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Editorial

With this fourth issue of The Clotting Times we have reached the end of 2011. In this issue we would like to start with a Focus Article that gives an overview and update on platelet function testing. A number of published guidelines are now available to guide practices, including test interpretation.

Since 2011 the ECAT Foundation has been running a programme for external quality control for monitoring both UFH and LMWH and we give you a summary of the results obtained so far.

Some clinical conditions are rare and therefore not always easy to diagnose. We thank Dr Katrien Devreeze from the University Hospital of Gent, Belgium, for providing a case report on a patient with acquired Glanzmann thrombasthenia . Her article provides more insight into the symptoms, diagnosis, and treatment.

In the rubric entitled “Literature Reviews” we would like to highlight the publication of Interference of rivaroxaban on one-stage and chromogenic factor VIII:C assays (V. Tichelaar et al. Thromb Haemost 2011; 106: 990 – 992).

The editorial board wishes you a healthy 2012 and hopes you will profit from reading this newsletter.

Yours sincerely,
Petra ter Hark

Focus Article:
Update on Platelet Aggregation Testing

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Summary

Many laboratories assess platelet function to help diagnose common and rare bleeding disorders. Tests that are validated to have important diagnostic usefulness for detecting bleeding disorders that are due to impaired platelet function include: aggregation assays, tests for dense granule deficiency and dense granule release defects, in addition to assays for less common disorders (e.g. genetic testing for selected conditions). Platelet function is commonly evaluated by testing aggregation responses to agonists by light transmittance aggregometry (LTA), using platelet -rich plasma (PRP),) or whole blood (impedance) methods. An evaluation of dense granule release by lumi-aggregometry assays with platelet-rich plasma (PRP) or whole blood improves the detection and subclassification of platelet function disorders. The interpretation of platelet function tests requires careful consideration of the pattern of abnormal responses, including potential false positives and false negatives findings. A number of published guidelines are now available to guide practices, including test interpretation. This article provides an overview and update on platelet function testing, with an emphasis on issues relevant to testing for diagnostic purposes.

What does platelet aggregometry measure?

Platelet aggregation assesses the ability of platelets to interact with each other after adding an activating agonist or ristocetin, while stirring the sample at low shear. Under low shear conditions, platelet-platelet interactions induced by activating agonists are mediated by binding interactions between the γ-chain of fibrinogen in plasma (or that is released from platelets) and the integrin receptor α IIb β 3 on adjacent platelets [1,2]. When the test is done with ristocetin, the ristocetin induces von Willebrand factor (VWF) binding to the platelet receptor glycoprotein Ib-IX-V [3,4] which is followed by platelet activation and then aggregation, mediated by α IIb β 3 [5,6]. With collagen, platelets first adhere to and are activated by the collagen fibrils, which requires the platelet integrin α 2 β 1 and glycoprotein VI, the platelet receptor important for collagen signaling. This leads to platelet shape change, which, as recorded on the aggregometer, an initial reduction in light transmittance that is followed by rapid aggregation. When platelets are aggregated by collagen and other strong agonists, dense granule secretion occurs independent of, but at the same time as, platelet aggregation [7].

Clinical aggregation tests are usually done with samples collected into sodium citrate anticoagulant, which reduces the extracellular ionized calcium concentration and increases aggregation responses to weak agonists, such as adenosine diphosphate (ADP) and epinephrine, which do not trigger dense granule secretion without aggregation [8,11]. Alternative anticoagulants have been explored to allow testing beyond the usual 3-4 hour limit for aggregation and release studies [12-15] but there is a need to determine if these are acceptable for bleeding disorder assessments.

With epinephrine, aggregation typically occurs in two phases: an initial or first wave of limited aggregation that is followed by faster, more extensive aggregation. Thromboxane generation, and the release of platelet dense granule ADP, provides positive feedback, increasing secondary aggregation with epinephrine, and other weak agonists, including low concentrations of collagen [8,10,16-18]. Accordingly, secondary aggregation responses to epinephrine and other weak agonists can be absent or reduced from drug effects (e.g. non-steroidal anti-inflammatory drugs including aspirin, alone or in combination with clopidogrel) or with severe dense granule deficiency [17-19].

A recent study of native and platelet count-adjusted samples illustrated that absent secondary aggregation can be considered a normal finding for platelet count-adjusted, sodium-citrated PRP but not for native PRP [20]. Deaggregation can be a normal finding for platelet count-adjusted PRP in tests using low concentrations of some agonists (e.g. 2.5 μ M ADP) whereas deaggregation with many agonists is an abnormality that often accompanies reduced maximal aggregation.

Many agonists used for aggregation testing (e.g. ADP, epinephrine, the thromboxane A_2 analogue U46619, and collagen) activate platelets by binding to an external platelet membrane receptor, leading to activation signals. An exception is arachidonic acid which is a substrate that the platelet enzymes cyclooxygenase 1 (COX 1) and thromboxane synthase must convert to thromboxane A_2 before it can trigger platelet activation by binding to thromboxane receptors. As a result, aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs) that block COX 1 impair aggregation responses to arachidonic acid, as do congenital defects in COX 1 or thromboxane synthase. The North American guidelines on LTA recommend comparing aggregation responses to arachidonic acid to those with thromboxane A_2 analogue U46619 to distinguish an aspirin-like defect from other platelet disorders [21], such as congenital thromboxane receptor defects (which impair aggregation responses to both arachidonic acid and thromboxane analogue U46619 [22]) and other platelet secretion defects [23].

Striking reductions in responses to ADP occur with $P2Y_{12}$ inhibitors such as clopidogrel, alone or in combination with aspirin. However, the most profound aggregation defects are seen in congenital and acquired Glanzmann thrombasthenia,

which cause either absent or markedly impaired aggregation with all agonists except ristocetin, due to the deficiency or dysfunction of α IIb β 3. Ristocetin-induced platelet aggregation (RIPA) is performed with low and high concentrations of ristocetin in order to assess gain-of-function, and loss-of-function, defects in VWF and its receptor, glycoprotein Ib-IX-V. The low concentration usually causes minimal platelet agglutination unless the person has type 2B or platelet-type von Willebrand disease (VWD). The high concentration of ristocetin triggers rapid agglutination of normal platelets, followed by platelet activation and aggregation mediated by α IIb β 3 [24,25]. Accordingly, RIPA can be delayed or reduced with loss of function defects from VWF deficiency or dysfunction [24], defects in glycoprotein Ib-IX-V, or when the aggregation phase is impaired by a platelet function disorder (e.g. Glanzmann thrombasthenia, an aspirin-like defect, a secretion defect, etc.).

Proper reporting of aggregation findings requires a qualitative review of the aggregation tracing and a quantitative analysis of the maximal aggregation (MA) responses to the tested agonists [21,26]. Additional parameters that can be assessed include: shape change [27]; the final aggregation at the end of the test; the amount of deaggregation (i.e. maximal minus final aggregation); the slope of the aggregation response; and the area under the aggregation curve [28].

The principle and uses of whole blood aggregation (WBA) have recently been reviewed [29]. In WBA assays, samples were tested after a 1:1 dilution in physiological saline, and uses changes in electrical impedance (measured in Ohms) to detect aggregation; specifically, the impedance is modified when platelets attach to the monolayer of platelets that first deposited on the test electrode during the procedure of setting the test baseline measurement [12,29,30]. For WBA, evaluated on lumi-aggregometers, the manufacturer recommends simultaneously measuring ATP release with aggregation for all agonists except ristocetin.

While there is a need for prospective studies comparing WBA and LTA findings, WBA and PRP aggregation findings are known to be different with some agonists [26,31,32]. Furthermore, the recommended concentrations of some agonists for WBA is different from LTA and epinephrine is recommended for PRP but not for WBA as no detectable response can be a normal finding in WBA [21,26,29]. WBA responses are influenced by the numbers of platelets, and leukocytes, in the sample [33] whereas the aggregation responses obtained using native PRP samples (which contain minimal leukocytes) have no or minimal correlation with the sample platelet count [20].

Recent practice surveys indicate that PRP aggregation tests are performed more commonly than WBA and that aggregation tests need to be improved through greater procedural control, including the use of standardized agonist panels, validated reference intervals and testing healthy control volunteer samples for quality monitoring [28,34-36]. The North American guidelines are unique as these guidelines include suggestions on how laboratories should interpret platelet aggregation test findings [21], as shown in Table 1 of this article. The British guidelines provide a listing of potential interfering drugs that may be useful when considering test findings [37].

LTA can be evaluated using native PRP (without platelet count adjustment) or PRP that is first adjusted to a standardized platelet count by adding autologous platelet-poor plasma (PPP) [26]. Both sample types have been validated for aggregation assays for bleeding disorder evaluations [20,23].

Laboratories should be aware of the recommended agonists and agonist concentrations for the two methods, which are summarized in Table 2. Laboratories should also be aware that the responses to some weak agonists (e.g. ADP and epinephrine) are different for native and platelet count-adjusted samples [20,38-41].

Laboratories should never refuse to test low platelet count samples as there is no accepted lower limit of PRP platelet counts for aggregation testing and the testing is needed to diagnose some thrombocytopenic disorders with aggregation defects (e.g. Bernard-Soulier syndrome, type 2B VWD) [42].

Table 1. Agonists for platelet aggregation studies, based on recent guidelines [21,26,37]. All concentrations shown are final, in PRP or whole blood.

Agonist and recommended single/multiple concentrations for testing	Aggregation method	
	Light transmission aggregometry with PRP	Impedance aggregometry using whole blood
ADP Test higher concentration(s) if abnormal with the lower concentration [21,26,37]	0.5-10 μM, ~5 μM to start [26] 2.0-10 μM [21] 2.5 μM [37]	5-20 μM [26]
Epinephrine Test with a single concentration as testing much higher concentrations is generally not helpful [37]	0.5-10 μM, ~5 μM to start [26] 5-10 μM [21] 5 μM [37]	Not recommended because some normal subjects have no response in whole blood [26]
Collagen (type 1 fibrillary or Horm collagen) Test higher concentration(s) if abnormal with the lower concentration [21,26]	1-5 μg/mL, ~2 μg/mL to start [26] Concentration that detects abnor- malities from NSAID [21] 1.25 μg/mL [37]	1-5 μg/mL [26]
Arachidonic acid Test at a single concentration [21,26]	0.5-1.6 mM [26] 0.5-1.64 mM [21] 1 mM [37]	0.5-1.0 mM [26]
Thromboxane analogue U46619 Test at a single concentration [21]	1-2 μM [26] 1 μM [21] 1 μM (extended panel only) [37]	Not provided
Ristocetin Low dose High dose	≤ 0.6 mg/mL [26] 0.5-0.6 mg/mL [21] 0.5-0.7 mg/mL [37] 0.8-1.5 mg/mL [26] 1.2-1.5 mg/mL [21] mg/mL [37]	0.25 mg/mL [26] 1.0 mg/mL [26]

What do assays of platelet dense granule release measure?

Dense granule release assays can be performed using PRP and whole blood. These assays help to detect some platelet function disorders with normal aggregation findings as well and providing information to subclassify defects in platelet function (e.g. to diagnose a platelet secretion disorder). Platelet dense granule release assays can be monitored as a stand-alone test, or as part of WBA or LTA assays [8,29,43-45].

The most commonly used method for assessing platelet-dense granule release is a bioluminescent assay, performed on a lumi-aggregometer. This assay uses D-luciferin and firefly luciferase to measure ATP released by activated platelets, usually simultaneously with aggregation [28,29,34,45,46]. The principle is as follows:

Table 2. Patterns of abnormal platelet aggregation and potential causes summarized from the North American guidelines [21].

Aggregation Findings	Potential Causes
Aggregation absent or markedly reduced with arachidonic acid but normal with thromboxane A_2 analogue U46619. Aggregation also reduced with lower concentrations of collagen and absent secondary aggregation with epinephrine.	These abnormalities are typical of aspirin-like defects, which can be caused by drugs that inhibit cyclooxygenase 1 and, less commonly, by inherited defects in thromboxane generation.
Aggregation is present with only ristocetin (or is markedly impaired with all agonists except ristocetin). Platelet count and size may be normal or there may be an associated macrothrombocytopenia.	This type of abnormality suggests possible Glanzmann thrombasthenia, due to inherited or acquired defects in integrin □IIb□3. Gain-of-function defects in this receptor should be considered if macrothrombocytopenia is present.
Aggregation absent with high concentrations of ristocetin and there is thrombocytopenia and also very large platelets.	Possible Bernard-Soulier syndrome, which can be caused by inherited or acquired abnormalities (e.g. from autoantibodies) in glycoprotein Ib-IX-V. von Willebrand disease should be excluded.
Aggregation is present but reduced with high concentrations of ristocetin, without an associated thrombocytopenia.	Possible von Willebrand disease or a defect in the glycoprotein Ib-IX-V. Ristocetin-induced platelet aggregation abnormalities usually reflect a significant deficiency or dysfunction of von Willebrand factor.
Aggregation is abnormally increased with low concentrations of ristocetin. Thrombocytopenia and platelet clumping may be present.	The findings suggest a gain-of-function defect in platelet-von Willebrand factor interactions, possibly due to type 2B or platelet-type von Willebrand disease. A false positive should be considered if there is borderline increased aggregation. The findings of the von Willebrand disease screen, including multimers, should be reviewed.
Aggregation is abnormal with multiple agonists and is markedly reduced with ADP, with significant deaggregation.	These abnormalities suggest a possible defect in the platelet ADP receptor $P2Y_{12}$, which can be inherited or drug-induced (e.g., from clopidogrel or prasugrel). The drug history should be reviewed.
Aggregation tests show abnormalities with two or more agonists that differ from the abnormalities described above.	These types of findings are common and suggest a platelet function disorder is present. Often these types of abnormalities are associated with defects in dense granule secretion and, less commonly, dense granule deficiency.
Abnormalities with only one agonist (excluding collagen or ristocetin).	Non-diagnostic findings that could represent a false positive. If the abnormality is seen only with epinephrine, the possibility of Quebec platelet disorder should be considered, particularly if there is a history of delayed bleeding.
Aggregation is normal with all agonists but is markedly reduced with collagen, tested at low and high concentrations.	A platelet collagen receptor defect, involving glycoprotein VI or $\alpha 2\beta 1$, should be considered.
Normal aggregation findings	There are some platelet function disorders that do not impair aggregation findings (e.g. mild dense granule deficiency and some dense granule release defects, Scott syndrome).

1) Platelet activation by an agonist stimulates the signaling pathways that trigger the release of dense granule ATP
2) ATP combines with the added D-luciferin, in the presence of luciferase, to generate inorganic pyrophosphate and luciferyl adenylate
3) Luciferyl adenylate combines with oxygen to generate the final reaction products: adenosine monophosphate, oxyluciferin plus light
4) The light emitted is quantified, using an ATP standard to calibrate each sample

Alternative methods use biochemical or radioactive methods to assess serotonin release as the endpoint (serotonin is a normal dense granule constituent). The radioactive method requires a pre-incubation of PRP, with radioactive serotonin to allow for uptake, before quantifying how much radioactive serotonin is released from platelets into the plasma following agonist stimulation [44,47].

Dense granule release is typically assessed with a panel of agonists such as thrombin, collagen, epinephrine, arachidonic acid and thromboxane analogue U46619 [48]. With strong agonists (e.g. collagen or thrombin), secretion occurs at the same time as aggregation whereas release is delayed until aggregation occurs with weak agonists (e.g. ADP, epinephrine) [8].

For diagnosing platelet disorders, the agonists that detect many common platelet function defects include: 6 μM epinephrine, 1 μM thromboxane analogue U46619 and 5 μg/mL Horm collagen [48]. Thrombin and collagen are useful because absent or reduced release with these agonists can be a clue to a deficiency of platelet dense granule contents. Repeat testing should always be considered to confirm abnormalities as dense granule release endpoints show variability [20,44,46,48]. Release assays should be considered as an adjunct, and not a replacement for aggregation tests as some platelet function disorders impair aggregation but not dense granule release and others impair release but not aggregation [48].

Laboratories that assess ATP release and aggregation simultaneously should be aware that the commercial D-luciferin/luciferase reagent contains magnesium that can potentiate submaximal PRP aggregation responses of sodium citrate-anticoagulated samples, which can increase the measured aggregation [49,50].

General practice points for the laboratory assessment of platelet function

Most coagulation parameters, including platelet aggregation and release data, do not show a symmetrical, bell-shaped distribution [42,48]. Accordingly, non-parametric statistical methods are preferred to estimate the reference interval for maximal aggregation and ATP release, using a minimum of 40 unique donor samples [21,42,48,51]. Reference intervals must be established for each laboratory's procedure, instrument and reagent combination in order to report the quantitative data on platelet function [21,26]. As the maximal aggregation responses to some agonists are different for platelet count-adjusted and native PRP, reference intervals need to be sample type-specific [20]. Samples from healthy subjects, who are not taking drugs that inhibit platelet function, should be tested in order to establish the reference intervals [52]. For children older than neonates, it is acceptable to use adult reference intervals for platelet function tests [21,53].

There are uncertainties about the limits of acceptable platelet counts for LTA and WBA assays. For WBA, samples with low platelet counts show reduced responses [33,52,54-56]. The PRP platelet count shows little relationship to maximal aggregation in samples with normal platelet counts [20,33,38,39,41,42,52]. When native PRP contains <200-250 x 10^9 platelets/L, the sample platelet count does influence findings, which have led some to recommend using data from diluted healthy control sample as the reference for low platelet count samples [42].

Aggregation and dense granule release tests show some within- and between-subject variability that is greater for platelet release than aggregation endpoints and affects reference intervals [20,44,48]. While most repeat aggregation tests confirm original findings [44], repeat testing is often important to confirm findings and exclude the possibility of a false positive finding, which

could be the result of a pre-examination or analytical artifact [21].

North American guidelines [21] recommend reporting platelet aggregation results with:

1) An overall interpretative comment,
2) Information on the agonists, and concentrations, tested
3) Information recorded on the maximal aggregation with each agonist
4) Reference intervals for the quantitative data (specific for the type of sample and procedure performed)

The maximal aggregation responses of native and platelet count-adjusted PRP are considerably different with some weak agonists, particularly with epinephrine and ADP [20,38-41]. While native PRP shows less variability than platelet count -adjusted PRP with weak agonists, platelet count-adjusted samples show less variable responses to ristocetin than native PRP [20]. Testing aggregation with native PRP is not inferior but also not superior to testing platelet count-adjusted PRP, although the adjusted PRP samples provide more sensitive detection of some abnormalities with weak agonists, such as epinephrine [20].

When reporting results of platelet function tests, it is important to consider that impaired aggregation, or release, with multiple agonists is more predictive than a single agonist abnormality [20,23,48]. The recommendations on how to report findings, described in the North American consensus guidelines [21], is helpful when considering the potential causes of abnormal aggregation findings when preparing a report. Many laboratories have difficulty with common abnormalities (e.g. single agonist abnormalities that could represent a false positive; distinguishing secretion defects from other abnormalities, such as aspirin-like defects). Other common errors are caused by being more specific than appropriate (e.g. suggesting reduced aggregation with ristocetin is due to von Willebrand disease when a defect in glycoprotein Ib-IX-V is also possible). For thrombocytopenic samples, it is a good strategy to first determine if there are abnormalities with ristocetin suggestive of either Bernard-Soulier Syndrome [57], type 2B VWD [58] or platelet-type VWD [25]. When there is absent or markedly impaired aggregation with all agonists except ristocetin, with or without thrombocytopenia, Glanzmann thrombasthenia should be considered as a potential cause (as some variants have thrombocytopenia [59]). If there is thrombocytopenia and the findings are abnormal with multiple agonists but not all agonists, many different conditions, including acquired defects and inherited platelet disorders should be considered.

For nonthrombocytopenic subjects, the aggregation findings should be evaluated first for "hallmark patterns" such as:

1) An aspirin-like defect (inherited or acquired): markedly reduced aggregation with arachidonic acid but normal aggregation with a thromboxane analogue, along with absent secondary responses to epinephrine and reduced aggregation with low concentrations of collagen.
2) Glanzmann thrombasthenia (inherited or acquired): aggregation is absent or markedly reduced with all agonists except ristocetin.
3) vWD or Bernard-Soulier Syndrome: aggregation is only abnormal with ristocetin.
4) A $P2Y_{12}$ defect (inherited or acquired): aggregation is markedly reduced and shows significant deaggregation with ADP, with additional abnormalities with agonists that are sensitive to ADP feedback [16,60,61].
5) A thromboxane receptor defect: reduced aggregation with thromboxane analogue and arachidonic acid.

However, the most common finding is an abnormality that differs from the above patterns, which is caused by either a platelet secretion defect or dense granule deficiency [17-19,21,62-65]. Sometimes, the aggregation findings are non-diagnostic (e.g. absent secondary aggregation with epinephrine and/or an aggregation abnormality with only one agonist), even when there is a definite platelet disorder (normal aggregation in a subject with definite impaired dense granule release or dense granule deficiency).

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ECAT Information:

Results of the surveys for heparin monitoring

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Introduction

The treatment of patients with heparin, both unfractionated heparin (UFH) and low-molecular-weight heparin (LMWH), is nowadays mostly monitored by the measurement of anti-Xa activity. Since 2011 the ECAT Foundation has been running a programme for external quality control for monitoring of both UFH and LMWH. For both modules plasma samples are used which are spiked with UFH or LMWH, respectively. The following is a summary of the results obtained so far.

UFH

For UFH, samples with a content in the range of 0.15 – 0.55 IU/mL were distributed to approximately 65 participants. The overall between-laboratory coefficient of variation (CVb) varied between 20 and 60%.

Figure 1 shows clearly that there is an inverse relationship between the heparin content in the sample and the CVb. It is yet unclear whether a lower CVb than 20% can be reached at higher UFH levels.

Figure 1. The relationship between the heparin concentration and the between-laboratory variation for Unfractionated Heparin (●) and Low-Molecular-Weight Heparin (▲).

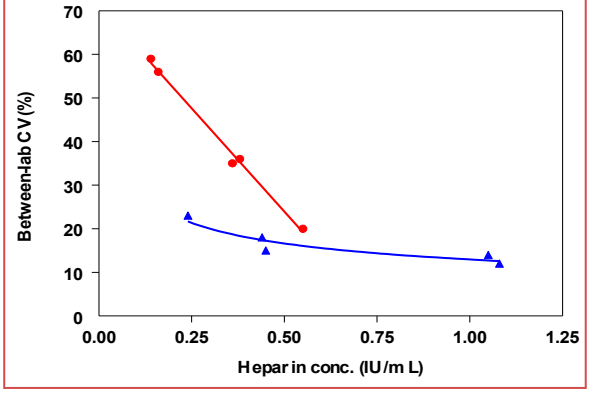

Table 1 shows an overview of the CVb for the most frequently (n ≥ 10) used methods. It is clear that, even within a method group, the variation in test results between laboratories could be considerable.

Table 1. The range of CVb for the different UFH-samples for the most frequently used methods.

Method	Average number of participants	Range of CVb (%)
Chromogenix/IL	10	22 – 42
Hyphen Biomed	16	16 – 28
Stago/Roche	24	13 – 47

Another interesting observation is related to the consensus values obtained for the most frequently used methods. Table 2 shows an overview of these consensus values.

It is clear that the consensus values obtained with the Rotachrom method are significantly lower than those for the Coamatic and Biophen methods. This is most likely explained by the fact that the Rotachrom method does not use dextran sulphate in the assay reagents while the other methods do. Dextran sulphate inhibits the binding of UFH to Platelet Factor 4 (PF4). Therefore the Rotachrom method is probably affected by the PF4 content of the normal plasma used for spiking the samples with UFH. This is a well-known phenomenon for *in vitro* spiked samples with UFH. It should be realised that this observation cannot be automatically translated into real patient samples.

From the surveys for UFH it can be concluded that there is still a considerable between-laboratory variation for the measurement of anti -Xa activity. This is specially the case in sample with a low UFH concentration.

Table 2. The consensus values (IU/mL) for the different UFH-samples for the most frequently used methods.

Sample	Chromogenix/IL Coamatic Heparin	Hyphen Biomed Biophen Heparin	Stago/Roche Rotachrom Heparin
Sample 1	0.22	0.18	0.07
Sample 2	0.21	0.22	0.09
Sample 3	0.43	0.47	0.27
Sample 4	0.45	0.47	0.27
Sample 5	0.60	0.62	0.48

LMWH

For LMWH, samples with a content in the range of 0.25 – 1.10 IU/mL were distributed to approximately 110 participants. The overall between-laboratory coefficient of variation (CVb) varied between 12 and 23%. This is significantly lower than for UFH. Figure 1 shows the relationship between the heparin content in the sample and the CVb. There is a small inverse relationship between the LMWH concentration and the CVb. However this is much less pronounced than for UFH.

In Table 3 an overview is given of the CVb of the most frequently (n ≥ 10) used methods. It is clear that, even within a method group, the variation in test results between laboratories could be considerable. Overall, it shows the Coamatic method to have the highest and the Biophen method the lowest CVb.

The consensus values obtained for the most frequently used methods are also evaluated (table 4).

The effect of UFH on the Rotachrom method was not observed for LMWH. The consensus values for the Rotachrom method are much more comparable to those of the other methods.

Table 3. The range of CVb for the different LMWH-samples for the most frequently used methods.

Method	Average number of participants	Range of CVb (%)
Chromogenix/IL Coamatic Heparin	17	14 – 23
I.L. HemosIL Liquid Heparin	10	7 – 22
Hyphen Biomed Biophen Heparin	19	5 – 15
Stago/Roche Rotachrom Heparin	45	6 - 23

On the other hand, slightly higher values are observed for the Biophen method. This is most likely a matter of calibration.

It can be concluded that for LMWH the between-laboratory variation for the measurement of anti-Xa activity is much lower than for UFH.

Final conclusion

The addition of the modules for the monitoring of UFH and LMWH by the measurement of anti-Xa activity are a useful expansion of the ECAT external quality control programme.

Table 4. The consensus values (IU/mL) for the different UFH-samples for the most frequently used methods.

Sample	Chromogenix/IL Coamatic Heparin	I.L. HemosIL Liquid Heparin	Hyphen Biomed Biophen Heparin	Stago/Roche Rotachrom Heparin
Sample 1	0.26	0.23	0.28	0.21
Sample 2	0.47	0.46	0.51	0.39
Sample 3	0.45	0.46	0.51	0.41
Sample 4	0.99	1.03	1.17	1.01
Sample 5	1.02	1.02	1.23	1.04

Case Report:
Acquired Glanzmann's thrombasthenia

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Case report

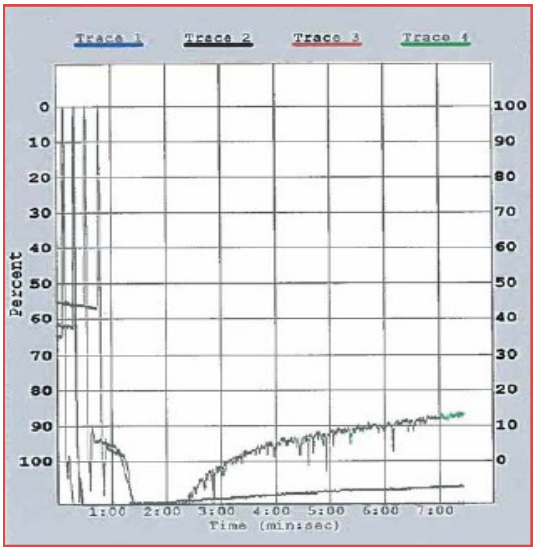
A 56-year-old man presented at the emergency department in April 2010 with gastrointestinal bleeding and mucosal haemorrhage (epistaxis and gingival bleeding). The patient is known to suffer from Evans' syndrome, a rare autoimmune disorder characterized by haemolytic anaemia and thrombocytopenia. The patient also suffered from pulmonary hypertension and liver dysfunction, for which he was treated with corticosteroids, azathioprin and bosentan (an endothelin receptor antagonist). His past medical history includes chronic digestive bleeding, due to intestinal angiomatosis, resulting in iron deficiency anaemia.

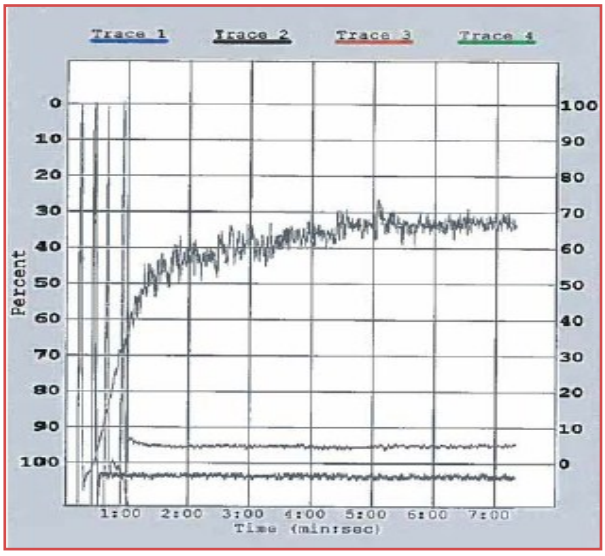
Gastroduodenoscopy at presentation exposed no bleeding focus. The complete blood count showed mild thrombocytopenia and mild anaemia (platelet count: 126 000/µl (ref. range 137 000 –370 000/µl), Hb: 13.0 g/dl (ref. range 13.3-17.7 g/dl), Hct: 38.1 % (ref. range 39.8-52.2 %)). The prothrombin time, activated partial thromboplastin time and fibrinogen were normal.

The patient was send home. However the day after, he presented with persistent melaena.

The patient was tested for acquired bleeding problems that suggested a possible defect in

Figure 1. Light transmission aggregometry of the patient. Platelet rich plasma and platelet poor plasma of the patient.
Absent or strongly reduced aggregation with all agonists, except ristocetin 1.5 mg/ml





Trace	1	2	3	4
Instrument	OPT	OPT	OPT	OPT
Reagent	ADP	ADP	Collageen	Collageen
Stirrer	2.5 µM	5 µM	2.5 µg/ml	5 µg/ml
Gain	1000	1000	1000	1000
Amplitude	0%	0%	0%	13%

Trace	1	2	3	4
Instrument	OPT	OPT	OPT	OPT
Reagent	Ristocetin	Ristocetin	Archidon	Epinephrin
Stirrer	1.5 mg/ml	0.5 mg/ml	1.5 mM	10 µM
Gain	1000	1000	1000	1000
Amplitude	79%	9%	1%	0%

primary haemostasis. The evaluation for a platelet disorder revealed an abnormal platelet function demonstrated by PFA-100 collagen/epinephrine and collagen/ADP closure times of > 300 sec. Investigations for von Willebrand's disease were negative. Light transmission aggregometry was performed. The platelet aggregation studies showed absent aggregation with ADP (2.5 and 5.0 µM), collagen (2.5 and 5.0µg/ml), epinephrine (10 µM) and arachidonic acid (1mM), but normal agglutination with ristocetin (0.5 mg/ml and 1.5 mg/ml) (Figure 1).

Because of the history of an acquired problem, with findings that suggested the diagnosis of Glanzmann's thrombasthenia, further testing was done for a defect in the platelet fibrinogen receptor GPIIb/IIIa (alphaIIb/beta3). The dysfunction of the GPIIb/IIIa receptor could not be explained by a decreased expression of the receptor on the platelets, as the expression of both the GPIIb/IIIa (CD41 and CD61) as well as GPIb/IX (CD42b) receptor with flow cytometry was normal.

Mixing studies of fresh donor platelets with the addition of patient's plasma was performed (Figure 2). The patient's plasma induced identical aggregation defects when incubated with control platelets: a partial inhibition of donor platelet aggregation with ADP, collagen, adrenaline and arachidonic acid and normal aggregation with ristocetin, confirming the blocking nature of the antibodies.

To identify the specificity of the platelet antibodies a solid phase enzyme-linked immunosorbent assay (ELISA) was used for the detection of antibodies to HLA class I antigens and to epitopes on the platelet glycoproteins IIb/IIIa, Ib/IX, Ia/IIa and IV (PAK-PLUS®, GTI). This ELISA revealed the presence of antibodies against the GPIIb/IIIa receptor, and in a lesser extent also against GPIa/IIa. Antibodies against HLA class I antigens were negative, as well as antibodies against GPIb/IX and GPIV. HPA-1a antibodies were excluded.

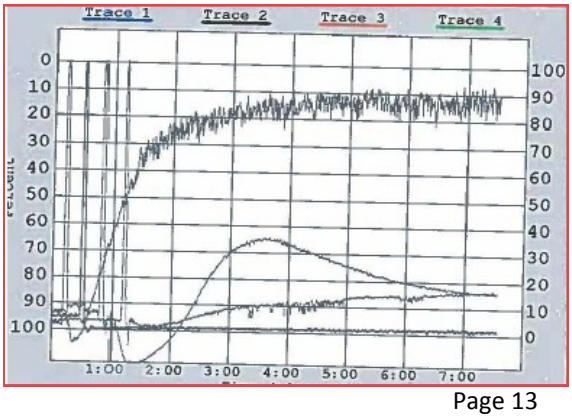
The presence of the antibodies against GPIIb/IIIa without a receptor deficiency is consistent with an acquired Glanzmann's thrombasthenia.

The patient was treated with intravenous immunoglobulins, corticosteroids and recombinant FVII (Novoseven®) resulting in a cessation of the bleeding, and recuperation from the platelet aggregation function and thrombocytopenia after two weeks of treatment.

Discussion

We describe a case of acquired Glanzmann's thrombasthenia in a patient with an underlying autoantibody syndrome, the Evans' syndrome. Acquired Glanzmann's thrombasthenia is a rare haemorrhagic disorder arising from the inhibition of the GPIIb/IIIa receptors by autoantibodies, alloantibodies or paraproteins [1]. Its diagnosis requires several laboratory assays and mixing studies [2]. Platelet aggregation studies reveal a characteristic pattern of partial or complete refractoriness of the patient's platelets to ADP, collagen, adrenaline and arachidonic acid. The GPIIb/IIIa complex, which is the receptor for

Figure 2. Light transmission aggregometry of the patient.
Platelet rich plasma of healthy donor and platelet poor plasma of the patient.
Inhibition aggregation (20%-80%) of donor platelets with all agonists, except ristocetin, by patient's plasma



fibrinogen, plays a major role in platelet function, binding plasma proteins in its activated state resulting in platelet-platelet adhesion and aggregation necessary for haemostasis. Platelet aggregation in response to ristocetin which acts through binding of Von Willebrand Factor to GPIb occurs normal. Mixing patient plasma with donor platelets resulting in a comparable platelet aggregation pattern indicates the presence of a circulating antibody against the GPIIb/IIIa receptor.

Flow cytometry may have diagnostic value for examining the platelet receptors by monoclonal antibodies. However, variable flow-cytrometric results for examining the GPIIb/IIIa expression have been described and might be due to a difference in the epitope specificity of the monoclonal antibodies used against GPIIb/IIIa [3, 4]. The normal expression of GPIIb/IIIa in one assay rules out the congenital deficiency of the glycoprotein receptor. In this case, the expression of GPIIb (CD41) and GPIIIa (CD 61) was normal. The blockade of those receptors by circulating antibodies (demonstrated by ELISA) was supported only by the mixing studies in light transmission aggregometry.

Because the patient had a history of one-platelet transfusion (transfused with single donor platelets in 2008 for severe thrombocytopenia), before the onset of the bleeding disorder, alloantibodies responsible for platelet dysfunction cannot be excluded. However, the rate of alloimmunization towards platelet-specific antigens (HPA) is low compared to alloantibodies induced by the HLA system. HLA antibodies and HPA-1a antibodies were excluded in this patient. Moreover, the patient had been diagnosed with Evans' syndrome with periods of anaemia and thrombocytopenia in the past without bleeding tendency. Hence, it is possible that an autoimmune mechanism may have resulted in the development of autoantibodies. The improvement of the patient's platelet function after immunosuppressive therapy implies a reduction of a putative autoantibody. Therefore, it appears reasonable to conclude to an aetiologic link between his thrombasthenia and the autoimmune Evans' syndrome.

Antibody-mediated loss of platelet function described in patients with antibodies specific for GPIIb/IIIa complex is usually not accompanied by thrombocytopenia [1]. In contrast to autoimmune thrombocytopenia (AITP) these antibodies do not destruct platelets (due to their subclass IgG2 or IgG4). In this patient thrombocytopenia had existed long before the bleeding history started, and therefore we can assume that it was not induced by circulating GPIIb/IIIa antibodies.

Acquired Glanzmann's thrombasthenia has been reported in association with lymphoproliferative diseases (Hodgkin and non-Hodgkin's lymphoma), acute leukaemia, drugs, HIV infection, other autoimmune disorders (e.g. immune thrombocytopenic purpura (ITP)), and also in otherwise healthy persons [5-8]. Patients present mainly with mucocutaneous bleeding. Bleeding can be severe, e.g. intracerebral haemorrhage is reported.

Response to immunosuppressive treatment and recombinant factor VII is variable [9]. In the acute phase, while awaiting the effects of immunosuppressive therapy, plasma exchange may be successful [10]. Treatment of the underlying disorder might induce remission. Spontaneous resolution is described in a minority of idiopathic cases [5].

Although it might be a rare event, one should be aware of acquired Glanzmann's thrombasthenia as a cause of an unexpected disorder in primary haemostasis in patients with underlying disease.

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Literature Review:
Interference of rivaroxaban in one-stage and chromogenic factor VIII:C assays

(V. Tichelaar *et al*. Thromb Haemost 2011; 106: 990 – 992)

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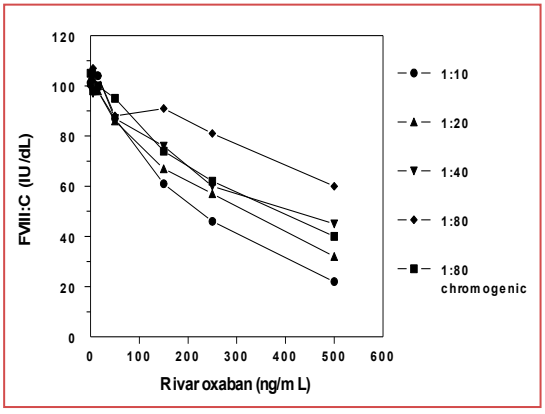
Rivaroxaban (Xarelto®) is one of the new anticoagulation drugs and is a direct competitive inhibitor of factor Xa. Rivaroxaban does not only bind to free factor Xa but also to factor Xa bound in the prothrombinase complex [1]. It has been reported that the use of Rivaroxaban can affect several laboratory tests, especially those in which factor Xa is involved [2-7]. In a recent publication Tichelaar *et al* described the interference of Rivaroxaban in the measurement of factor VIII activity (FVIII:C) by using both a one-stage clotting assay and a chromogenic assay [8]. In their study they noticed that FVIII:C was more often lower in patients on Rivaroxaban than in patients on low-molecular-weight heparin in combination with vitamin K antagonists. They looked in more detail into the interference of Rivaroxaban in the FVIII:C assays by spiking normal citrated plasma with different Rivaroxaban concentrations (0 – 500 ng/mL). FVIII:C was measured with the one-stage clotting assay in 1:10, 1:20, 1:40 and 1:80 sample dilutions. The chromogenic assay was performed with 1:80 diluted samples.

Figure 1 The *in-vitro* relationship between the Rivaroxaban concentration and FVIII:C measured at different dilutions.



In figure 1 an overview of the results obtained is given (for details see table 1 of ref. 8).

On the basis of the results obtained the authors indicated that a dose-dependent interference with Rivaroxaban concentrations higher than 50 ng/mL was observed in the one-stage clotting assay and with a Rivaroxaban concentration higher than 150 ng/mL in the chromogenic assay.

They also investigated the effect in 6 volunteers after an oral intake of 20 mg Rivaroxaban. After 4 hrs of intake the average decrease in FVIII:C was 33 IU/dL with a 1:10 dilution in the one-stage clotting assay and 18 IU/dL in the

chromogenic assay. However there was a large individual difference in the observed effect, with one volunteer showing almost no effect. The authors concluded that it should be realized that Rivaroxaban could influence any FXa-dependent coagulation assay, including the one-stage and chromogenic FVIII:C measurements.

From this publication it is clear that awareness of the interference of the new oral anticoagulation drugs in general and Rivaroxaban in particular in coagulation assays is very important.

For further details about this publication see reference 8.

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CLOT-ED Website: "Focus Articles"

In the educational part of the CLOT-ED website the item **Focus Articles** can be found. This item contains a diversity of in-depth articles in the field of Hemostasis and Thrombosis.

The articles are divided in the following topics: Pre-analytical Phase, Analytical Phase, Post-analytical Phase, Disorders of Hemostasis & Thrombosis and Articles of General Interest.

Articles related to the Pre-analytical, Analytical or Post-analytical Phase describe several aspects, such as biological or methodological variation, which may have significant impact on patient outcome as regards diagnosis, treatment, and therapeutic monitoring. Understanding how these variations may affect coagulation laboratory testing will lead to more reliable test results and will therefore lay a foundation for quality improvement.

The topic "Disorders of Hemostasis and Thrombosis" gives more insights into specific diseases and treatments. For instance, here you can find an article on the role of ADAMTS13 in Thrombotic Thrombocytopenic Purpara or the role of direct Thrombin Inhibitor Anticoagulants (DTIs) in the Management of Patients with Heparin-Induced Thrombocytopenia (HIT).

Finally in "Articles of General Interest" the articles describe general issues, such as Aspirin Resistance, establishing therapeutic ranges for heparin and a general article about the coagulation cascade.

Reading these Focus Articles should provide laboratory professionals with substantial information relevant to their daily practice. Enjoy!

Announcements

New modules

At the beginning of 2012 we start with 5 new modules on the quantitative measurement of new anticoagulation drugs. The following new modules are beginning: Dabigatran, Argatroban, Orgaran, Fondaparinux and Rivaroxaban. Samples for these modules will be sent out 2 times a year (survey 2012-2 and 2012-4). If you are interested and want to participate or need more information, please contact the ECAT Foundation at info@ecat.nl.

Special issue The Clotting Times

At the end of 2011 we distributed to each participant in the ECAT external quality assessment programme the special issue for 2011 of The Clotting Times. This issue focuses on acquired coagulation inhibitors, clinical and laboratory aspects. There is still a limited number of these booklets available. You can obtain an extra copy (or copies) for € 10,= (excl VAT) per booklet. If you are interested please contact the ECAT Foundation at info@ecat.nl.

Previous issues of The Clotting Times

All issues of The Clotting Times are available on the educational section CLOT-ED of the ECAT website.
http://www.ecat.nl/the-clotting-times-2

ECAT EQA Programme information

Detailed background information about the ECAT external quality assessment programme is described in the Programme Manual. This manual can be downloaded from our website and is available in the “Information” section.

Upcoming Events

* May. 03 - 05, 2012:
  THSNA 2012
  www.thsna.org
* Jun. 07 - 11, 2012:
  Platelets 2012 International Symposium.
  www.platelets2012.org
* Jun. 27 - 30, 2012:
  58th Annual SSC meeting.
  www.ssc2012.org
* Jul. 01 - 05, 2012:
  21st International Congress of the ISFP.
  www.isfp2012.com
* Nov. 08 - 09, 2012:
  8th ECAT International Symposium 2012.
  www.ecat.nl
* See for more events the Calendar on
  www.ecat.nl/calendar-2

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