CLOT-ED

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Editorial

After two years of producing The Clotting Times we at the ECAT have now chosen a brand new, fresh layout. In the content you can see that every rubric from now on has its own colour. In this way you can easy recognize a particular item in the pages which follow.

Every issue will start with a "focus article" which in this issue gives you an update on pre-analytical issues in platelet function testing. This article seeks to heighten awareness that lack of attention to pre-analytical conditions can significantly impact results from platelet function testing.

The next section is "ECAT information". Based on results from the ECAT surveys, the effect of Rivaroxaban on haemostasis assays is described in this issue.

In the section entitled "Literature Review" we would like to present highlights from recent publications. The current issue gives a synopsis of the publication, *Lupus anticoagulant testing: analyzing fresh samples after a single centrifugation and after a 6-8 hr delay* (P. Froom and M. Barak. Clin Chem Lab Med 2012; 50: 367 – 370).

The editorial board hopes that you will appreciate our new design and enjoy reading this and future issues.

Yours sincerely, Petra ter Hark

Content

News

ECAT accredited.

In 25 April 2012 the External Quality Assessment (EQA) programme of the ECAT Foundation received its accreditation according to ISO Guide 17043. ECAT is accredited by the Dutch Accreditation Council (RvA), which is by law appointed as the national accreditation body for The Netherlands. The accreditation was based on an assessment according to the requirements of ISO/IEC Guide 17043:2010. This ISO Guide, entitled: Conformity assessment – general requirements for proficiency testing, is the international standard for accreditation of EQA and proficiency-testing (PT) programmes.

Accreditation means that ECAT has been demonstrated to be able to organize EQA surveys in a competent manner. For further details see our website.

PROF. TESTING
RvA R 018

Questionnaire on ECAT programme

The ECAT is very much interested in your opinion about any further extension to the ECAT EQA programme. We would therefore greatly appreciate if you would complete the online questionnaire on this topic which can be found in the member section of the ECAT website. Completion of this questionnaire takes only 5 minutes. Please complete the questionnaire before **30 June 2012**.

ECAT symposium 2012

On **8 and 9 November 2012** our **8th ECAT symposium** will be held in Leiden, The Netherlands.

As usual the major focus of the programme of this symposium will be on diagnostic issues and the quality of laboratory test performance in relation to the medical need. The programme will include sessions on pre/analytical variables, reference values, haemophilia, anti-coagulation, lupus anticoagulant, POCT and "prolonged-APTT". Also the interactive discussion of clinical cases will be included.

In conjunction with this symposium a course on the interpretation of EQA results as well as a course workshop on "prolonged-APTT" and inhibitor assessment will be organised on **7 November 2012**. Further details will be available soon on the ECAT website.

Focus Article:

Pre-Analytical Issues in Platelet Function Testing - An Update

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This article seeks to raise awareness of the numerous pre-analytical (also referred to as pre-examination) variables affecting platelet function testing by critically examining the impact that variability and inattentiveness to it can have on test results. Variability can be introduced in two ways: 1) endogenous biological issues as they relate to the patient and 2) exogenous variability resulting from specimen collection & transport and sample process & handling. The latter will be the focus of this updated presentation.

Various technologies are available for evaluating platelet function. Table 1 lists those most commonly used by a clinical laboratory and denotes their specimen/sample requirements. For all methods, the specimen (tube obtained directly from the patient) is whole blood. However, the sample (product used for actual testing) differs between methods.

Terms / Abbreviations Used	
Abbreviation	Name
CT	Closure Time
IA	Impedance Aggregometry
LTA	Light Transmittance Aggregometry
OTC	Over-The-Counter
PPP	Platelet Poor Plasma
PRP	Platelet Rich Plasma
PRP AGG	Platelet Rich Plasma Aggregation
RIPA	Ristocetin Induced Platelet Aggregation
WB	Whole Blood
WBA	Whole blood Aggregation
Platelet Agonists	
Adenosine Diphosphate (ADP)	
Arachidonic Acid (AA)	
Collagen (COL)	
Epinephrine (EPI)	
Ristocetin (RISTO) [High & Low doses]	
Thrombin (α-Thrombin)	
Thrombin Receptor Agonist Peptide (TRAP)	
Thromboxane Analogue U46619	

Table 1. Platelet Function Testing

Methods	Specimen	Sample
Low Shear Systems		
• Light Transmittance Aggregometry (LTA)	WB	PRP
• Impedance Aggregometry (IA)		
– PRP AGG	WB	PRP
– WBA	WB	WB
High Shear System		
• PFA 100®	WB	WB

Platelet function testing is used for the diagnostic evaluation of bleeding disorders, whether due to congenital or acquired disorders of platelet function. Physicians must compile a careful clinical and family bleeding history as well as medication history prior to a laboratory evaluation for a platelet function defect. Though platelet function testing can determine the efficacy of anti-platelet agents, the use of light transmittance aggregometry (LTA) is discouraged. Should a pharmacological agent or agents require laboratory monitoring, physicians must have a clear understanding of what inhibitory endpoints are acceptable for any given low- or high-shear system.

For the evaluation of a bleeding disorder, a patient must be drug-free prior to undergoing phlebotomy. The list of substances that can adversely affect platelet function is long [1,2,3]. Drugs frequently encountered by the coagulation laboratory are listed in Table 2. Other agents include: 1) prescription drugs and over-the-counter drugs in which many the presence of aspirin is ubiquitous, 2) therapeutic inhibitors of platelet function, 3) antimicrobials, 4) certain chemotherapeutic agents, 5) psychotropic drugs, and 6) food and herb supplements. A complete turn-over of platelets occurs every 7-10 days (based on platelet life-span), therefore patients should abstain from any products affecting platelet function for at least 10-14 days[4,5].

A patient's platelet count should be considered prior to testing. Sufficient platelets (as recommended by the aggregometer manufacturer) must be present in order to yield a functional response that falls within the threshold limitations of the instrument. Therefore severely thrombocytopenic specimens cannot be used (see section entitled "Sample Preparation", below).

Table 2. Agents Affecting
Anticoagulants
Heparin
Warfarin (Coumadin)
Direct Thrombin Inhibitors
Cardiovascular Agents
β–Adrenergic Blockers (Propranolol)
Vasodilators (Nitropursside, Nitroglycerin)
Diuretics (Furosemide)
Calcium Channel Blockers
COX-1 Inhibitors
Aspirin and all proprietary or OTC preparations
COX-1 & COX-2 Inhibitors
Ibuprofen (Motrin)
Indomethacin (Indocin), Naproxen (Naprosyn, Aleve)
Mefenamic Acid (Furadantin)
COX-2 Inhibitors (Coxibs)
Celecoxib (Celebrex)
Inhibitors of Platelet Receptors
Abciximab (ReoPro) [αIIbβ3]
Clopidogrel (Plavix) [P2Y12]
Phosphodiesterase Inhibitors
Dipyridamole (Persantine)
Cilostazole (Pletal)
RGD Peptomimetics
Eptifibatide (Integrelin)
Tirofiban (Aggrastat)

Specimen Collection

It is important that the venipuncture is non-traumatic and blood should flow uninterruptedly through the needle. Specimen collection should follow general guidelines presented in CLSI document H3-A6 [6]. Specifics as they relate to platelet function testing are addressed in the next sections.

Needle Gauge

A needle with a diameter greater than 1 mm (19 gauge) may be traumatic for a vein and adversely affect hemostasis. Needles with diameters under 0.7 mm (22 gauge or higher) prolong blood collection and increase the pressure gradient in the needle, which could lead to hemolysis and platelet activation [7]. However, Carcao noted that blood specimens obtained from children using 23 versus 21 gauge needle sizes showed no significant differences in PFA-100 closure times [8]. The use of butterfly cannulae systems (for children or adults with difficult veins) has been discouraged because they potentially reduce blood flow and by that increase the risk of platelet activation [7]. Mani and colleagues demonstrated that differences could not be shown with either PRP AGG responses or PFA-100 CT when samples were collected in the same subjects using 21 gauge ordinary needle systems or 21 gauge butterfly cannulae systems [9].

Stasis due to a cuff or tourniquet should be minimized; nonetheless, studies have shown no differences in PRP AGG responses to ADP or EPI when stasis was produced by a cuff at 60 mm Hg pressure for 10 minutes [10]. For routine blood collection, a tourniquet is released as soon as blood begins to flow [6,7].

Evacuated Tubes versus Syringes

Though concerns have been raised that the use of evacuated tubes may lead to platelet activation, comparison studies between their use and that of syringes showed minimal differences in aggregation studies [10,11]. Likewise, little difference was noted when using either a one- or two-syringe technique for blood collection [12]. Since syringe draws are sensitive to operator technique and proficiency, platelets can be exposed to variable shear forces during the phlebotomy [13]. Should a syringe be used for blood collection and it does not contain citrate anticoagulant, then the specimen will need to be transferred to either a polypropylene or siliconized coated glass tube. Proper transfer requires that 1) the needle be removed from the syringe, 2) blood added gently down the side wall of the opened tube, and 3) the ratio of blood to anticoagulant be adhered to strictly [12].

Anticoagulants

Trisodium citrate dihydrate is the sodium salt of citric acid with the chemical formula of $Na_3C_6H_5O_7$. The recommended concentration for platelet function testing is 105–109 mmol/L (3.2%) [1,5]. Sodium citrate chelates calcium ions, hence its anticoagulant effect. However, some unbound calcium must be present in WB and PRP in order for aggregation to occur. Therefore higher trisodium citrate concentrations, such as 3.8%, will bind more calcium ions than the 3.2% concentration [10]. Han and Ardlie demonstrated that ADP responses were blunted when using 3.8% versus 3.2% citrate [14]. On the other hand, von Pape and colleagues, when using the PFA-100, which is a high-shear system, demonstrated that 3.8% versus 3.2% buffered sodium citrate

showed greater sensitivity (at 1 hour post collection) in discerning the presence of aspirin [15]. Commercially available collection tubes contain either buffered or non-buffered sodium citrate. Buffering with citric acid allows for blood and/or plasma to maintain a pH that is in the physiological range. Heilmann demonstrated that buffered versus non-buffered sodium citrate (either 3.2% or 3.8%) specimens resulted in less flow obstructions (caused by micro-thrombi) when using the PFA-100 [16].

The anticoagulant to blood ratio must be 1 part trisodium citrate to 9 parts blood. Chelation of calcium ions will be impacted if this nominal ratio is not used [17]. If specimen tubes are under-filled, then more anticoagulant is available for calcium chelation thereby reducing or eliminating the availability of unbound calcium for *in vitro* platelet function. The net effect is the blunting (reduction) of aggregation responses with EPI and ADP agonists [18]. On the other hand, fewer calcium ions are chelated if evacuated tubes are over-filled. Over-filling of evacuated tubes results from practices (such as opening a tube and filling it with a syringe previously used for blood drawing) that do not adhere to manufacturers' specifications [7,17].

PPACK (D - phenylalanine - proline - arginine chloromethyl ketone) is an inhibitor of "-thrombin. PPACK anticoagulant is used in pharmacological studies that determine the efficacy of glycoprotein IIb/IIIa (GPIIb/IIIa or αIIbβ3) inhibitors. Clinical trials using eptifibatide showed that sodium citrate removed calcium ions from GPIIb/IIIa and falsely enhanced the inhibitory activity of the drug. Specimens collected in PPACK did not show this aberration [19].

Anticoagulants to avoid when testing for platelet function include:

- Etheylenediaminetetraacetic acid (EDTA) because it removes ten times more calcium from blood than citrate solutions thereby leaving no unbound calcium ions available for *in vitro* platelet function [10,18].
- Heparin exerts its anticoagulant effect via accelerated inhibition of thrombin by antithrombin thus ongoing thrombin, generated in the test system, is inhibited. Though aggregation responses to ADP are greater with heparin versus sodium citrate, responses to COL and secondary responses to EPI are blunted [20]. Spontaneous platelet aggregation may occur in heparinized plasma [10].
- Acid-Citrate-Dextrose (ACD) acidifies the pH of PRP to 6.5. Platelets do not aggregate below a pH of 6.4 [10,12].

Specimen Hematocrits (HCT)

Hardisty noted that individuals with higher hematocrit values required higher concentrations of an agonist to elicit a response [21]. Tubes from individuals with a high HCT have excess citrate present in the plasma compartment, an outcome similar to that observed with over-filled specimen tubes. Hence the recommendation that the amount of citrate in a collection tube be adjusted to compensate for patient HCT values greater or lesser than 0.45 L/L (45%) [4,5,10,12]. Without adjustment, the quantity of free calcium may not be optimal to achieve maximal aggregation responses. CLSI document H21-A5 provides a nomogram for determining volumes of anticoagulant and blood that can be used for various HCT values [17].

For high-shear systems such as the PFA-100, specimens with HCT values below 0.20 L/L (20%) cannot be used because an occluding platelet plug will not form [22]. With increasing HCT values, CT decreases, however, values above 0.50 L/L (50%) may yield erratic CT measurements [22]. Abbate noted no effect of HCT on WBA [23]. In contrast Mackie noted significant differences in COL-induced WBA with hematocrits ranging from 0.10 to 0.60 L/L (10%-60%). However, a concern is that "hematocrit" adjustments were made by the *in vitro* manipulation of WB with saline rather than using native blood with various hematocrits [24].

Specimen Handling

Specimen Transport

The manner in which a whole blood specimen is transported from the patient to the testing site can significantly affect a specimen's integrity [13,17,25]. Issues to consider are:

- Mode of transportation
  - Pneumatic tube systems are to be avoided [26,27]
  - Carrying by hand is preferred
  - Vehicular transport may be unavoidable due to the physical location of the referring laboratory
- Duration of transport (must allow sufficient time subsequent to transport for test performance)
- Position of tubes (standing upright is preferable to laying on side) [28]
- No traumatic handling such as vibration, shaking, or agitation (all can lead to hemolysis and subsequent platelet activation) [28]
- Temperature [4,11,12,29,30]
  - Maintain at room temperature
  - No exposure to severe cold such as refrigeration, cool/ice packs, or winter temperatures
  - No exposure to heat (summer temperatures)

Age of Specimen

Specimen age is the timeframe in which the whole blood remains stable. The overall time is dictated by the time re-

quired for processing and testing. If these steps require a few minutes, then storage time can be "longer". In contrast, processing/testing that requires one hour or more will reduce the time that a specimen can be transported/ stored. Sufficient time must be allotted in the "stability window" to perform the assay. Studies performed with the PFA-100 indicate that specimen storage time is between 4 to 6 hours [11,29,30] From their observations, Sweeney and colleagues stated that a whole blood specimen collected with an evacuated tube, maintained at room temperature, and tested by WBA was stable for 3 hours [31].

Sample Preparation

For the PFA-100, specimen and sample are synonymous. Whole blood specimens with platelet counts below 50 x 109/L (50,000/µL) cannot be used as the PFA-100 closure times become abnormally prolonged [32]. Very elevated platelet counts may adversely affect the CT (cause immediate closure).

For IA using whole blood, specimen is synonymous with sample. Generally, if the WB platelet count falls below 50 x 109/L (50,000/µL), then the specimen should not be used for testing. Platelet counts must be greater than 100 x 109/L (100,000/µL) in order to use the agonist ADP [33,34].

Platelet-rich plasma and platelet-poor plasma are required for PRP AGG studies performed by either LTA or IA. PRP and PPP can be obtained by room temperature centrifugation of a whole blood specimen. The goal is to obtain PRP in which only platelets are retained and from which red and white blood cells have been removed. Centrifugation speeds are noted in relative centrifugal force (rcf), also known as g-force. Rcf is calculated by knowing the rotating radius of a centrifuge and its rotational speed (revolutions per minute [rpm]). A rcf nomogram is provided in CLSI document H18-A4.25 In order to minimize remixing of plasma and red cells, a swing-out bucket (angle) rotor should be used and the brake not applied at the end of centrifugation. Representative centrifugation speeds and times noted in the literature for the preparation of PRP are as follows: 100g for 10 minutes [34], 135g for 15 minutes [12], 150g for 30 minutes [35], 150 - 200g for 10 - 15 minutes [4], 180g for 10 minutes [36,37], 180g for 15 minutes [38,39] and 250g for 10 minutes [40].

The optical density of PRP is directly proportional to the concentration of platelets. As the number of platelets increase in a sample, the opportunities for platelet collisions in the test cuvette increase, which result in 1) an increasing rate of aggregation and 2) a relatively greater change in optical density [10]. It is for this reason that "standardization" of the PRP platelet count by PPP has been performed. On the other hand, lower limits for PRP platelet counts are determined by the linearity of an aggregometer (for example, a differential of 50,000 or more platelets should exist between PRP and PPP). This lower limit value may be defined either by an aggregometer manufacturer or by respective laboratories performing their own in-house linearity studies.

If the platelet count of PRP is adjusted, then traditionally autologous PPP (versus buffer) is used. Subsequent to centrifugation, PRP is removed using a plastic pipette, then placed in a polypropylene tube with limited surface area-to-volume ratio, and capped. The remainder of the residual blood is centrifuged at a higher rcf to obtain PPP. PPP should be platelet "free" (a residual platelet count of less than 10 x 109/L [(10,000/µL]). A simple method for determining the rcf required to achieve this residual platelet count is to check the platelet count of PPP using an automated cell counter. Likewise the platelet count of the PRP is determined in order to calculate how much PPP will be needed to dilute the initial PRP to a target PRP count/range for testing. PRP with counts below an assigned target value should be used undiluted.

The literature varies as to target platelet counts for the diluted PRP: 200 x 109/L (200,000 µL) [37], 250 x 109/L (250,000 µL) [12,34], 200 - 350 x 109/L (200,000 – 350,000 µL) [36]. In contrast other authors suggest that there should be no adjustment of the PRP platelet count [35,38,40]. This concept has been supported by more recent publications [41-46]. Cattaneo and colleagues showed that: 1) the extent to which platelet aggregation is inhibited by PPP is a function of the dilution factor and that 2) adenine nucleotides in PPP inhibit aggregation responses to ADP and COL. The inhibitory phenomenon was abated when platelets were washed and resuspended in buffer or when PRP was diluted with PPP in the presence of apyrase. Apyrase degrades adenine nucleotides thus preventing desensitization of ADP receptors. The authors concluded that platelet counts in the range of 200-600 x 109/L) should not be adjusted with autologous PPP [42].

PRP and PPP should be examined for interfering substances. Lipemia impacts the baseline turbidity of the sample. Though the PPP blank, in relation to the PRP, should compensate for the presence of lipids, testing on lipemic samples should be avoided. Lipemia does not affect IA [47]. Hemolysis, due to an improper venipuncture or resulting from exposure to excessive heat or agitation, leads to the release of nucleotides from the disrupted red cells and subsequent activation/desensitization (particularly to ADP) of platelets. Red cell contamination of PRP can occur due to improper centrifugation, braking of centrifuge, or disturbing the cellular component of the centrifuged sample when attempting to pipette PRP. Red cells, due to their large size,

can absorb more of the transmitted light in an optical aggregometer and because of this result in a falsely depressed aggregation response [10,12].

Sample Storage Prior to Testing

For the PFA-100 and WBA, sample is synonymous with specimen, hence all requirements for appropriate handling remain the same. For PRP AGG (tested by either LTA or IA) the sample is PRP.

pH

Commercial trisodium citrate blood collection tubes are buffered with citric acid, to a pH of 5.1–5.3, which maintains the pH of a plasma sample between 7.3 and 7.45 (near the physiological pH of 7.36 for venous blood) [7,48,49]. When non-buffered citrate is used, the pH of a plasma sample will rapidly increase to non-physiological levels because plasma has lost the buffering capacity of hemoglobin (found in red blood cells), which was removed by centrifugation in order to prepare PRP [48]. Han and Ardlie demonstrated that the effect of pH on aggregation is mediated by a decrease in calcium. As pH rises, the calcium-citrate complex dissociates less readily. Furthermore the binding of calcium to albumin is best between pH 7 - 8. The net outcome of both events is that as pH rises, less calcium is available to participate in the aggregation reaction [14].

Studies on the effect of pH changes on platelet aggregation have only been performed using PRP. Aggregation does not occur if the pH is below 6.4 or above 10. PRP exposed to air (tube is not capped) undergoes a rise in pH due to the diffusion of CO2 from plasma into the ambient atmosphere. Immediately upon preparation, the pH of PRP is approximately 7.5 [50]. For PRP, stored at room temperature, optimal aggregation occurs at pH ~8.0 for ADP and pH 7.7 for EPI [4,10,51]. RIPA is also pH dependent with PRP aggregation responses diminishing as plasma pH increases (from a maximal response at pH 7.6 to no response at pH 8.2) [50]. Appropriate pH can be maintained by 1) capping the test tube containing PRP, 2) limiting the surface area-to-volume ratio (use large volume of PRP in a small size test tube), 3) avoiding frequent mixing/agitation of PRP, and 4) introducing PRP directly into the tube and not allowing it to flow down the sides [4,10,50]. Reducing CO2 diffusion for PPP must also be taken into consideration; therefore, if PPP is used for PRP platelet count adjustment then it should be capped in order to minimize pH changes [14,18].

Temperature of Sample

Irrespective of sample type (WB or PRP), storage should be at ambient room temperature prior to testing.

- Cold temperature (0-4°C)
  - Affect platelets by causing them to become contracted, rounded, granular, and lose their microtubular system. These changes can be partly reversed when platelets are chilled for less than one hour and then restored to 37°C. Platelets undergo spontaneous aggregation if stored in the cold. However if chilled platelets are warmed for one hour at 37°C, no spontaneous aggregation occurs and the subsequent response to agonists (ADP & EPI) is significantly higher than in samples stored at room temperature or 37°C [52,53].
- Elevated temperature (37°C)
  - O'Brien showed that capped PRP stored at 37°C for 90 minutes failed to respond to EPI. Likewise, responses to thrombin and ADP were blunted at approximately 200 minutes and to ADP after 180-240 minutes [54]. Similarly Han and Ardlie noted loss of platelet responsiveness to ADP [14].
- Room temperature (20—25°C)
  - Platelets held at room temperature are more sensitive to various aggregating agents, particularly ADP, than those stored at 37°C. When platelets are stored at room temperature there is little change in responsiveness for the first 2 hours (phlebotomy to sample preparation to testing) [10,14].

Silver and colleagues compared PRP samples stored at 25°C to those stored at 37°C. Subsequent to storage for two hours at these respective temperatures, the authors showed that the effect on peak aggregation responses was not significantly different for ADP. However responses were substantially lower for both EPI and COL when stored at 37°C versus 25°C [55].

Age of Sample

Sample stability for testing by the PFA-100 or WBA is the same as for specimen stability since specimen and sample are synonymous for these two methodologies.

For PRP samples, discrepancies regarding the effects of time are evident in the literature. Most studies examining this issue were performed in the mid-1970s [10]. These studies were done either prior to or at approximately the same time as studies by Han & Ardlie and Coller & Gralnick [14,50]. Work by these two groups showed that the effects of time were related to changes in pH and that those changes were directly related to the escape of CO2 from the PRP sample tube. A comprehensive study performed by Roper in 1977 showed that PRP samples processed 90 minutes after incubation at room temperature consistently showed values lower than those processed within 30 minutes of PRP/PPP dilu-

Table 3. Guidelines and Pre-Analytical Issues

Pre-Analytical Issue	BCSH[1] (1988)	CLSI[5] (2008)	NASCOLA[59] (2010)
Time off Medications		14 days	
Fasting/Not Fasting		Fasting	
Needle Gauge		19—21	
Sodium Citrate Concentration	3.2%	3.2%	
Transport via Pneumatic Tube System		No	
Specimen/Sample Storage			
Temperature	20—25 °C	20—25 °C	
Capping of Sample Tube	Yes	Yes	
For LTA			
PRP Platelet Count Adjustment	200 x 10^9/L	200—250 x 10^9/L	200—300 x 10^9/L
PRP Adjustment with Autologous PPP	Yes	yes	Yes

Legend: CLSI, Clinical Laboratory Standards Institute ; BCSH, British Committee for Standards in Haematology; NASCOLA, North American Specialized Coagulation Laboratory Association

tion [56]. Unfortunately they did not indicate if their sample tubes had been capped and by that account for any potential pH effect.

In 1975 Rossi and Louis clearly showed the refractoriness of platelets to EPI when PRP samples tested within 30 minutes of venipuncture were used [10,57]. This was verified by Warlow [58] and subsequently cited by others as the rationale for not testing PRP within the first 30 minutes after phlebotomy [4,10,35,56]. Zucker suggested that this initial platelet refractoriness and subsequent gain of function may occur because centrifugation releases ADP from red blood cells and platelets [4].

Studies seem to reach a better level of agreement as to the maximal time intervals between venipuncture and testing [4,14,18,54-55]. Based on their findings, Silver and colleagues recommended that a PRP sample be maintained at room temperature and used between 2 and 4 hours after platelet donation [55]. This time interval is supported by their data showing that responses to most agonists declined between 2 and 4 hours (120-240 minutes) but these changes were not as significant as the substantial decreases noted between 4 and 6 hours. The caveat is that samples must be stored in such a way that pH changes are minimized.

Guidelines

Guidelines for platelet function testing have been published [1,5,59]. A summary of pertinent points as they relate to pre-analytical concerns are noted in Table 3.

Summary

Endogenous and exogenous pre-anayltical issues can dramatically affect results from platelet function testing. Consideration of the following initial components is critical: 1) patient drug/food history, 2) patient platelet count and HCT, 3) phlebotomy technique, and 4) anticoagulant concentration & buffering. For a screening system such as the PFA-100 or for complete profiling with WB AGG, specimen transport and handling are crucial. These concerns are also true for PRP AGG but PRP sample preparation demands further vigilance. Extreme care must be taken in the interpretation of platelet function test results in light of the pre-analytical concerns presented in this article.

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ECAT Information:

The effect of Rivaroxaban on haemostasis assays; results from ECAT surveys

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Rivaroxaban is an oral anticoagulant acting as a direct factor Xa inhibitor. It is used for the prevention of venous thromboembolism (VTE) in patients who have undergone total hip replacement or total knee replacement surgery as well as for stroke prophylaxis in patients with non-valvular atrial fibrillation.

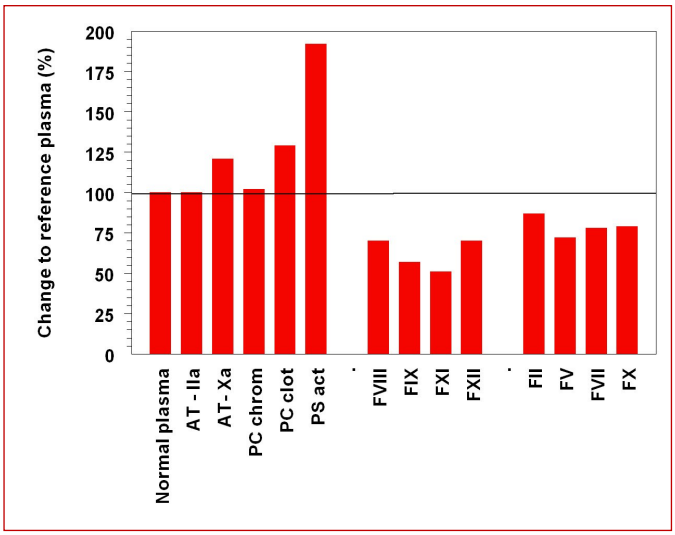
Rivaroxaban may affect haemostasis assays because of the effect on Factor Xa. This was investigated in a number of different studies [1-5]. These studies have clearly shown that samples with rivaroxaban taken from volunteers as well as in -vitro spiked samples affect both global and specific haemostasis assays.

In haemostasis laboratories samples could be presented from patients under treatment with Rivaroxaban without any information available about this treatment. It is therefore important that technical personnel as well as clinical chemists are aware of the potential effect of Rivaroxaban on several haemostasis assays. For that reason the ECAT has distributed in several of their surveys a normal pooled plasma spiked with approx. 200 ng/mL Rivaroxaban. This concentration is within the therapeutic range and shown to have impact on several haemostasis assays [1-5].

In the 2011-2 survey such a sample was used in both the thrombophilia and intrinsic coagulation factor module, while in the 2011-3 survey a similar sample was used in the extrinsic coagulation factor module. Here a summary of the observations is given. The effect of Rivaroxaban is expressed as the relative change in activity in comparison to a similar normal pooled plasma, except for the APC Resistance ratio results. The variation in concentration and activities of haemostasis factors between different normal pooled plasmas from the same producer are less than 5%. This allows us to compare the results of the Rivaroxaban-enriched plasma with a comparable normal pooled plasma without Rivaroxaban. All samples were produced by Technoclone, Vienna, Austria.

The activities of haemostasis factors investigated in the spiked plasma were corrected for the dilution factor of the plasma as a result of the addition of Rivaroxaban.

Figure 1. The effect of Rivaroxaban (≈ 200 ng/mL) on the measurement of se veral haemostasis parameters expressed as a deviation from a normal pooled plasma without Rivaroxaban (= 100%).



Antithrombin, protein C and protein S activity assays

The effect of Rivaroxaban on antithrombin activity (both IIa and Xa-based assays), protein C activity (both chromogenic and clotting assays) and protein S activity is shown in figure 1. It can be observed that there is no effect of Rivaroxaban on the anti-IIa Antithrombin assay and the chromogenic protein C assay. A significant effect of Rivaroxaban can be observed for the anti-Xa antithrombin assay, the protein C clotting activity assay and the protein S activity assays. The results for antithrombin are in line with those published in the literature [2, 5]. To our knowledge, no studies have yet specifically looked at the effect of Rivaroxaban on protein C and S activity. However, there is an effect of Rivaroxaban reported on the APTT assay [1, 2, 4, 5] resulting in a prolonged APTT . Because both the protein C clotting activity assay and the protein S activity assay are APTT-based assays an effect could be expected. The effect on the protein S activity assay is especially remarkable (almost a factor 2).

APC Resistance

Table 1 shows the effect of Rivaroxaban on APC Resistance testing for the most frequently used methods. It can be observed that for all methods, except the Chromogenix Coatest APC Resistance test, an increase in the APC ratio

Table 1. The effect of Rivaroxaban (≈ 200 ng/mL) on the measurement of APC Resistance.

Method	APC ratio - Rivaroxaban	APC ratio + Rivaroxaban
Chromogenix Coatest APC Resistance (global test)	3.58	3.15
Chromogenix APCR-V / I.L. HemosIL FVL (specific test)	2.58	3.03
Siemens ProC AcR (global test)	2.29	2.66
Siemens PC Global/FV (specific test)	2.14	2.74
Pentapharm Pefakit APC-R FVL (specific test)	3.83	5.17

occurs in the presence of Rivaroxaban. Such an effect was also observed in the study of Hillarp and co-workers [5]. They show a concentration-dependent increase of the APC Resistance ratio. This implies that in principal a heterozygous Factor V Leiden patient under the treatment with Rivaroxaban could have a ratio close to normal.

The reason why an opposite effect was observed for the Chromogenix Coatest APC Resistance test is unclear.

Intrinsic clotting factors

Figure 1 also shows the effect of Rivaroxaban on the intrinsic clotting factors (FVII, FIX, FXI and FXII). At the level of approximately 200 ng/mL Rivaroxaban a decrease of these clotting factors of about 30 – 50% can be observed (see table 2). This is in line with data published in the literature [4]. For Factor XII no data is available from the literature. These clotting factors are measured by APTT-based assays. It was shown that Rivaroxaban affected the measurement of APTT [1, 2, 5]. This may result in the clotting factor analysis in falsely decreased levels up to the level of a mild deficiency.

It was also shown in the literature that even with the application of a chromogenic Factor VIII assay decreased Factor VIII levels can be observed after the administration of Rivaroxaban [3].

Table 2. The effect of Rivaroxaban (≈ 200 ng/mL) on the measurement of intrinsic and extrinsic clotting factors.

Parameter	Percentage decrease in ECAT survey [Rivaroxaban] ≈ 200 ng/mL	Percentage decrease in literature (ref. 4) [Rivaroxaban] ≈ 100 - 115 ng/mL
Factor II	13%	10%
Factor V	28%	13%
Factor VII	22%	11%
Factor VIII	30%	26%
Factor IX	43%	25%
Factor X	21%	14%
Factor XI	49%	32%

Extrinsic clotting factors

There is even an effect of Rivaroxaban on the extrinsic clotting factors, FII, FV, FVII and FX (see fig 1). At the level used in the survey (approx. 200 ng/mL) a reduction of 10 – 30% can be observed (table 2). This is a less pronounced effect than observed for the intrinsic clotting factors. These clotting factors are measured by PT-based assays. Because Rivaroxaban affected the measurement of PT [1, 2, 5] an effect on the measurement of the extrinsic clotting factors could be expected as well.

It should be noticed that the absolute effect on the APTT and PT could be reagent-dependent [1, 2, 4, 5]. Each laboratory should therefore carefully investigate the effect of Rivaroxaban on their own test system as long as no data for that test system is available from the literature.

Conclusion

Data in the literature as well as observations from ECAT surveys using a sample enriched with Rivaroxaban show a significant effect on several haemostasis assays. Laboratories should be aware of this phenomenon to interpret appropriately results from samples of a patient under treatment with Rivaroxaban.

Educational surveys like those performed in 2011 are meant to assist laboratories in the awareness of such potential analytical problems in the haemostasis laboratories.

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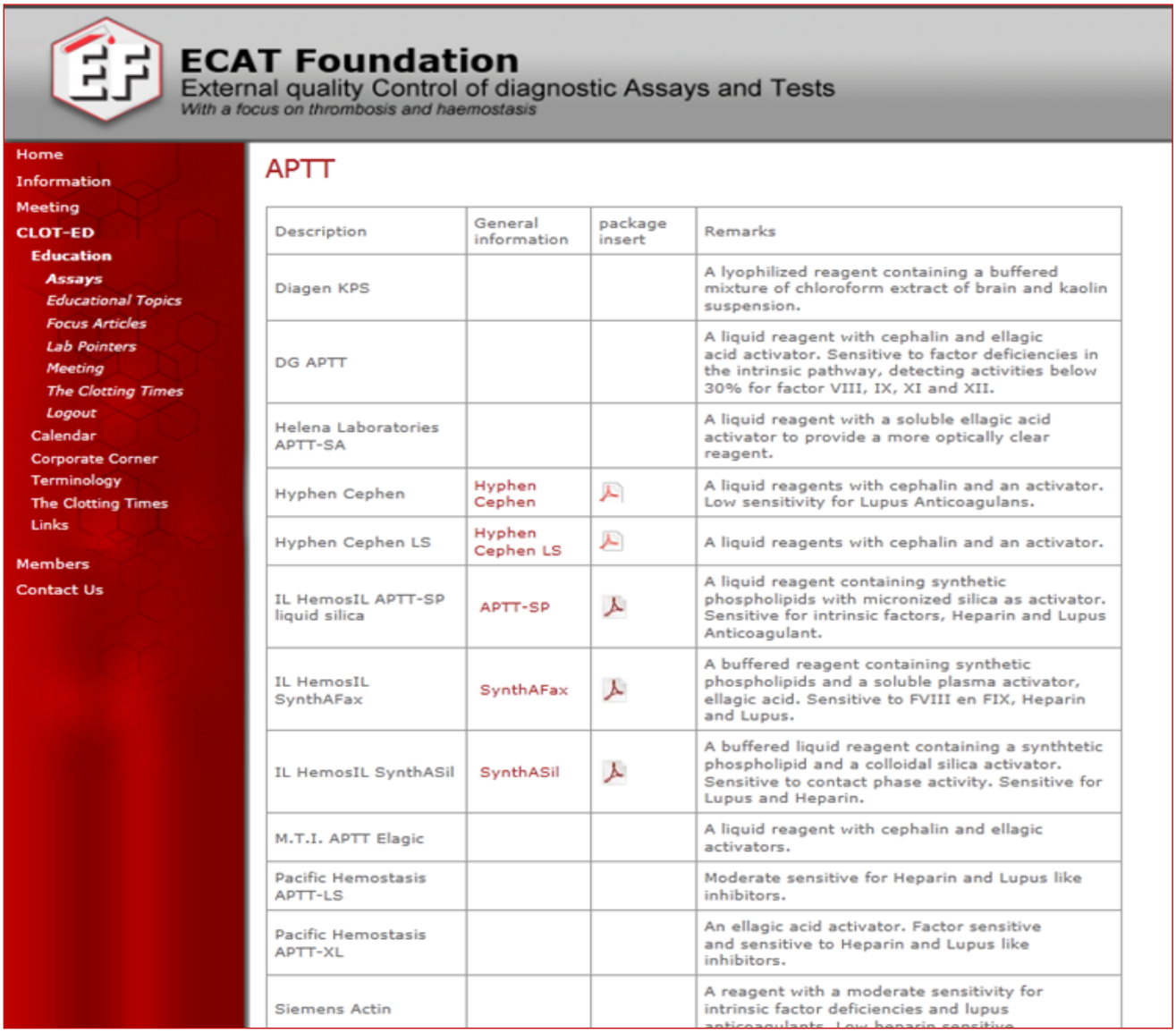
ECAT Information:

CLOT-ED: Assays

The educational website of ECAT is named CLOT-ED. The aim of this educational website is to provide laboratories with a variety of information about laboratory-related issues in the field of thrombosis and haemostasis. It exists as an open and a pass-word protected part which is only accessible to ECAT participants. In the pass-word protected section there is a new item, named “Assays”. “Assays” gives the laboratory professional an overview of which reagents are available for different haemostasis assays. An example of the APTT reagent list is shown in the figure.

The assay lists are defined according to the following categories: Screening, Thrombophilia, Intrinsic Clotting factors (VIII, IX, XI, XII|), Extrinsic Clotting factors (II, VII, IX, X), von Willebrand Factor, Factor XIII, Factor VIII inhibitor, Heparin-Induced Thrombocytopenia, Fibrinolysis and Homocysteine. If you select the link of a specific assay you find the reagents which are available and specific remarks. For companies that have an advanced-level status in the Corporate Corner a direct link to the reagent-specific information on their own website and, if available, a direct link to the package insert can be accessed.

The aim is to provide practical and some key information for each test or reagent to facilitate daily laboratory practice. You can find the item “Assays” with the following link: http://www.ecat.nl/assays/ .
Enjoy!



Case report:

Hermansky-Pudlak syndrome

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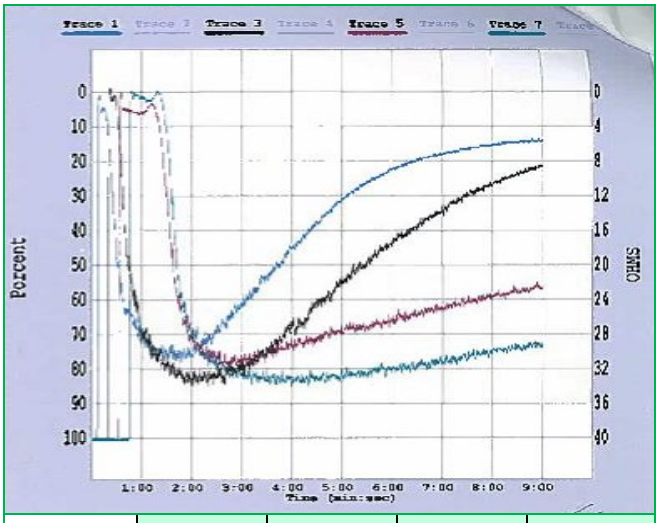
A six-year-old girl presented with ocular albinism and nystagmus. She had a history of easy bruising but superficially with minor trauma, without any history of internal organ bleeding. Epistaxis had seldom occurred in the past. In the family history there was no generalized or ocular albinism, nor any history of bleeding tendency. She was the first child of a non-related Caucasian mother and an African father.

Physical examination demonstrated a girl with a small bruise on the forehead and the thorax in good general condition. No other clinical abnormalities were found except for the ocular manifestations.

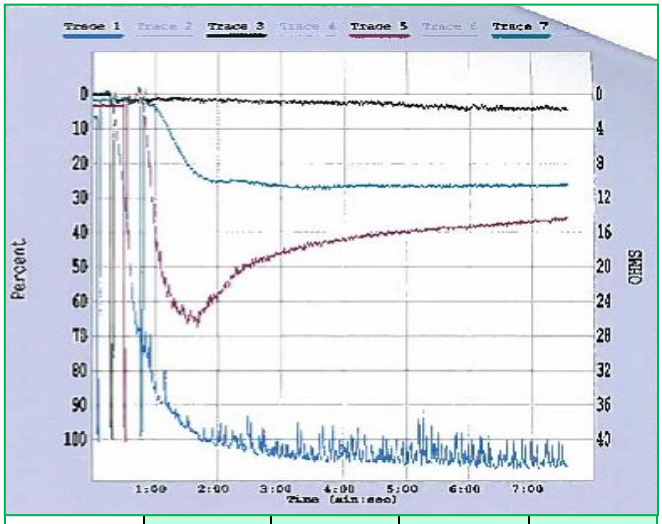
On the first occasion, laboratory studies showed a normal platelet function analysis (PFA), abnormal aggregation studies and decreased ATP secretion measured by lumi-aggregometry.

The light transmission aggregometry showed a deaggregation with low and high concentrations of ADP (2.5 and 5 µM), a normal aggregation with collagen (2.5 and 5 µg/ml), normal aggregation with ristocetin (1.5 and 0.5 mg/ml), reduced aggregation and deaggreagtion with arachidonic acid (1.5 mM) and thromboxan A2 analogue U46619 (1µM), and reduced aggregation with epinephrine (10µM). See Figure 1.

Figure 1. Light transmission platelet aggregation of the patient



	Channel 1		Channel 2		Channel 3		Channel 4	
	Trace		Trace		Trace		Trace	
	1	2	3	4	5	6	7	8
Instrument	Opt	N/A	Opt	N/A	Opt	N/A	Opt	N/A
Reagent	ADP 2.5 µM		ADP 5 µM		Collageen 2.5 µg/ml		Collageen 5 µg/ml	
Stirrer Gain	1000 2/104		1000		1000		1000 2/55	
Amplitude	75%		83%		76%		85%	
Slope	189		197		156		180	
Lag Time	0		0		0		0	
Area Under	0		0		0		0	



	Channel 1		Channel 2		Channel 3		Channel 4	
	Trace		Trace		Trace		Trace	
	1	2	3	4	5	6	7	8
Instrument	Opt	N/A	Opt	N/A	Opt	N/A	Opt	N/A
Reagent	Ristocetin 1.5mg/mL		Ristocetin 0.5mg/mL		A.A. 1.5 mM		Epinephrin 10 µM	
Stirrer Gain	1000 2/104		1000		1000		1000 2/55	
Amplitude	108%		2%		65%		25%	
Slope	189		6		173		37	
Lag Time	0:22		0:23		0:49		0:54	
Area Under	510.8		17.1		223.2		120.3	

There was no ATP secretion with 1 and 2 units/ml of thrombin. Prothrombin time, activated partial thromboplastin time and platelet count were normal. On a second occasion, one month later, these laboratory results were confirmed, except for a less marked deaggregation with ADP and a slightly prolonged closure time with the epinephrine/collagen cartridge (160 seconds, cut-off value 150 seconds) of the PFA. Electron microscopy of the patient's platelets confirmed that the patient's storage pool disease was due to dense body deficiency (see Figure 2B). The clinical and laboratory features were consistent with Hermansky-Pudlak syndrome.

The Hermansky-Pudlak syndrome

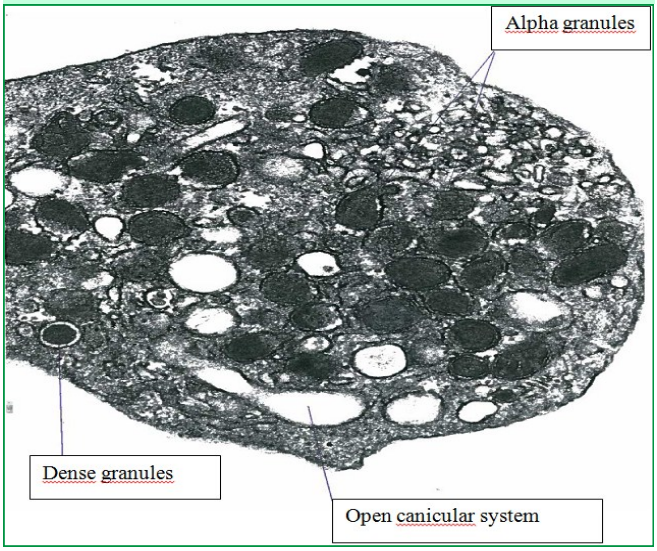
Hermansky-Pudlack syndrome is an inherited platelet disorder. Inherited platelet disorders constitute a large group of genetic defects that can lead to bleeding symptoms of varying severity. Besides defects in platelet surface membrane glycoproteins (e.g. Bernard Soulier syndrome and Glanzmann Thrombasthenia), defects in platelet receptors ($P2Y_{12}$, TXA_2), defects in platelet-signalling pathways (Wiskott-Aldrich syndrome), defects in platelet-derived procoagulant activity (Scott and Stormorken syndrome) and defects of platelet granules can be characterized [1]. See Figure 3 [2].

Defects in the α-granules (Gray platelet syndrome) or in the dense (δ) granules (Hermansky-Pudlak and Chediak-Higashi syndrome) are not always restricted to platelet dysfunction and are part of a more complex condition affecting other organ systems, as is the case in the Hermansky-Pudlak and Chediak-Higashi syndromes where other cytoplasmic organelles such as melanosomes are also involved [1]. These disorders are due to defects in genes that encode proteins whose function extends to several cell types and the "haemostatic defect" mostly concerns secretion-dependent aggregation [3]. The Hermansky-Pudlak syndrome is classified as a Storage Pool Disease (SPD). These qualitative platelet disorders are characterized by a deficiency in the number of granules, granule content or their release mechanisms upon stimulation. In many cases, these defects are associated with reduced platelet aggregation and consequently bleeding tendency, but also with other associated symptoms caused by defects in other cells containing cytoplasmic organelles [1]. They lead to clearly defined phenotypes where melanosomal defects cause a lack of pigmentation of the skin and hair which is the case in the Hermansky-Pudlak, Chediak-Higashi and Griscelli syndromes [3].

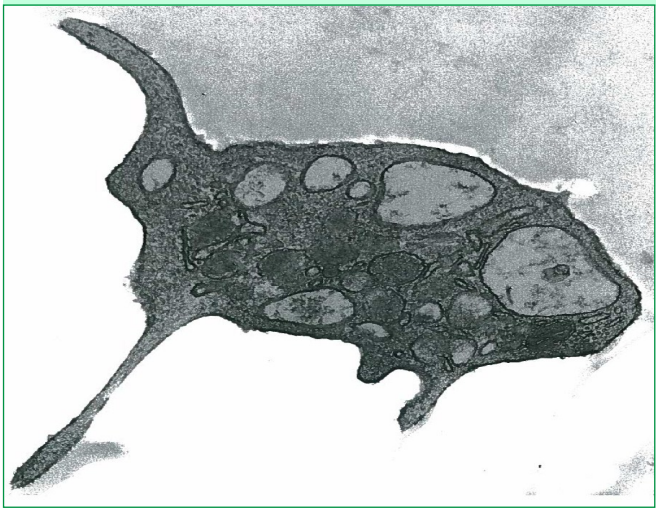
The Hermansk-Pudlak syndrome was first described in 1959 by Hermansky and Pudlak in two patients with oculocutaneous albinism who had bleeding diathesis [4]. The syndrome is characterized by a deficiency of the dense granules accompanied by a lifelong bleeding tendency, oculocutaneous albinism and defects in lysosomal-related organelles including melanosomes and lysosomes [5]. Ceroid-lipofuscin storage in the reticulo-endothelial system, granulomatous colitis or fatal pulmonary fibrosis may occur in some cases [5]. Albinism is accompanied by horizontal nystagmus with lateral eye movement with a decrease in pigmentation allowing iris transillumination [6]. Due to albinism patients have a reduced visual activity and photophobia. Oculocutaneous albinism is a defining aspect of the disorder but varies widely in the degree of hypopigmentation as well as correlation between retinal pigmentation and hair/skin pigmentation [7]. Pulmonary fibrosis is the most serious complication,

Figure 2. Electron microscopy of platelets.

A: normal platelet

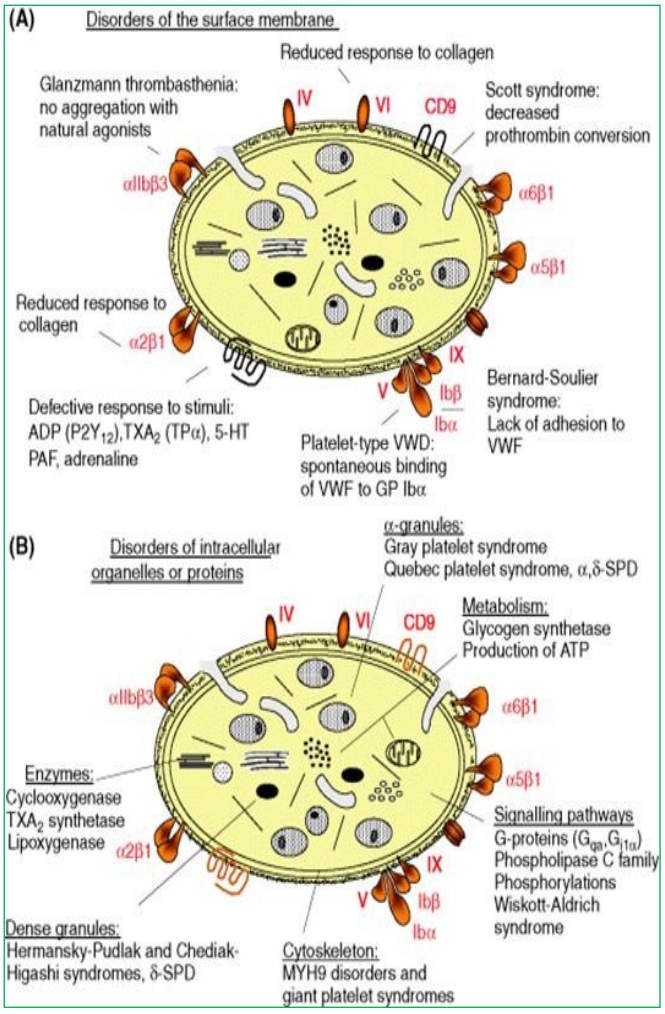


B: platelet with lack of dense granules



With thanks to Dr. Anne De Mulder (Laboratory of Hematology and Haemostasis, CHU Brugmann, Brussels, Belgium) for performing the eletron microscopy and providing the pictures.

Figure 3. Cartoon showing the most common inherited defects as they affect (A) the surface membrane and (B) intracellular constituents of platelets [2]



usually presenting in the fourth or fifth decade and accounts for 50% of the morbidity [8].

Worldwide the disease is extremely rare, but in Puerto Rico it is found in five out of every six albinos [9, 10]. It is an autosomal recessive disorder associated with multiple genes [3] with a role in the regulation of pheomelanin/melanin production in melanocytes or in the regulation of membrane/vesicle and protein trafficking or in organelle biosynthesis. Defects in at least eight genes (HPS-1 to HPS-8) are known to cause distinct subtypes of the disease [11]. HPS-1 is the most common subtype, it is also the most common subtype found in Puerto Rican patients. The genotype of HPS -1 represents the most severe of the known mutations and accounts for a high risk of pulmonary disease, haemorrhages and granulomatous colitis. One subtype (HPS-2) may be associated with innate immunity defects [3, 12]. The syndrome has a wide variety of phenotypic appearances. Platelet aggregation with ADP, epinephrine and collagen is reduced, with usually a prolonged bleeding time [5]. Electron microscopy shows that platelets have a smaller quantity of dense bodies. Dense bodies are needed for the second phase of the platelet aggregation.

The Chediak-Higashi syndrome also presents with a failure in platelet aggregation and oculocutaneous albinism. However, these patients also suffer from severe immunological deficiency and progressive neurological dysfunction [5] [1]. Most of them do not survive childhood [13] because of an accelerated phase with fatal lymphohistiocytosis. The hallmark of Chediak-Higashi syndrome is the presence of giant inclusion bodies in a variety of granule containing cells including platelets [3].

Patients with Griscelli syndrome have partial albinism and silver hair. Different subtypes present with neurological defects and/or severe immunodeficiency sometimes complicated with a fatal haemophagocytic syndrome [3]. A differential diagnosis of Hermansky-Pudlak syndrome can be difficult to establish, associated as it is with bleeding and an impaired secretion-dependent platelet aggregation [14]. However, major bleeding is rare and platelet-dense granules are little studied in this syndrome [6].

Patients with the δ-storage pool disease are diagnosed with a mild bleeding disorder and easy bruising due to a defect in the dense granules but not accompanied by albinism. Platelet aggregation are not always impaired [15].

Diagnosis

The diagnosis of the dense granule disorders relies on the clinical picture together with the demonstration of the defect in platelet dense granule content and/or release [16]. Dense granules are rich in serotonin, ADP, ATP, calcium, pyrophosphate and histamine. Upon platelet activation the platelet granules content is secreted, further enhancing both platelet adhesion and activation. The granule deficiency may be severe or partial.

Largely due to the abnormal secretion of ADP, SPD affecting dense granules causes a defective secretion-

dependent aggregation [6]. Platelet-dense granule disorders may result in defects in platelet aggregation that range from an abnormal response to all agonists to more subtle changes only seen with low concentrations of agonists [16]. Characteristic features are: (i) the absence of second-wave aggregation to epinephrine (however, this can been seen in a proportion of normal subjects); (ii) a delayed and reduced response to collagen; (iii) impaired aggregation of low concentrations of agonists, such as arachidonic acid and TRAP; and (iv) high concentrations of ADP provoke full irreversible aggregation [16]. Mild dense granule deficiency may not impair aggregation findings [17].

Platelet-function analyser (PFA) measurements may be sensitive to SPD, primary secretion defects and Hermansky-Pudlak syndrome, though false-negative results occur in patients with all these disorders [18]. Patients whose history

suggests platelet-function disorders will need further investigations whether the PFA is normal or abnormal.

A marked reduction in both content and ratio of ADP to ATP or absence of release of ATP measured by lumi-aggregometry indicates a platelet-dense granule disorder.

Reduced numbers or absence of dense granules can be confirmed by electron microscopy. The calcium present in the granules gives them an intrinsic electron density and dark appearance in electron microscopy [6]. See Figure 2.

Management

The bleeding symptoms of dense granule disorders should be managed as for other mild platelet disorders [16]. In the milder platelet dysfunctions bleeding is less often spontaneous, but trauma-related bleeding can be a problem for instance during surgery. Antifibrinolytic agents (e.g. tranexaminic acid) are useful for the control of menorrhagia and other mild mucocutaneous bleedings, such as epistaxis. Desmopressin (DDAVP) is often used preventively and may be the agent of choice for mild bleeding problems where tranexaminic acid alone is not effective. Administration of DDAVP results in an increasing von Willebrand factor secretion from endothelial cells and may be sufficient to reduce the bleeding tendency. Patients with Storage Pool Disorders usually (but not always) respond. It is also not clear whether laboratory correction (e.g. of the bleeding time or PFA) will correlate with clinical efficacy. The effect is better assessed by the clinical response instead of doing a DDAVP correction test.

Patients with inherited platelet disorders are treated during severe bleeding periods with the major goal of providing sufficient numbers of active platelets to assure a minimal haemostatic function [16]. Platelet transfusion should be limited and reserved for situations where other agents have failed, due to the risk of transfusion-transmitted infections, allergic reactions and allo-immunisation.

Recombinant factor VIIa is an alternative therapeutic agent whose use is being evaluated. It is licensed for Glanzmann thrombasthenia but not for other platelet disorders.

Haematopoietic stem cell transplantation is recommended for children with severe diseases such as Chediak-Higashi syndrome [6].

In summary

Hermansky-Pudlak syndrome is a rare congenital bleeding disorder. Patients usually present with easy bruising or mucocutaneous bleedings (gynaecological, dental extraction, epistaxis). Hermansky-Pudlak syndrome is a subtype of platelet storage pool disease, especially a delta granule defect. The platelets are characterized by an abnormally low content of dense granules. Patients have a normal platelet count with abnormal platelet function assays. The diagnosis is confirmed by demonstrating a reduced ATP secretion and electron microscopy illustrating the reduction in dense granules. HPS-1 is the most common subtype and also the most severe subtype and accounts for a high risk of pulmonary fibrosis, haemorrhages and granulomatous colitis. Bleeding can be managed by antifibrinolytica, desmopressin and platelet transfusion.

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Literature review:

Lupus anticoagulant testing: analyzing fresh samples after a single centrifugation and after a 6-8 hr delay

(P. Froom and M. Barak. Clin Chem Lab Med 2012; 50: 367 – 370)

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Proper sample preparation is important for reliable Lupus Anticoagulant testing. Platelet contamination of the plasma may interfere in laboratory tests. This is even more important when samples are frozen for batch-wise analysis [1-6]. With an ongoing process of automation in clinical laboratories also testing for Lupus Anticoagulant (LA) is increasingly performed as single-sample testing instead of batchwise-testing. This means that LA testing is performed on single-centrifuged fresh plasma samples. Furthermore, centrifugation within 3 hrs and analyzing within 4 hrs after blood collection is not always possible. Therefore Froom and Barak [7] performed a study to investigate whether single centrifugation and delayed centrifugation (6-8 hr) result in reliable LA test results.

For the comparison of one or two centrifugation steps they included 50 different plasma samples. For the comparison of testing within 4 hrs or testing at between 6 -8 hrs they used 40 different samples.

LA testing was performed using a Silica Clotting Time (SCT) integrated test system (screen and confirm) as well as a diluted Russel Viper venom Test (dRVVT) (screen and confirm). Both tests were from Instrumentation Laboratory.

The average ratio between one or two centrifugation steps is for the SCT test 1.03 ± 0.08 and for the dRVVT test 1.01 ± 0.05. This shows that there is no significant difference in the test result between one or two centrifugation steps.

The average ratio between testing within 4 hrs and testing at between 6 – 8 hrs is for the SCT test 1.00 ± 0.07 and for the dRVVT test 0.97 ± 0.06. Also here no significant difference could be observed.

For both study variables only a few discordant results were observed.

The authors concluded that LA testing can be performed on single centrifuged fresh plasma samples even with a delay in testing of up to 8 hr after blood collection.

This is of course a small study with only two different test systems used on one single analyzer (Sysmex CA-6000). But it indicates that implementation of LA testing in laboratory automation is possible. Each laboratory should, of course, validate for its own situation (used LA tests and analyzer) whether, with the centrifugation conditions and time interval used, automation is possible.

For further details about this publication see reference 7.

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