.4; SD=9.4; Fig.5-1-1-7) while the use of our corrected formula delivered a meaningful reference range for PLCR-RI (mean=20.6%; SD= 9.1; Tab.4-3-2-2.), as illustrated in Fig.4-3-2-2 and Fig.5-1-1-7.

Establishing reliable reference ranges, for the first time, for PLCR-RI and the new RIs and addressing their mutual correlation with PLT-RI was essential for: a.) understanding of the specificity and the accuracy of RTH for screening PLT function (p.5-2-9), b.) interpreting PLT-RI measurements and enlarging the clinical significance of RTH (p.5-4-4 and 5.), and c.) setting a protocol for the quality assurance of RTH measurements (p.5-4-6).

5-4-3-2. Impact of the modality of RTH-II measurements on the reliability of the assay

The use of different modes of measurements for the reference range of RTH-II demonstrated: (1) marked increase in the means and the variations of PLT-RI by practicing two a.F measurements instead of one a.F measurement (Tab.4-3-4), and (2) critical value for the significance of the increase of the means of 2.a.F subgroup compared with the 1a.F ones ($p=0.067$), as revealed by the pairwise comparisons. These findings could be attributed either to bioindividual variations between these subgroups and/or to systemic increase in the 2nd in-double a.F measurements compared with the 1st ones due to unacceptable formation of PLT particles relevant to aggressive re-mixing of the blood by vortex. The control by phase-contrast microscopy did not regularly but occasionally demonstrated such effect. These effects, however, were not strong enough to disturb the reliability of applying these 3 modes of measurements for establishing the reference range of PLT-RI since the differences between the means were not significant ($p>0.1$; Anova test) and the variances were statistically homogeneous ($p>0.1$; Levene test). Nevertheless, the undesirable occurrence of PLT particles by vortex of the a.F blood was mainly eliminated by the basic improvements of RTH-II (implicating SOP-3; p.5-1-2-3 and 4), but it is at least not completely preventable. Thus, we recommend: **a.)** Performing the a.F measurements in-double for assuring the quality of the measurements and the interpretation of the results, as described in p.5-4-6-1. Significant decrease of the 2nd a.F PLT count compared with the 1st one could be an indicator for unacceptable occurrence of PLT particles. **b.)** For expertise personnel, performing the a.F measurements one time instead of twice (unless the 1st measurement is suspected) may be justified to spare time for delivering the results. **c.)** A protocol for the quality assurance of RTH-II results is essential for preserving the reliability of RTH-II assay, as described in p.5-4-6.

5-4-3-3. Impact of using different automatic cell counters on the reliability of RTH-II measurements

None of the 3 different Sysmex cell counters revealed any sign of systemic or specific influence on the accuracy of RTH-II measurements. The differences between the means of PLT-RI or PLCR-RI in the counter's subgroups were not significant and the variances of RIs were homogeneous, as described in Tab.4-3-5. Nevertheless, the precision study demonstrated marked decreases in the variations of PLT-RI by the use of more modern cell counters for RTH measurements ($p<0.05$ for: series 2 vs. 1, series 3 vs. 1 and 2; Tab.4-2-4-2-1). Thus we may suggest that despite Sysmex counters deliver comparable accuracy for RTH-II assay performed on cWB, using modern counters may improve the precision of this assay, especially the use of Sysmex SF-3000.

5-4-4. Fundamental improvement in the clinical significance of RTH-II for screening PLT function

5-4-4-1. Enlarged and reliable specificity on PLT function

Our study (p.5-2-7 and p.5-2-9-A) proved that: **A.)** The retention of PLT (PLT-RI) measured under the standardized conditions of RTH-II demonstrates proportionally and reliably the response of PLT to activation by shear stress forcing the blood through the inter-connected micro pores of the RTH filter

system. In contrast, the serious errors in the accuracy and the precision of RTH (p.5-4-1) disrupt its reliability of measuring PLT-RI. **B.)** The response of PLT to activation is comparable to the physiologic reaction of circulating PLT at a disturbed endothelium and/or subendothelium. This response is demonstrated mainly by: (1) extensive adherence on the athrombogenic surface of RTH filter (2) extreme PLT spreading and shape changes, (3) aggregation/agglutination bridging the opposite surfaces of its micropores associated with extremely prolonged pseudopodia, and (4) mild PLT degranulation. Thus, RTH-II assay can assess directly multiple responses of PLT to activation by shear stress which enlarge its significance for screening PLT function. The specificity of RTH was related initially to PLT stickiness [Wenzel 2000] and later also to aggregation [Schenk 2002, Wienefoet 2003].

5-4-4-2. Reliability of the reference range for assessing the pathological variations

The unreliability and variability of the reference ranges of RTH disrupted its clinical significance in contrast to RTH-II (p.5-4-2). In addition, the majority of the reference ranges of PLT-RI were addressed by using cPRP [Krischek 2000, Schenk 2002]. Only one reference range was defined by using cWB [Krischek 2000]. But the mean value was too high (mean=70.2%) for evaluating PLT hyperactivity which was rather the main pathological concern for RTH assay [Wenzel 2000, Schenk 2001 and 2002, Wieding 2003]. The too small range of biovariability of this reference range (SD=2.1) throws doubts on the reliability of the measurements. Whilst the reference range of RTH-II addressed on cWB deliver acceptable biovariability with adequate ranges for assessing mainly hyper- and possibly hypo-PLT activation (mean= 25.2%, SD= 6.25; Tab.4-3-2-2). High PLT-RI values indicate increased PLT activation and high stickiness which could be interpreted as pathologic and might represent a thrombophilic risk proportional. Low PLT-RI values indicate poor PLT response to activation and could be a sign of a serious loss in PLT function and a risk of bleeding potency. Abnormal results, however, suggest performing further specific inspection of PLT function.

5-4-4-3. Nobel clinical significance on PLT size distribution and further quality assurance

The novel identification of new retention indices (RIs) for the PLT size distribution (PDW-RI and MPV-RI) and the correction of PLCR-RI formula added novel significance for RTH-II assay since none of the previous RTH procedures or any other PLT retention tests included these RIs. The reliability and the significance of these RIs in clinical practice were established by: a.) their reliable measurements (Tab.4-2-4-2-1, Fig.4-1-2-3-2-10 and 4-2-4-2-3 & 4; p.5-3-1), b.) well-defined reference range for each RI (Tab.4-3-2-2; p.5-4-3-1), and c.) the definition the mutual correlations of these RIs with PLT-RI within their reference ranges as well as within the in-series measurements of the precision (Fig.4-1-2-3-2-10 and Fig.4-2-4-2-7). Despite further investigations are required to address the promising clinical significance of the RIs of PLT size distribution, our study shows evidently that the incorporation of the determinations of these RIs in RTH-II assay enlarges its clinical significance for evaluating the heterogeneity of PLT function and enhances further the quality assurance of RTH-II measurements, as described as follows:

A.) Incorporation of PLCR-RI:

The measurements of PLCR-RI and their correlations with PLT-RI played a beneficial role in:

- a.) Defining and validating the errors of RTH measurements and setting the related modifications. This became evident in many investigations e.g.: (1) the PLT response to the reactivating reagent

(Fig.4-1-1-2-B-2), as explained in p.5-1-1-3; and (2) addressing to the PLT particles phenomenon leading to set the standard vortex (Fig.4-1-2-4-3-a1, a2, b, and c; p.5-1-2-3), (3) validating the corrections delivered by using the standard vortex (Fig.4-1-2-3-2-7; p.5-1-2-4-B, and p.5-1-2-5).

b.) Setting the key directions of the quality assurance of RTH-II measurements (p.5-4-6-1 and 2).

The reliability and the significance of PLCR-RI measurements and their correlations with PLT-RI were further enhanced by providing direct insights into the specific characteristics of large PLT function and their interaction with RTH filter which were demonstrated as follows:

- 1) The systemically and significantly lower levels of PLCR-RI versus PLT-RI revealed by the reference ranges of RTH (n=70: mean= 20.5% vs. 25.2%; Tab.4-3-2-3: Fig.4-3-2-1 and 2) as well as throughout the investigations of our study using cWB (as explained in p.5-1-1-7 and 5-1-2-5-B) confirm strongly that large PLT are less active than the normal sized PLT.
- 2) The decrease of PLCR-RI compared with PLT-RI (RI-Diff) implicate proportional mild shift of PLT size to the right after passing RTH filter. The negative mean of the reference range of MPV-RI (-1.97%; Tab.4-3-2-2) supports this conclusion. The significant increase of RI-Diff by the occurrence of PLT particles phenomenon confirm also the lower response of large PLT to inconvenient vortex of the blood after passing RTH filter compared with normal sized PLT, as explained in p.5-1-2-5-A).
- 3) The constantly strong positive correlation of PLCR-RI with PLT-RI in our divers investigations (using cWB, cPRP, EC, our PLT standard: $r>0.75$; $p>0.0005$) confirm that the activity of large PLT is, however, intimately proportional to that of PLT.

Addressing these phenomena or implications was not possible by using the old formula of PLCR-RI used in RTH (Fig.5-1-1-7 and Fig.5-1-2-5-C), as explained in p.5-1-1-7 and p.5-1-2-5-C. The systemic difference between PLT-RI and PLCR-RI values and the pattern of their strong correlation within their reference ranges as well as the in-series measurements of the precision play essential role in the quality assurance of RTH-II measurements, as described in p.5-1-2-4-B, p.5-1-2-5-A and p.5-4-6-1-d & 2. This enhances the reliability and consequently the significance of RTH-II for screening PLT function.

B.) Incorporation of PDW-RI and MPV-RI:

In concordance with PLCR-RI, the reference ranges of PDW-RI and MPV-RI (Tab.4-3-2-2) demonstrated negative means that reflect the mild shift of PLT size distribution to the right after passing the RTH filter, as already explained (p.5-4-4-3-A). In addition, the variations of the levels of these RIs and their patterns of correlation with PLT-RI could reflect the increase of this shift attributed to PLT hyperactivation by inconvenient vortex as well as the necessity of applying the standardized vortex method (Fig.4-1-2-3-2-10), as explained in p.5-1-2-6-B. The measurements of PDW-RI and MPV-RI are interpretable in regard of their reference ranges together with their patterns of mutual correlation with PLT-RI and PLCR-RI (Fig.4-3-3-3 to 6). Obtaining either of MPV-RI or PDW-RI value significantly apart from its reference range and/or normal pattern of correlation with PLT-RI or PLCR-RI is a sign of probable abnormal PLT function or the unacceptable occurrence of PLT particles phenomenon relevant to hyperactivation (p.5-1-2-7-B and C). The use of PLCR-RI seems to be more reliable for estimating the function of large PLT than MPV-RI since the later demonstrated insignificant correlation with PLT-RI in the healthy individuals in contrast to PLCR-RI ($r= 0.021$, $p>0.05$ vs. $r=0.689$, $p<0.0005$: Fig.4-3-3-2 and 5).

Nevertheless, the integration of PDW-RI and MPV-RI in RTH-II along with PLCR-RI may contribute to further enhancement of the reliability and the clinical significance of RTH-II.

5-4-5. Expanding the clinical significance of RTH-II to the other blood cells:

Incorporation of WBC-RI and RBC-RI

The reference ranges for WBC-RI and RBC-RI enhance the reliability of RTH-II for screening PLT function and extend its clinical significance further to the other blood cells. None of previous RTH procedures or any other PLT retention test identified RIs for leucocytes and/or erythrocytes. Obtaining WBC-RI and/or RBC-RI values that are significantly out of their normal ranges should be interpreted also in light of their correlation with PLT-RI (Fig.4-3-3-7 and 8) and the probable inadequate mixing of the blood after passing RTH filter or unacceptable occurrence of PLT particles phenomenon (p.5-4-6-1-b and c). In this respect, the determination of these RIs enhances the quality assurance of PLT-RI and RTH-II measurements and consequently the reliability of the test, as evidently revealed by the investigations of improving RTH (p.5-1-2-6-A). Otherwise, abnormal findings of WBC-RI and/or RBC-RI could be seen as indicators of pathologic disorders in leukocytes or erythrocytes respectively. Beyond it, several observations underline probable specific interactions of blood cells with RTH filter:

A.) The blood cells were able to pass through RTH filter despite they were bigger in size than the pores of RTH filter (16-22 μm). This was evident in the followings:

- a.) The different investigations of our study demonstrated considerable counts for WBC and RBC after passing RTH filter reflected by significant WBC-RI and RBC-RI respectively.
- b.) The reliable reference ranges of WBC-RI and RBC-RI.
- c.) The adherence of leucocytes and erythrocytes on the filter was less significant than platelets, as shown by electron microscopy (p.4-2-6-3-A).

B.) The electron microscopic examination demonstrated the activation of the blood cells passing RTH filter by shear stress. This was evident by the leukocyte degranulation (Fig.4-2-6-3-c-2) as well as the erythrocyte agglutinations and formations of coin-rolls (rouleaux Fig.4-2-6-3-b) which reflect the activation of these cells [Leone 2001, Lee 2004, Arese 2005, Falanga 2005, and Lang 2005]. Interestingly, these cells demonstrated direct adherence on the PU surfaces of the filter (Fig.4-2-6-3-b, and Fig.4-2-6-3-c-1 and 2).

C.) The inter-correlations of WBC-RI and RBC-RI with PLT-RI in cWB from healthy blood donors (Fig.4-3-3-7 and 8) as well as in the in-series precision demonstrated constant patterns (Fig.4-2-4-2-7; p.4-1-2-3-2-4 and 6).

Despite leucocytes and erythrocytes seem to be retained by RTH filter mainly “unspecifically” due to their sizes (Fig.5-2-7-7-B and p.5-2-8-A), these observations refer to an interesting probable specific interaction between these cells and RTH filter. Such specific effect should be further investigated especially for the leukocytes as they demonstrate positive RI level (mean= 4.25%; Fig.4-3-2-5). In addition, since the monocytes demonstrate contacts with erythrocytes compared to that found in vivo after activation (Fig.4-2-6-3-b) [Stamenkovic 1990], this phenomenon should be followed up. The “specific” interaction of blood cells with RTH filter implicates additional hypothetic uses of RTH-II assay

such as to exclude erythrocyte rigidity and to find out erythrocyte deformability respectively. At least the filter itself might be prepared in a way to measure RBC-RI sensitively. Such applications of RTH-II should be of interest for physicians working in the field of microcirculation. Additional investigations are required therefore to address further the clinical significance of these RIs.

5-4-6. Quality assurance protocol: Essential enhancement of RTH-II reliability and satisfaction

The availability of quality assurance protocols for the analyses in the clinical laboratory is mandatory for preserving the reliability of their measurements as well as enhancing the excellency and of the analyses with greater satisfaction especially in the routine clinical laboratory in respect to the heavy everyday run and the variable experience of the laboratory personnel [Ohman 1997, Carr 2004, Franchini and Veneri 2005]. None of the previous guidelines of RTH assay included a clear quality assurance protocol [Wenzel 2000, Wienefoet 2003]. Despite the basic improvements of the procedures of RTH addressed to RTH-II have eliminated the main sources of the variability and unreliability of this assay (p.5-4-1-1), the errors attributed to the delicate mixing of the a.F blood (after passing RTH filter) by vortex before performing the a.F measurements seem not to be completely preventable. The marked increases in the 2nd a.F measurements for the reference range of PLT-RI probably linked to the unacceptable occurrence of PLT particles (p.5-4-2-2) represent at least a critical example of such possible errors. A quality assurance protocol for RTH-II is therefore essential to enhance the satisfaction with its performance especially in the clinical practice in respect to the heavy everyday run and the variable experience of the laboratory personnel. Based on the relevant conclusions of the investigations of developing and validating RTH (p.5-1 to 3), we could address a comprehensive protocol for the quality assurance of RTH-II measurements considering 3 main axes:

5-4-6-1. Key directions for the quality assurance and interpretation of RTH-II measurements

- (1) The RIs for the 1st (RI-1) and the 2nd (RI-2) in-double measurements should be calculated and the means represent the final values of RIs. The reference ranges (>30 healthy individuals) of PLT-RI and the other RIs as well as the mutual correlation between PLT-RI and the other RIs should become available for reliable interpretation. Alternatively, the user may depend on the reference ranges obtained by our study (Tab.4-3-2-2, Fig.4-3-3-2 to 8; p.4-3).
- (2) Elevated level¹⁷ of RBC-RI, especially if associated with highly elevated level of PLT-RI and normal MPV-RI/PDW-RI values, could be a sign of inadequate mixing of the a.F blood (after passing RTH filter) i.e., before performing the 1st a.F measurements. The a.F blood should be re-mixed thoroughly and a second a.F measurement has to be performed. A significant decline of RBC-RI in 2nd measurement compared with the 1st one confirms the initial inadequate mixing especially if associated with significant decline of PLT-RI. In such case, the RTH-II test should be repeated.
- (3) PLT-RI values should be interpreted in view of their reference range and the correlation of PLT-RI with the other RIs. The interpretation of abnormal values should take into consideration the occurrence of possible errors (mainly the PLT particles) as well as the pathological variation. This

¹⁷ The term elevated or reduced RI level refers to the values that are remarkably above or under the upper or the lower discriminator of the reference range of the corresponding RI respectively.

is specifically important for elevated PLT-RI values, especially when associated with reduced WBC-RI and/or MPV-RI/PDW-RI values that could be attributed to the occurrence of PLT particles due to aggressive mixing of the a.F blood (p.5-1-2-6) which disrupts the reliability of RTH-II measurement.

- (4) Significant increase of the difference between PLT-RI and PLCR-RI (i.e., increase of RI-Diff) could be either pathologic or an indicator of possible “PLT particles” error, as explained in p.5-1-2-5-A.
- (5) Significant increase of the PLT-RI-1 values compared with PLT-RI-2 is an indicator for aggressive mixing of the blood before the 2nd a.F measurements leading to unacceptable formation of PLT particles related to PLT hyperactivation. Only the 1st measurement (PLT-RI-1) should be considered for calculating the final PLT-RI value in such a case (p.5-4-2). In the case that significant trend of systemic or frequent elevation of PLT-RI-1 levels compared with PLT-RI-2 is observed, the reliability of performing RTH-II has to be controlled by in-series measurements (p.5-4-6-2).

5-4-6-2. Controlling the precision of RTH-II: Significance of the correlations of PLT-RI levels and variations with the other RIs

The protocol for assessing the precision of RTH-II is based on the following observations:

1. As explained in p.5-1-2-4, 6A-2 and 6B, the unacceptably high variability and falsely elevated levels of the in-series and horizontal measurements of PLT-RI obtained by using RTH (in stages 1-3 of the improving protocol) were *negatively* correlated with the falsely decreased levels of the secondary RIs. These “*error patterns*” of correlations were attributed to PLT particles phenomenon and they were corrected substantially to *positive* correlations i.e. “*correct patterns*” by using RTH-II (stage 4) which was associated with acceptable variations and accuracy of PLT-RI and the other RIs, as demonstrated in Fig.4-1-2-3-2-1 to 6 and Fig.4-1-2-3-2-9 and 10.
2. PLCR-RI preserved positive correlations throughout the whole stages (1-4) of the improving protocol. The impact of the basic correction of the accuracy and the precision of PLCR-RI by using RTH-II compared with RTH was demonstrated by the significant shift of the PLT size distribution to the left, as explained in p.5-1-2-5 and shown in Fig.4-1-2-3-2-7.
3. The significant positive strong correlation between the in-series measurements of PLT-RI and the time intervals in RTH converted to insignificant weak negative correlation in RTH-II (Fig.4-1-2-3-2-2; as explained in p.5-1-2-1).
4. Despite the precision study (using RTH-II; p.5-3-1) demonstrated further significant reduction in the variability of PLT-RI and the other RIs, the values of PLT-RI preserved the same “*correct patterns*” of correlations – with both the other RIs and the time intervals – compared with stage 4 of the improving protocol, as demonstrated in Fig.4-2-4-2-7.

The patterns of the mutual correlations of PLT-RI with these RIs as well as the time intervals may therefore, and besides the levels of RIs, be used as useful indicators for controlling the precision and the accuracy of RTH-II, as described in the following table:

Table 5-4-6-2: Indicators of the reliability of the serial precision of RTH-II based on the variations of RIs besides the patterns of PLT-RI correlations (*r*) with time and RIs by using cWB.

		Time	PLT-RI	PLCR-RI	PDW-RI	MPV-RI	WBC-RI	RBC-RI	HGB-RI	HCT-RI
SD ^a :	Mean		2.9	4.6	3.7	1.7	2.1	1.0	1.0	1.0
	Upper limit		4.1	4.9	4.1	2.4	2.2	1.5	1.3	1.5
Correlation with PLT-RI " <i>r</i> " ^b	Correct ^c	-0.015	–	0.836	0.203	0.195	0.288	0.405	0.454	0.419

^a the means and the upper limits of the standard deviations (SD) of the retention indices (RIs) are based on the results of our study of the precision by using cWB (Tab.4-2-4-2-1). ^b the correlation coefficient (*r*: by spearman's rho test). ^c Correct patterns of correlations are based on the data of the in-series measurements associated with acceptable precision and high accuracy which were performed on: a) 3 series in stage 4 of the improving protocol (stage 4; p.5-1-2-4, 6-A-2 and 6-B) and b.) 3 series in the precision study (p.4-2-4-2). ^d the "error patterns" of correlations are based on the data of the measurements performed on 9 series from different healthy blood donors in stages 1-3 of the improving protocol using RTH procedures which were associated with unacceptably high variability and seriously false results (p.5-1-2-4, 6-A-2 and 6-B.). Note: the "*r*" values described in this table should be considered as approximate discriminators; the RTH-II measurements are more suspicious when the resulting "*r*" values are closer to the "error patterns" whereas they are supposed to be more reliable by pronouncing "*r*" values closer to the correct patterns.

A regular control of the in-series precision of PLT-RI by using blood from healthy individuals is necessary to assure the reliability of RTH-II. This is mandatory especially in the case of frequent incidences of the above mentioned potential errors in RTH-II measurements (p.5-4-6-1-b to e). Two main cases could be distinguished in the assessment of the in series precision:

- A.** If the variation (SD) of PLT-RI exceeds significantly its acceptable ranges (Tab.5-4-6-2), the reliability of performing RTH-II assay is under question. In such cases, the patterns of the correlations demonstrated by PLT-RI with the other RIs may give insights into the reason of the variation as follows: **(1)** Obtaining "negative patterns" of correlations similar to those attributed with the PLT particles phenomenon (Tab.5-4-6-2; Fig.4-1-2-3-2-1, 3, 5, 9 indicate inconvenient mixing of the blood after passing the filter and consequently unreliable performance of RTH-II by the responsible technician (also explained in p.5-1-2-4, 6-A-2 and 6-B). The performance of RTH-II should be then improved to deliver acceptable in-series variation and correct patterns of the RIs correlations with PLT-RI. **b.)** Obtaining correct patterns of RIs correlation excludes mainly the responsibility of the technician. The quality of RTH filter in that lot of the kit should be then inspected by quantity assessment, as described in the next section (p.5-4-6-3).
- B.** If the values of PLT-RI are within the reference range (Tab.4-3-2-2) and their variations (SD) are within the acceptable limits (Tab.5-4-6-2), the performance of RTH-II is reliable. The inter-correlation of PLT-RI with the other RIs are supposed to be in concordance with the "correct patterns" found in healthy individuals (Tab.5-4-6-2), otherwise the reliability of the performance is suspicious.

5-4-6-3. Quantity assessment of the reliability of RTH filter

The high precision of the weight of RTH filter (CV= 0.8-3.1%) and the weight retention index (wRI) (SD= 0.1-0.2) revealed by the precision study (Tab.3-2-4-2-1 and 2) besides their well-defined reference ranges with low variability by using cWB as well as EC enhance firmly the reliability of using these parameters as essential indicators for possible errors in the integrity of RTH filter and the or the reliability of RTH measurements under the centrifugation conditions of 110 g/ 5 min, as described p.5-2-10-2 and 3. The quantity assessments of wRI and the weight of RTH filter control mainly:

a. In-series precision: The variations of the in-series measurements (n=10) should not exceed significantly the acceptable limits addressed by our precision study (wRI: SD<0.2, RTH filter weight: CV<3.1; Tab.4-2-4-2-1 and 2). The in-series precision of the wRI is controlled preferably by using cWB from healthy individuals and possibly by using Eightcheck control (EC).

b. Accuracy of the measurements: The measurements should be within the upper and lower limits of the reference ranges (Tab.4-2-3-2 and Tab.5-2-8).

Disrupting the precision or the accuracy of these parameters for a given lot of RTH-II kit implicates unacceptable quality of the RTH filters in that lot. In the clinical practice, however, measuring these parameters is difficult. Controlling these parameters is actually mandatory only in the case of serious errors in RI measurements that are probably related to RTH filter reliability or integrity rather than to mal-performance by the laboratory personnel, as already explained in p.5.4-6-2.

Additional quality control of RTH-II assay includes the quantity assessment of the blood amount added to RTH tube since it enhances the reliability of RTH measurements, as explained in p.5-2-10-1. The values should be within the reference range (Tab.4-2-3-2 and Tab.5-2-8) and not significantly exceed the limits of the variations defined in our study (=3%; Tab.4-2-4-2-1 and 2).

5-4-7. Excellency and satisfaction in clinical practice and in POC systems

Compared with RTH, the excellency of RTH-II was substantially improved by the basic development in: **(1)** the reliability (accuracy and precision) of RTH-II, **(2)** the definition of unified SOP for RTH-II which represents itself a prerequisite for the widespread use of the assay. This was missed in the variable procedures in RTH, **(3)** the clinical significance supported by evidence based and expanded specificity as well as well defined reference ranges. **(4)** Setting a quality assurance protocol for RTH-II measurements for the first time. This not only enhances the reliability of RTH-II but also attain the satisfaction of its use in the clinical laboratory [Carr 2004, Franchini and Veneri 2005].

In addition, the development of RTH-II implicated additional basic improvements in the excellency of RTH-II (Tab.5-4-7) comprising of: **A.)** basic shortening in the turnaround time (TAT= 35-53 min vs. 5.5-6 min; p.5-4-7-1); **B.)** essential reduction of the performance costs (p.5-4-7-2); and **C.)** performing the assay significantly more simple and with greater convenience (p.5-4-7-3).

The essential improvements in the promptness, the costs, the convenience, and the simplicity of RTH-II improve essentially the cost effectiveness of this assay and address evidently the excellency of RTH-II in clinical practice as a simple and reliable assay for screening PLT function in short time. This is also of specific advantage for the application in the ICU (intensive care units) as well as at the POC (point of care) methods which are increasingly applied for further excellence in patient care [Despotis 1997, Harrison 2000, Calatzis 2003, Gilbert 2004, Kayser 2005, Shore 2005]

5-4-7-1. Basic shortening of the turnaround time

In association with our fundamental modifications on RTH (SOP-1, 2, 3; p.5-1) addressed to RTH-II, the turnaround time (TAT) of RTH (35-53 min: using cPRP; p.3-4-3-2) was basically shortened in RTH-II to 5.5-6 min, as shown in Tab.5-4-7. This essential reduction ranged from 84% up to 89%

according to the variable procedures used in RTH (Tab.5-4-7), as described as follows: **A.)** By using cWB as the tested material of choice for applying RTH-II in the clinical practice instead of cPRP for RTH, the time required for preparing and transferring cPRP (>16 min; p.5-1-1-1) was excluded from RTH-II. **B.)** The exclusion of the addition of the reactivating reagent saved the time required for preparing and applying it (>6 min; p.3-3-3 and p.5-1-1-3). **C.)** Avoiding the incubation step (p.4-1-1-5) saved 3-10 min. **D.)** Applying the b.F measurements (in-double) on the materials in the original collecting tube (p.5-1-1-4) allowed saving at least 1 min of the time of the assay by performing these measurements during the centrifugation of RTH tubes. **E.)** Standardizing the centrifugation time at 5 min instead of up to 16 min (p.5-1-1-2) shortened 11 min out of the TAT. **F.)** Standardizing the vortex steps (p.4-1-2-4 and p.5-1-2-2) reduced the requested time from 3 min to <3 sec (p.4-1-2-5). **G.)** The possibility of performing the a.F measurements for one time (RTH-II) instead of twice (RTH) dropped out the related time (1 min; p.5-4-3-2).

Table 5-4-7: Essential improvements in the excellency of RTH-II compared with RTH.

Procedures	RTH		RTH-II		Improved Excellency in RTH-II	
	Time ¹ (min)	Modifications ²	Time (min)	Saved Time (min)	Saved Costs/ Material	Greater Convenience and Ease
Prepare cPRP ³	16	Not used ⁴	0	16	15 min Centrifuge	+++
Add 450 µL cPRP or cWB in RTH tube ² "Adjust micropipette"	0.3	500 µL cWB	0	0.3	Adjustable pipette	+++ "Readily adjusted pi- pette"
Add 50 µL reactivating reagent "Preparation, Adjust pipette"	6	Non	0	6	Reagent, Pipette, Tips	+++
Incubation	3-10	Excluded	0	3-7		+++
b.F measurements "in-double"	1	In parallel with centrifugation	1	0		+++
Centrifugation of RTH tube	5-16	5 min	5	5-11	+++	+++
Vortex of cWB	3	Slight vortex	<3 sec	3	+++	+++
a.F measurements "in-double"	1	One time preferably	0.5-1	0-0.5	+++	+++
Turnaround time (sec)	>35-53		5.5-6	84-89%	+++	+++

¹ Time intervals differ according to the variable procedures used in RTH. ² Relevant modifications on SOP-1, SOP-2 and SOP-3 addressing the final SOP of RTH (p.5-4-1-2). ³ cPRP was typically used in RTH as the "tested material" of choice in the clinical practice, whilst cWB was used occasionally. ⁴ Not used for the routine application in the clinical practice (p.5-1-1-1).
+++ = significant improvement in the corresponding field.

5-4-7-2. Substantial reduction in the costs of performing the assay

This reduction resulted from using less equipment (only the standard disposable RTH tube is required with a convenient centrifuge) with no additives and less labor intensive which were attributed to:

(1) Using cWB instead of cPRP (5-1-1-1) avoids the costs of operating the centrifuge besides consuming the used materials (e.g., extra tips) and saving the required labor. (2) The exclusion of the addition of the reactivating reagent (p.5-1-1-3) saves its relatively expensive costs in addition to the costs of the tips and micropipette required for its preparation and application. (3) By standardizing the amount of the tested materials at 500 μ L and excluding any additives (p.5-1-1-3 and 4), only one readily adjusted micropipette is required instead of a more expensive adjustable one for adding 450 μ L of cWB and another micropipette for adding 50 μ L of the reactivating reagent. (4) Standardizing the centrifugation and the vortex steps reduced the operation costs of these instruments significantly since the time of their consumption was essentially shortened in RTH-II compared with RTH e.g., the centrifuge: 5 min vs. up to 16 min, vortex: 2-3sec. vs. 3 min (Tab.5-4-7). (5) The basic shortening of the TAT (up to 89%) reduces the labor costs relevantly.

5-4-7-3. Greater simplicity and convenient application

Due to the substantial improvement in the simplicity and convenience of RTH-II compared with RTH, the RTH-II assay became less labor and equipment intensive and could be performed with greater ease as described as follows: 1.) Using cWB instead of cPRP (p.5-1-1-1) avoids the procedures of preparing and transferring cPRP to the test tube and minimizes the amount of tested blood from >1.5 mL to 0.5 mL. 2.) By applying 500 μ L of the tested materials instead of 450 μ L due to standardizing their amount in RTH-II (p.5-1-1-4), a micropipette readily adjusted at 500 μ L could be used instead of adjusting an adjustable micropipette at 450 μ L. 3.) Excluding any additives and consequently the required preparation procedures e.g., the reactivating reagent (p.5-1-1-3). 4.) Performing the b.F measurements in-double on the original collecting tubes (SM) (p.5-1-1-3) is practically easier to practice than on RTH tubes in the heavy run of the clinical laboratory. This is attributed to the smaller size of RTH tube compared with the collection one (p.3-2-2 and p.3-3-1) as well as to the specific care required in order not to wound the RTH filter. 5.) Minimizing the time of the manual vortex of RTH tube from 3 min to 2-3sec (p.4-1-2-4) is essential for the convenience of the laboratory personnel. 6.) The Basic shortening (up to 89%) of the TAT of RTH-II assay down to 5.5-6 min (Tab.5-4-7).

5-4-8. Automation of RTH-II measurements and reporting: Online quality assurance and interpretation with greater satisfaction

The automated cell counters could be set for online reporting of RTH-II measurements including:

- A.) Calculation of RIs based on the means of the b.F and a.F measurements.
- B.) Demonstration of the correlation of PLT-RI with the other RIs on convenient scatters. Only the normal results (e.g., last 100 sample) are kept in these scatters as a "reference chart". The demonstration of the results (RIs) of a new RTH-II measurement on the reference charts allows direct and rapid online interpretation as well as quality assurance of these results.
- C.) In parallel with (B.), the RIs of the 1st and the 2nd in-double measurements which are critical for the quality assurance of RTH-II (p.5-4-6-1) are calculated and demonstrated on convenient charts.

The automation of RTH-II measurements allows: **(1)** Online reporting includes the numerical results of RIs and the demonstration on their corresponding reference charts. **(2)** Further satisfaction for the patients especially in ICU by shortening the time required for calculating and reporting the results. **(3)** Most importantly, online evaluation of the results that minimizes substantially the time required for the rapid interpretation and quality assurance and offers simultaneously greater ease and convenience. This is especially evident by delivering the reference charts along with the charts of the 1st and the 2nd measurements when compared with the inconvenient procedures for similar manual reporting. **(4)** Setting the cell counter for automated reporting of the in-series measurements of RIs along with the scatters of the mutual correlations between the variations of PLT-RI and the other RIs would make performing the precision control of RTH-II more convenient and allow therefore for further enhancement of the reliability of RTH-II. **(5)** The possibility of online display of the results (numerical and charts) on the screen of the automated cell counter enhance further the above mentioned advantages.

The adaptation of the automatic cell counters for RTH-II measurements promises of even further essential improvements in the quality assurance and the promptness of RTH-II and enhances further the satisfaction and the convenience that are necessary for its performance as a simple and reliable assay for the rapid evaluation of PLT function in the clinical practice and especially for the ICU and POC applications [Massari 1998, Moris 2001, Gilbert 2004, Shore 2005 and Franchini 2005].

5-4-9. Applicability in researches using different materials (cWB, cPRP, iPLT, EC, PLT Std):

Providing reliable, informative, and nobel insights into PLT function and characteristics

The measurements of RTH-II applied on the diverse materials used in our investigations for validating and characterizing the specificity, the accuracy and the precision of RTH-II showed evident compatibility with the multiple morphological controls as summarized as follows:

- A.)** PLT spreading applied on cWB and PLT isolated by gel-filtration (iPLT) (Tab.4-2-2-1 and 2 compared with Tab.4-2-2-3; p.5-2-2).
- B.)** Phase contrast microscopy applied on: (1) cWB in the morphological pilot study (Tab.4-1-2-4-3-a to c; p.5-1-2-3-B) and in the reference range (p.5-2-9-A), and (2) the fixed mediums of Eightcheck control (EC; p.5-2-8) as well as the standard of washed (Sw) and unwashed (Suw) PLT (Tab.4-2-5-5-1 and 2; p.5-2-3 and 6).
- C.)** Electron microscopy applied on cWB and EC (explained in: p.5-2-7 and 9).

This evident compatibility between the measurements of RTH-II and the multiple morphological controls proves the accuracy, the specificity and the precision of RTH-II for analyzing PLT function in the materials used in these studies. Further enhancement to the reliability of using RTH-II for evaluating PLT function in research was evident by its ability to:

1. Triggering and answering the questions addressing to the "PLT-particles" phenomenon: This was possible only by the reliable measurements of PLT-RI besides the interacted RIs (WBC-RI, RBC-RI etc.) and their compatibility with the associated PLT aggregates/agglutinates and PLT degradation investigated in diverse materials/phases (cWB, iPLT, EC, Sw, and Suw), as explained in p.5-1-2-7.

2. Exploring the variability of the effect of the reactivating reagent on PLT retention and putting out the question of identifying the cut-off point for further studies (p.5-1-2-4): Identifying this effect by using cPRP underlines the reliable applicability of RTH-II on cPRP.

3. Reflecting the state of tested platelets in different biological phases accurately and precisely: An evident example is demonstrating the low and invariable activity of the fixed PLT in EC compared with sustained PLT in cWB throughout the study of the in-series precision (Fig.4-2-4-2-1 to 6) as well as the reference range of RTH-II (Fig.5-2-9-A).

4. Providing novel insights into the interaction of blood components with PLT: The measurements of RTH-II in our investigations on iPLT and our PLT standards triggered the hypothesis of PLT predisposition for hyperactivation by the partial absence of other blood components. The significance of RTH-II assay exceeded, in this case, that of the morphological tests (PLT spreading and phase-contrast microscopy) which had just a confirmatory role, as explained in p.5-2-3.

5. Validation of the precision of the washed and unwashed PLT standard (prepared from fixed cPRP) by using RTH-II: This validation was reliable, informative and more beneficial for the development of these standards than the use of CBC (e.g., Fig.4-2-5-5-1 vs. 4; Tab.4-2-5-5-1).

The crucial role of RTH-II for triggering and answering the key questions addressing these effects in the different used materials (cWB, cPRP, iPLT, EC, Sw, and Suw) or putting out key questions for further investigations as well as the evidence based compatibility with the different morphological controls confirm together strongly the reliability of using RTH-II assay in researches for screening and evaluating PLT function specifically, accurately, and precisely in diverse biological phases.

VI CONCLUSION

The evaluation and the development of the variant procedures of RTH led to basic improvements on the reliability, the convenience, and the significance of the test implicating the definition of a new standardized assay on citrated whole blood (cWB) which was introduced as RTH-II. The stepwise investigations validating, improving and standardizing the methodology of the procedures of RTH resulted in extensive modifications and basic identifications that were set and incorporated in RTH-II (Tab.5-4-1):

(1) The cWB was defined as the tested material of choice in clinical practice instead of cPRP used mainly in RTH. This was essential to avoid the artifact variability attributed to PLT activation by the separation steps as well as to the variability attributed to the diverse interactions of the variant PLT gradients (after preparation by centrifugation) with their medium in the plasma. In addition, the whole blood reflects the physiological status more accurately than plasma and reduces the probable errors related to PLT hyperactivation (by e.g., aggressive mixing). **(2)** The variable amounts of the tested materials used in RTH were standardized at 500 μ L in RTH-II. **(3)** The addition of the “reactivating reagent” in RTH (Ca^{2+} with hirudin) was excluded since it was not justified. Also the resulted PLT reaction was found to be unstandardizable, convertible (from positive to negative) and vary inter-individually. **(4)** The incubation step (3-10 min for the tested materials in RTH tube) was excluded in RTH-II to avoid possible PLT activation by long contact with the filter and the consequent variability of PLT response related with the variant incubation periods and the inter-individual differences. **(5)** The b.F measurements (before the filter passage filter) were applied on the materials in the collection tube (RTH-II) instead of the RTH tube after the incubation step (RTH). The amount of the tested material passing RTH filter was thus standardized at 500 μ L by avoiding the variability related to the variant amounts of the samples aspirated by the cell counters (100-400 μ L). The blood amount passing the filter became subsequently sufficient for performing the a.F measurements (after the passage of the filter) “in-double” as well as for additional control tests. Putting the collection tube on a rotator assure the homogeneity of the tested material in contrast to the variant rates of blood sedimentations during the stand of RTH tube which leads to unacceptable variability in the measurements. **(6)** The extremely variable centrifugation conditions used in RTH-I (79-160 g/ 5-16 min) were standardized at 110 g/ 5 min which allows acceptable PLT distribution in the filter and delivers reliable measurements. Surprisingly, the centrifugation conditions were inadequately specified in some previous RTH studies. **(7)** The blood collected in the lower part of RTH tube (after centrifugation) was mixed by vortex (3 min in RTH) before performing the measurements. Standardized slight vortex (=3sec) was applied in RTH-II in order to avoid probable unacceptable PLT aggregations, agglutinations, and/or disruption that damage the reliability of the measurements. **(8)** The a.F measurements should be performed within 2 min after centrifugation to assure the accuracy (preserve the blood homogeneity and minimize the errors attributed to probable occurrence of “PLT particles phenomenon”). **(9)** The measurements should better be performed “in-double” to assure their reliability. The patterns of the variations between the 1st and the 2nd in-double measurements were also defined as rapid indicators for probable errors (induced by e.g., inadequate or inconvenient mixing). In-double or in-series measurements should be performed by using new tips for each pipetting instead of the same tip in order to avoid unstandardized variation (not specified in RTH

related studies). **(10)** The formula of calculating the large platelet retention index (PLCR-RI) was corrected by using the absolute values of large PLT instead of their ratio (%). The resulted reference range and its correlation with the other retention indices (RIs) provided significant meanings/conclusions in contrast to those obtained by using the original formula. Such errors of RTH put also serious questions on the reliability of related previous studies. **(11)** Nobel identification of additional retention indices (RIs) for leucocytes (WBC-RI), erythrocytes (RBC-RI) and for the PLT size parameters (platelet derived width "PDW-RI" and mean platelet volume "MPV-RI") was set for the first time in any retention test system. Defining these RIs (including PLCR-RI) besides their correlations with the PLT retention index (PLT-RI) was essential for: *a.)* defining the errors and the sources of variability of RTH, *b.)* setting and validating necessary modifications, *c.)* getting comprehensive interpretation for the accuracy, the precision and the specificity of RTH-II, and *d.)* setting for the first time a key protocol for the interpretation and the quality assurance of RTH-II measurements. **(12)** Nobel identification of the weight retention index (wRI: the weight of the blood retained in the filter) was set. This index was essential for standardizing and validating the centrifugation conditions as well as the quality assurance of the integrity and the reliability of RTH filter. **(13)** "PLT particles" phenomenon was identified as the main source of errors. PLT activated by shear stress passing through RTH filter are opposed to hyper-activation by inconvenient vortex (RTH) leading to degradable PLT aggregates/ agglutinates (PLT particles). These particles are misread by the cell counters as large PLT, erythrocytes and/or leucocytes (whenever the PLT particles are within their size discriminators) and are thus demonstrated by: *(a)* proportional false decreases in RBC-RI and WBC-RI, *(b)* a false shift to the left for the RIs of PLT size, and *(c)* relevant false increase in PLT-RI. Occasional PLT hyperactivation may occur before passing the filter if the blood is inconveniently handled leading to inverse patterns of false variations for the RIs. The final values of the RIs vary according to the total of the false variations before and after the passage of the filter. PLT hyperactivation leads also to rosette forms associated with PLT decrease. The identification of PLT particles phenomenon besides the new RIs was crucial for defining RTH errors and setting the related modifications. The correlations between PLT-RI and the other RIs (except PLC-RI) convert from defined positive patterns to negative ones by the occurrence of PLT particles after passing the filter. Despite RTH-II avoids the occurrence of this phenomenon, it is not completely preventable. Understanding the implications of this phenomenon is therefore mandatory for the right interpretation and quality assurance of RTH-II measurements. **(14)** The room temperature was defined as a standard condition for performing RTH-II. **(15)** The time of performing the assay was standardized at <120 min which preserves the reliability of the measurements. Exceeding this limit up to 180 min used in RTH may be associated with marked decrease in the values of PLT-RI.

The diverse approaches used in our investigations for validating and characterizing the accuracy, the precision and the specificity of RTH-II with the use of multiple morphological control (PLT spreading, phase-contrast as well as screening and transmission electron microscopy) proved the accuracy, confirm the acceptable precision of RTH-II and provided evidence based interpretation for the specificity of the assay. The accuracy of PLT-RI was for the first time studied in normal individuals. The extreme falsely elevated levels (mean=2.2 folds) and their increase by time (up to 61%) were substantially corrected. The unacceptably high in-series variation by RTH was reduced by at least 80% in RTH-II

which delivered precise values (SD: >14 vs. 2.9). The reliability of RTH-II measurements is dependent on precise and accurate PLT count and the biological variability, whilst the errors induced by additional sources of variability in RTH (e.g., PLT particles) increase the variability and false results unacceptably. Using more modern cell counters proved to improve the precision of the measurements remarkably but showed no impact on the accuracy. The day-by-day precision which was evaluated for periods longer than ever before (up to 10 days) using normal level Eightcheck control (EC) revealed also acceptable low variation for PLT-RI (SD<2.9).

Thus, in 2004 RTH-II could be established. For the first time, a reliable reference range for PLT-RI in cWB with acceptable biovariability (n= 70: mean= 25.2%, SD=6.25) was defined. The impact of different modes of measurements on the accuracy of the results was inspected. In parallel with PLT-RI, the precision and the accuracy of the other RIs were substantially corrected and reliable reference range for each retention index (RI) was for the first time defined. The accuracy of the RIs of PLT and the other blood cells was proved and characterized by phase contrast and electron microscopy. It was further proved that:

A.) RTH filter is rather athrombogenic in contrast to the other “filter systems” used in previous PLT retention assays (e.g., Hellem 1960).

B.) Under the standardized conditions of RTH-II using cWB, the retention values of the leukocytes (WBC-RI=4.2%) and erythrocytes (RBC-RI=-3.6%) are related to their sizes which are bigger than the pores of the filter. Whilst platelets which are smaller in size than the pores of the filter interact specifically with it demonstrating basically higher retention values than the blood cells. PLT-RI reflects directly the response of PLT to activation by shear stress during the passage of the filter. The PLT activation is demonstrated by shape change, extreme adherence and spreading on the filter with remarkable degranulation and excessive formation of wall-bound aggregates that bridge the opposite polyurethane surfaces of the interconnected micro pores, especially in the upper part of the filter. PLT may occasionally form free aggregates clumping within the lumina of the pores. The PLT response to activation is comparable to the physiological reaction of circulating PLT at a disturbed endothelium and/or subendothelium. The RTH-II assay may therefore reliably screen the multiple function of PLT in hemostasis and thrombosis. The upper and lower limits of the reference range of PLT-RI in cWB allow measuring PLT hyper- and hypo-activation which is in contrast to the previous study of Krschek [2000]. The clinical significance of RTH-II was further promoted by several observations e.g., the remarkable negative correlation of PLT-RI with PLT count suggests that the activity of PLT increases in association with the decrease of their count in healthy individuals.

C.) The specificity and reliability of RTH-II was further established by its ability to demonstrate accurately and precisely the inhibited function of PLT in the fixed medium of EC (PLT-RI= 2.6%; SD=2.1) compared with the biovariability of PLT activity in cWB and compatibly with the morphological control. Platelets in EC were not activated (spherical) during the passage of the filter and demonstrated no real adhesion on it but only occasional agglutinates with selective contacts to the filter. Whilst RTH studies delivered unexplainably high PLT-RI levels in fixed PLT control (>21%; Krschek 2000). The comparability between the retention values of the blood cells in cWB and that in the fixed medium of EC enhances linking the retention of these cells in cWB mainly to their size.

D.) The determinations of the RIs of PLT size parameters (especially for large PLT “PLCR-RI”) expand the significance of RTH-II assay to the PLT size distribution and show PLT functional heterogeneity. This was evident by e.g., demonstrating the lower activity of large PLT compared with normal sized PLT in normal individuals and their lower response to hyperactivation.

E.) The determinations of the RIs of leucocytes (WBC-RI) and erythrocytes (RBC-RI) could further extend the significance of RTH-II to the other blood cells which showed some specific signs of activation and interaction with the filter. This may implicate additional hypothetical uses of RTH-II assay such as to find out erythrocyte rigidity and deformability.

The reliability of RTH-II in researches was established by its applicability on the different materials (cWB, cPRP, gel-filtered PLT, EC, and washed and unwashed prefixed cPRP) used in our investigations for validating the reliability and specificity of RTH-II with evident compatibility with the multiple morphological control. For example, the RTH-II assay was able to demonstrate the influence of the partial absence of the normal medium of platelets on their predisposition to hyperactivation and stickiness (e.g., forming aggregates and agglutinates) which was basically much less compensated in fixed medium than in cWB. This was proved by the ca. 2 folds increase of PLT-RI levels in gel-filtered platelets compared with cWB (57.4% vs. 30.5%) and the basically higher increase of PLT-RI in the washed prefixed PLT standards up to 5 folds compared with the unwashed ones (5.7% vs. 33.5%). The RTH-II played essential role in identifying and characterizing different new phenomena of PLT function (e.g., PLT particles, PLT response to Ca^{2+} with hirudin) besides providing specific beneficial insights for the development of our standards for washed and unwashed prefixed PLT.

The development of the reliability of RTH-II compared with RTH was further associated with substantial improvement in the excellency and the convenience of the test. No preparation for plasma or any reagent is required and no additives are used in RTH-II. The blood amount required for the test was reduced from >1.5 mL to 0.5 mL. The turnaround time was shortened extensively up to 89% (35-53 min vs. 5.5-6 min). The assay became significantly simpler and less labor intensive. It is performed with greater ease, less equipment (only the standard disposable RTH tube is required with a convenient centrifuge) and higher cost effectiveness (Tab.5-4-7). In addition, setting a quality assurance protocol for RTH-II assay for the first time not only enhances the reliability of RTH-II measurements and interpretation but also attains the satisfaction of its use in the clinical laboratory. Our suggestion to automate RTH-II by using conventional electronic cell counter allows on-line reporting, interpretation and quality assurance which enhance further the convenience and cost effectiveness as well as the reliability and quality assurance of RTH-II measurements with greater satisfaction.

Despite previous “adhesion and retention” tests proved to be meaningful, they could not be established in clinical practice because they are, in contrast to RTH-II, poorly standardized and require experienced personnel and additional equipments (e.g., Høllm-II 1970, O’Brien 1987). Nevertheless, the perspective for setting further improvement in the reliability of RTH-II assay could be seen in view of several observations in our study: e.g.: a.) the negative correlation of the weight of RTH filter with the weight retention index (wRI) and with the PLT-RI, b.) the occasional disruption of PLT after the passage of the RTH filter, and c.) the delicacy of mixing the activated PLT in blood after the passage of the filter that

represents the main drawback of RTH-II application in clinical practice since it requires experienced technicians. This might be overcome e.g., by developing more standardized filter system.

In regard of the absence of simple and standardized assays for screening PLT function reliably [Breddine 2003, Rand 2003, Tsiara 2003, Haubelt 2005, Levi 2005, Tan 2005, and Reiningger 2006], the competency of RTH-II assay is evident as a simple and rapid assay for screening PLT function reliably and cost effectively. The essential advantages of RTH-II assay urge for further serious investigations addressing its clinical significance including mainly the response of PLT-RI to pathological variations and the associated implications on PLT size distribution. The significance of the RIs of leucocytes and erythrocytes in the interpretation and the quality assurance of RTH-II measurements is established, but their clinical significance requires further basic researches. The RTH-II assay can be useful test in researches of PLT function as well as in clinical practice as an ideal adjunctive to refine the tools for the assessment of PLT response and activity in physiology, pathophysiology, and drug monitoring. Several studies on RTH referred to some beneficial uses for screening PLT function. But the unreliability and variability of RTH assay disrupt the reproducibility of data. The recent introduction of RTH-II by our institute as a standardized, reliable and simple assay implicating reliable reference range and evidence-based specificity delivers the right platform for further clinical studies. As a first example for this is the recent study of Klinkhardt [2006] on using RTH-II for monitoring "antiplatelet" therapy. The study concluded that RTH-II may be "a simple and easy for monitoring effects on P2Y(12)-inhibitors on platelet degranulation". The reliability and the convenience of RTH-II promise major improvement in the medical care delivered for the satisfaction of the patients in the clinical practice as well as in the point of care systems.

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IX CURRICULUM VITAE

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School: (always listed on principals honor roll)

09/1972-06/1978 Basic and elementary stages in the French School, Damascus, Syria
 09/1979-06/1981 Preparatory stage school in Iben-Alatheer School, Damascus, Syria
 09/1982-06/1985 Secondary stage concluded by Baccalaureate in general sciences, Iben-Khaldoon Secondary School, Damascus, Syria

High Education:

09/1985- 09/1990 Bachelor degree (B.Sc.) in pharmacy and medicinal chemistry, Faculty of Pharmacy, Damascus University, Syria (*ranking the top of my class*; 450 students).
 09/1993- 09/1995 Diploma degree (2 years) in clinical biochemistry and laboratory diagnosis, Faculty of Pharmacy, Damascus University, Syria
 10/1995- 04/1996 Intensive English course in the Center of the languages, the High Education Board, Damascus University, as pre-requisites obligatory for the proceedings of the Master degree.
 08/1996- 04/1998 Master of Science (M.Sc.) in clinical biochemistry and laboratory diagnosis, Faculty of Pharmacy – Damascus University, Syria, in cooperation with Würzburg University, Germany (excellent degree). As *special honor for my work*, the University determined, for the first time in its history, to publish the thesis on its expense and distribute it to the Faculties of Medicine and Pharmacy in the Middle East. Also, I got for my master degree a full recognition by the German academic exchange service “DAAD” (Referat: 425, PKZ: A/99/03292) and was *awarded a scholarship to get my Ph.D. in Germany*.

Experience:

- Up 04/2002: Lecturer in the seminars and symposiums held in the Institute of Clinical Hemostaseology and Transfusion Medicine, University of Saarland, Germany.
- Up 04/2001: Resident and lecturer in the laboratories of the Institute of Clinical Hemostaseology and Transfusion Medicine, University Clinics of Saarland, Germany.
- 1999-2000: Foundation and direction of my pharmacy in *center Damascus*; the capital of Syria.
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- 1995-2000: Lecturer in the seminars and symposiums held in the Dept. of Clinical Biochemistry – Faculty of Pharmacy, Al-Assad and Congenital University Hospitals, and Tishreen Central Military Hospital, and the Association of Clinical Biochemistry in Damascus, Syria.
- 1994-2000: Residency (part-time) in the Laboratories of Al-Assad University Hospital, Damascus University, Syria.
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- 1991-1993: Military service. Supervisor in the Pharmacy (1 year) and then in the Clinical Laboratory (1 year) of Yousef Al-Azmeh Military Hospital, Damascus, Syria.
- 1990-1991: Supervisor and seller in Al-Masri pharmacy, Damascus, Syria.
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- 07/1998: One-week intensive course on PCR techniques in the Laboratory of Molecular Genetics, University Clinics of Würzburg, Germany.
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- 07/1996: Supervisor on 3week voluntary training course for the clinical and the first aid management, Faculty of Pharmacy, Damascus University, Syria.
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Languages

Excellent command on Arabic (native language), fluent in English, good in German, understand French fairly.

X GLOSSARY

Ab = abandon number.

a.C = the measurements performed on the blood in Eppendorf tubes (p.3-2-2) after centrifugation.

acc= according to.

a.F = after centrifugation and passing RTH filter.

a.F measurements = the measurements performed on the tested materials (e.g., blood) in RTH tubes after passing RTH filter i.e., after centrifugation.

Aggr = platelets aggregates or aggregation.

Aggl = platelets agglutinates or agglutination.

ATU= Anti-Thrombin Unit.

BD = blood donor (p.3-1).

b.F = before centrifugation and passing RTH filter.

b.F measurements = the measurements performed on the tested materials (e.g., blood) before passing RTH filter i.e., before centrifugation.

CBC = the complete blood cell counting (p.3-2-4).

CH = chapter i.e., the number of t chapter.

CPRP = citrated platelet rich plasma (p.3-4-2).

cWB= citrated whole blood (p.3-4-1).

D.W. = distilled water (p.3-3-4).

EC = the normal level of Eightcheck control (p.3-3-2).

e.g., = for example.

Epp = Eppendorf tube (p.3-2-2).

Fbr = fibrinogen.

Fig. = figure.

HGB = hemoglobine or hemoglobine concentration (g/dL).

HGB-RI = the retention index of HGB (%).

HCT = hematocrete or hematocrete rate (%).

HCT-RI = the retention index of HCT (%).

h.V = relatively high vortex mode (rate).

ICU = intensive care unit.

i.e., = that means.

iPLT = isolated PLT (platelets isolated by gel-filtration using Sepharose gel; p.4-2-2-1).

KS = Kolmogorov-Smirnov test (p.3-6).

MPV= Mean platelet volume (fL).

MPV-RI = the retention index of MPV (%).

m.V = relatively moderate vortex mode (rate).

N or n = number of a certain group e.g., the number of the individuals recruited in the reference range.

NA = not available e.g., the cell counter was not able to determine the value of this measurement.

ND = not determined by the cell counter.

p = the probability value (statistics; p. 3-7).

p. = refer to the respective paragraph/section or chapter. e.g., p.4-2-3-1 refers to the first section of chapter 4-2-3.

PBS = phosphate buffer saline (p.4-2-5-1).

PDW= platelet distribution width value (fL).

PDW-RI = the retention index of PDW (%).

PLCR = the ratio of platelet large cell (%).

PLCR-RI = the retention index of PLCR.

PLT = platelet or platelet count ($\times 10^3/\mu\text{L}$).

PLT-RI = the retention index of PLT.

POC = point of care systems or methods.

RBC = red blood cell or red blood cell count ($\times 10^6/\mu\text{L}$).

RBC-RI = the retention index of RBC.

RI= = the retention index of a specific parameter.

RI= the retention indices of RTH assay.

RTH measurements= the b.F and a.F measurements.

RTH tube = the standardized Eppendorf retention tube for performing RTH assay (p.3-2-1).

sec = second.

SEM= scanning electron microscopy.

SM = Sarstedt monovettes (used in blood collection; p.3-4-1).

SOP = standard operation procedures.

SOP-1, SOP-2, SOP-3 = the 1st (p.3-4-3-2), the 2nd (p.4-1-1-7) and the 3rd SOP (p.4-1-2-5) of RTH assay.

Std = standard.

s.V = relatively slight vortex mode (rate).

Tab. = table.

TEM= transmission electron microscopy.

V = mixing the blood by vortex.

vs. = versus.

WB = whole blood.

WBC = white blood cell or white blood cell count
($\times 10^3/\mu\text{L}$).

WBC-RI = the retention index of WBC.

wRI = the weight retention index of RTH (the percentage
“%” of the weight of the blood trapped in RTH filter;
p.4-2-3-1-1-B).

>h.V = applying further high vortex.

& = and.

