BRITISH JOURNAL OF BIOMEDICAL SCIENCE, 2016
VOL. 73, NO. 1, 10–20
http://dx.doi.org/10.1080/09674845.2016.1156865

Platelet function testing: from routine to specialist testing

David Gurney

Department of Haematology, University Hospital Bristol, Bristol, UK

ABSTRACT

Platelets have many functions within the haemostatic system, and when these actions are diminished for whatever reason, a bleeding tendency can manifest. Unravelling the reason(s) for this bleeding can be complex due to the multiple roles platelets perform. This review seeks to explain each level of platelet testing moving from those performed at local hospital laboratories to those performed by specialist centres and university research departments. It will examine the testing available and discuss when to move on to additional testing.

ARTICLE HISTORY
Received 1 December 2015
Accepted 17 February 2016

KEYWORDS
Platelet; function; aggregation; analysis; analytical techniques

Introduction

In 2006, Lord Carter published his review of NHS pathology services [1] outlining ways by which laboratories could improve efficiency and ultimately save money. One of these proposals was to establish specialist referral laboratories for specialised analyses. However, all testing begins at local level and this review aims to look at how the initial testing phase for platelet disorders can be operated, and when to move on to more advanced screening.

Normal platelet function

Platelets are anucleate fragments produced from megakaryocytes, found in the bone marrow. The megakaryocytes produce 10^{11} platelets a day with a lifespan of approximately 10 days, with a normal adult platelet count being 150–400 x 10^9/L. The resting platelet is discoid in shape and about 4 µM in diameter. This state is maintained by intact vessel endothelial cells producing nitric oxide (NO) and prostacyclin (PGI_2). Once there is a breach in the vasculature, the concentration of NO and PGI2 reduces rapidly. Collagen, laminin, fibronectin and von Willebrand factor (VWF) are also exposed at the site of injury. Activation of the platelet is viewed in distinct phases. Initially, the platelets and VWF agglutinate via glycoprotein (GP) Ib. This process is reversible and when analysing platelet function tests is referred to as 'first phase'. As platelet activation continues, they anchor to the damaged area via the collagen exposed from the sub-endothelial matrix, resulting in exposure of receptors on the platelet surface and release of activators from internal storage pools. This process is irreversible and is the initiation phase for thrombus formation. The activated receptor GPIIb/IIIa cross-links with fibrinogen to stabilise the agglutinating platelets. This phase is aggregation or second phase. These processes are not happening in isolation, the platelet is moving in a rolling like motion along the break until the cross-linked fibrinogen/fibrin anchors it securely to the site of injury. During this process, the storage pool contents are locally recruiting further platelets to increase the size of the thrombus and leucocytes to help prevent infection. This process is primary haemostasis.

In conjunction with this, the platelet membrane acts as a phospholipid surface for the activation components prothrombinase and tenase factor complexes to accelerate the coagulation process. This whole process plugs the break in blood vessels, thus preventing blood loss and barring pathogens from entering the bloodstream.

Platelet disorders

The first step to accurate platelet function testing is to ensure that the sample is fit for purpose. Pre-analytical variability, such as activation,[2] clots and interfering substances, should be discounted as early as possible. It is recommended that a pre-analytical interview and questionnaire (such as the one detailed in [3]) are carried out by a suitably qualified clinician to minimise this interference, as a number of over the counter medications (primarily aspirin) and foods [4–6] have been implicated in the reduction in platelet efficacy. Hereditary platelet function disorders are a heterogeneous group of common bleeding disorders that require specialist technical

CONTACT David Gurney david.gurney@uhbristol.nhs.uk

and clinical expertise. Those disorders can be categorised into two sub-groups; those that are a product of the number of platelets and those that are a product of the platelet function. Disorders resulting in a reduction of platelet numbers can also have functional aspects. This is particularly evident in macrothrombocytopenias such as Bernard Soulier Syndrome (BSS) and the Myosin Heavy chain gene 9 related disorders (MYH9RDS).

Functional disorders can further be divided into those affecting the external receptors such as Glanzmanns thrombasthenia (GPIIb/IIIa) or BSS (GPIb), those affecting the internal biochemical pathways (Scott's and Stormorken syndrome) and disorders affecting the internal organelles (storage pools disorders such as Hermansky–Pudlak syndrome).

Testing for platelet disorders

There has been recent guidance from both the British Society for Haemostasis and Thrombosis [7] and the International Society of Thrombosis and Haemostasis [8] regarding platelet function testing, both producing useful algorithms for diagnosis.

Number and size

The first line laboratory test for a suspected platelet disorder is the full blood count (FBC). This automated measure includes leucocyte count, haemoglobin, erythrocyte count, red cell parameters and platelet count. This will provide the clinician with a platelet count and platelet size, although this is not without its limitations [9,10] and all laboratories performing automated FBCs should be aware of these limitations. Modern analysers also provide parameters such as optical platelet counts O-PLT), mean platelet volume (MPV), platelet distribution width (PDW) and immature platelet fractions (IPF).[11,12] Small red cells with a low mean cell volume (MCV), such as those found in iron deficiency anaemia, or blast fragments in a newly diagnosed leukaemia, may be counted as platelets leading to spurious elevation of the platelet count. Macrothrombocytopenias with a raised MPV and a right shift in PDW can lead to falsely elevated erythrocyte counts and reduced platelet counts as platelets are counted within the analysers red cell gates (Figures 1 and 2). In larger laboratories, access to fluorescence flow cytometry analysers may give more accurate platelet counts at a lower level such as those below 20×10^9/L [13,14]; however, less specialised laboratories would still be able to produce a manual platelet count estimate (the inter-observer coefficient of variation (CV) on manual counting is high (10–20%) [15]), using a Neubauer haemocytometer. Patients with thrombocytopenia may just require platelet transfusions to increase the patients' platelet count [16]; however, when functional defects are suspected, further analysis may be required.

Platelet function screening tests

Establishments without the workload or laboratory experience to perform full platelet function testing may wish to employ one of the number of point of care (POC) analysers available. Whilst convenient, staff need to be aware of the analysers' methodology and ensure they are used within their technical constraints. The most common POC analyser available is the Platelet Function Analyser [17] (PFA-100/PFA-200) from Siemens (Siemens UK, Frimley, Surrey). This analyser uses a cartridge-based system to pass a whole blood sample through a capillary at arterial pressure passing through an aperture covered by a membrane coated with collagen and either adenosine diphosphate (ADP) or epinephrine. The time taken for the platelets to occlude the aperture and produce a blocking force opposite to that of the pressure from the analyser is measured as the closure time. This measurement is only valid when the platelet count and packed cell volume (PCV) are within the given parameters,[18] and it is advised that a FBC should be run in parallel with this assay to check these criteria are being met. The PFA-100 can yield useful results when used to predict pre-procedure bleeding, where a negative result will discount an overt platelet defect. It is known to be lacking in diagnosing or monitoring bleeding disorders and their management regardless of result,[19] to such an extent that the current guidelines list it as an optional screening test [20] due to the sensitivity being 86% and the specificity being 89%.[21]

Another popular POC device is the VerifyNow (Accriva distributed in the UK by Elitech, Berkhamstead, Herts). This device had been designed for monitoring anti-platelet medication,[22] unlike the PFA-100 which is marketed as a diagnostic tool. The VerifyNow analyser is also a cartridge-based system, each cartridge being specific for the anti-platelet agent on test. Cartridges are available for aspirin, $P2Y_{12}$ blockers and GPIIb/IIIa antagonists. Blood passes across beads coated with agonists specific for the drug being monitored, for example to measure GPIIb/IIIa antagonists the beads are coated with fibrinogen, aspirin has cationic propyl-gallate which is a novel COX-1 activator and $P2Y_{12}$ has a novel pathway activators. As the blood passes through the cartridge across the beads, they agglutinate and more light passes through the cartridge. When the light reaches a given value the assay stops. The time taken to reach the stop point is reported. If the anti-platelet agent is present, it prevents the beads from aggregation and the timed response is longer.

Laboratory staff should be aware of POC platelet testing outside of the laboratory, as there could be the possibility of quality assurance issues. The most common of these are the rotational elastomeric devices, the rotational elastomeric analyser (ROTEM) (TEM UK Ltd., Hartlepool, UK) and the thromboelastograph (TEG) (Haemonetics, Coventry, UK).[23] These devices measure clot strength using torsion, with blood in a stationary

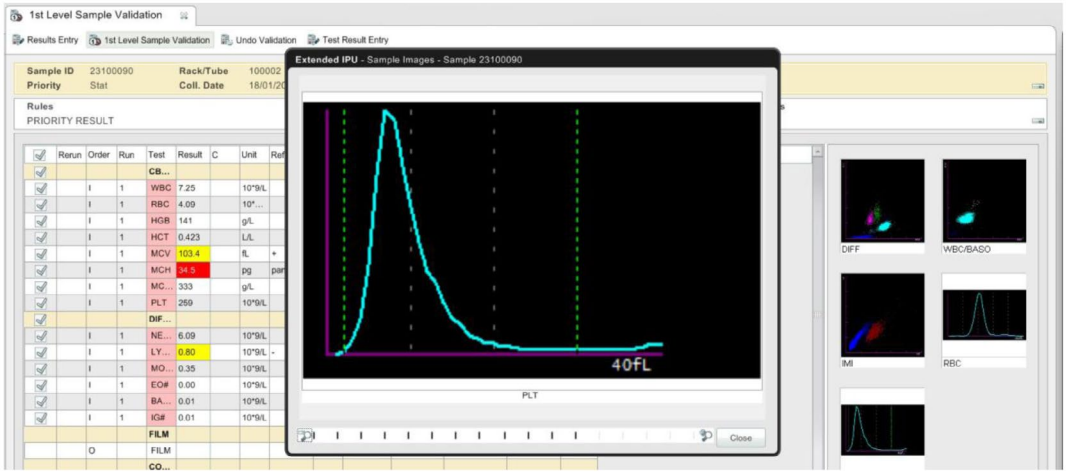

Figure 1. Normal platelet histogram produced by a Sysmex XE analyser showing the normal FBC parameters, scatter plots and the 40fL marker. (Sysmex, Bucks, UK).

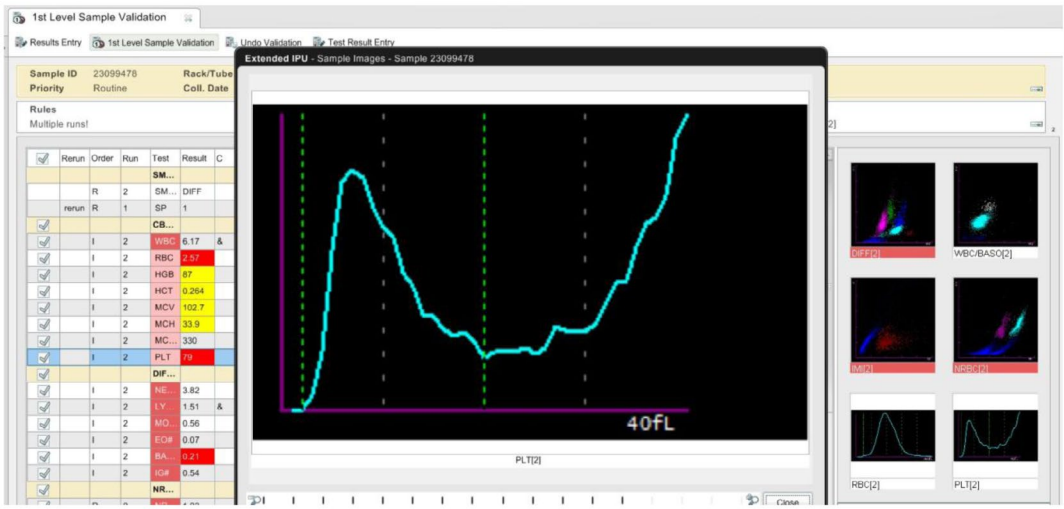

Figure 2. Platelet histogram showing small red cells/large platelets produced by a Sysmex XE analyser also displaying the normal FBC parameters, scatter plots and the 40fL marker (Sysmex, Bucks, UK).

cup with a rotating pin, or a rotating cup and a stationary wire. As the clot forms the pin is exposed to increasing resistance. This resistance is converted into a trace (Figure 3) from which information such as speed of clot formation, strength of the clot and breakdown can be derived. The way the parameters compare across analysers and the influences are shown in Table 1.[24]

Other devices are available, but have a small market share, such as Cone and Plate(let), Xylum clot analyser and the haemostatometer [25–27] and tend to be the preserve of research laboratories.

If these assays do not provide enough data and there is still a strong clinical suspicion of a platelet defect, then further laboratory testing is required.

Specialist platelet function testing

The next stage of analysis for platelet disorders is to move from global assessment of the platelet within the haemostatic system to testing specific functionality of the patients' platelets. There are currently two main methods for analysing platelet function, light transmission aggregometry (LTA) sometimes called Born aggregometry after its developer [28] and whole blood aggregometry (WBA), also called multiple electrode aggregometry (MEA), after the assay device. As its name suggests, LTA works by passing light through the platelet suspension, known as platelet-rich plasma (PRP). PRP is prepared from whole blood, and centrifuging slowly so the red and white cells are removed, but the platelets retained in suspension. If the patient has an increased MPV these platelets may be removed with the red cells producing a PRP platelet count $<150 \times 10^9$/L below the current recommended threshold.[29] If the patient is already thrombocytopenic with a FBC platelet count $<100 \times 10^9$/L producing a viable PRP will be problematic. If the PRP platelet count is below the recommended value, proceeding with platelet aggregation, it should be discussed with the requesting clinician.

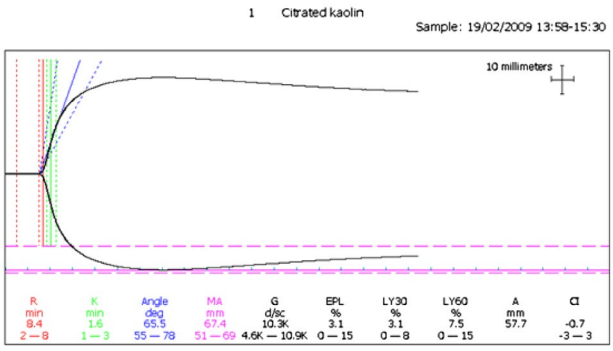

Figure 3. Normal visco-elastic trace (TEG/ROTEM) showing reaction time (*R*), kinetic time (*K*), alpha angle (Angle deg), maximum amplitude (MA), Lysis 30 (LY30) and Lysis 60 (LY60).

Table 1. How the ROTEM/TEG parameters equate and their parameters and modifiers. Adapted from [24].

TEG	ROTEM	Measurement of	Major influence by
Reaction time (R)	Clot time (CT)	Initial fibrin formation	Coagulation factors
Kinetics (K)	Clot formation time (CFT)	Speed of clot formation	Fibrinogen, Factor XIII, and platelets
Alpha angle (α)	Alpha angle (α)	Speed of clot formation	Factor XIII, platelets, and fibrinogen
Maximum amplitude (MA)	Maximum clot firmness (MCF)	Maximal clot strength	Platelets and fibrinogen
Lysis (LY30, LY60)	Lysis (LY30, LY60)	Fibrinolysis	Plasmin

LTA compares the PRP with a plasma sample in which there are no platelets, known as platelet poor plasma (PPP). Chemicals known to modify specific areas of platelet response are referred to as agonists. These agonists (either singularly or in combination) are added to the reaction cuvette and as the platelets are activated they aggregate, as the resulting aggregates are removed from suspension, more light passes through the cuvette and the analyser creates a trace. An example of LTA platelet aggregometer trace can be seen in Figures 4 and 5. The wide variation of disorders and trace possibilities is shown in Table 2,[3] an example of a storage pool disorder trace is shown in Figure 6. MEA can utilise either PRP or whole blood, the aggregating platelets are detected by a change in resistance across two parallel wires. As the aggregate increases so does the resistance signal, this signal is translated into a tracing, an example of which is shown in Figure 5. Both assays measure the speed to aggregation denoted by the primary slope (PS) and the when this reaction slows, primary aggregation (PA). Further aggregation may take place in which case a secondary slope (SS) and aggregation (SA) will be displayed. Maximum aggregation (MA) is the point at which the aggregation is strongest and final aggregation (FA) is the aggregation value when the assay is complete. If the aggregates are stable then the MA and the FA will be equal. If there is, disaggregation of the platelet clumps the MA could be greater than the FA.

Agonists used routinely for both systems are ADP, collagen, arachidonate, epinephrine and ristocetin. [4,7,20,30] The three main ADP receptors on the platelet surface are $P2Y_1$, $P2Y_{12}$ and $P2X_1$. Activation of both $P2Y_1$ and $P2Y_{12}$ receptors is needed for a full response to ADP and the mechanism plays a role in the conformational change and activation of GPIIb/IIIa (also known as integrin $\alpha_{IIb}\beta_3$ and CD41/61). The $P2X_1$ receptor is an ion channel involved in calcium flux, regulating calcium flow needed for full aggregation. ADP only gives a first phase response at low concentrations such as <1 µM with higher levels, such as 10 µM, the first and second phase reaction shows a continuous full aggregation trace. Collagen activates the platelets via four glycoproteins; the roles of GPIa/IIa ($\alpha_2\beta_1$, CD49b/CD29) and [31] GPVI (p62) [32] are well defined, but those of GPIV (CD36) [33] and GPIIb/IIIa [34] are less well understood. In the platelet, arachidonate (arachidonic acid) is converted by cyclo-oxygenase to thromboxane A_2, which is released via granule secretion to amplify localised platelet activation. Epinephrine interacts with $\alpha_2$andregenic receptors on the platelet surface, enhancing the effects of other agonists. Ristocetin (an antibiotic developed in the 1950s but no longer used) causes *in vivo* mediated agglutination of platelets via VWF, and so can be utilised as an *in vitro* platelet agonist for assessing VWF/platelet interactions.

Testing can be done with other agonists which probe the platelet response further than those used routinely. Thrombin receptor agonist peptide (TRAP) is a small peptide, either six or fourteen amino acids long that activates the thrombin receptor on platelets known as the protease-activated receptor 1 (PAR-1). This is a very useful tool, as using native thrombin to activate the receptor would cause the fibrinogen to fibrin conversion in the PRP mixture, causing white thrombus formation in the reaction vessel. Elucidation of a poor response to arachidonate can be determined by investigating the reaction product, thromboxane A_2 and its receptor. As thromboxane A_2 has an extremely short half-life (measured in milliseconds), a stable thromboxane mimetic 9,11 dideoxy-9α,11α-epoxymethano-prostaglandin $F_{2\alpha}$ commonly referred to as U46619 is used. U46619 will

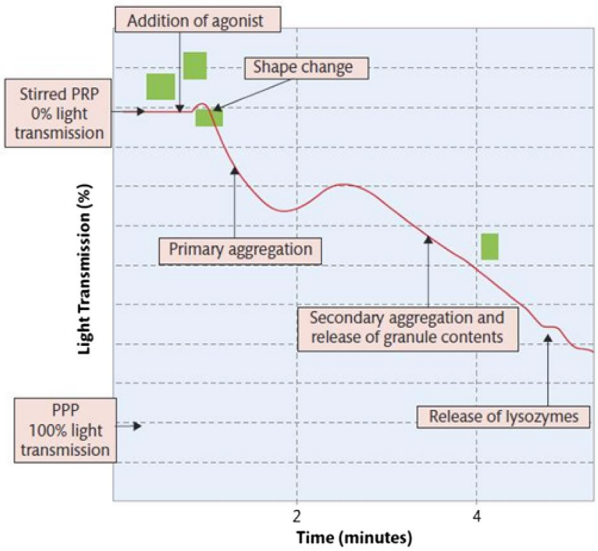

Figure 4. Stages of platelet agglutination and aggregation. Used with permission [3].

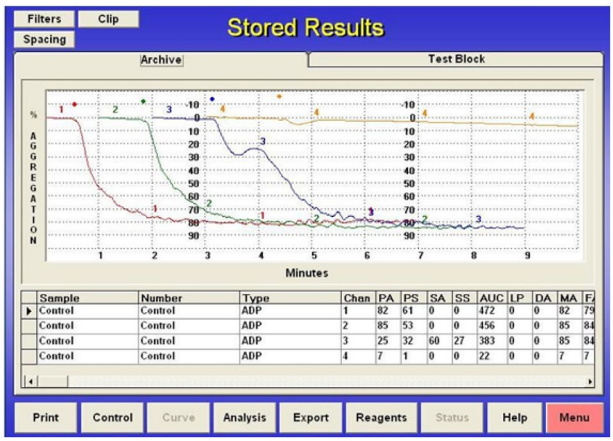

Figure 5. Normal platelet responses for given concentrations of ADP displayed on a PAP-8E platelet aggregometer showing values for primary aggregation (PA), primary slope (PS), secondary aggregation (SA), secondary slope (SS), area under the curve (AUC), lag phase (LP), disaggregation (DA), maximal aggregation (MA) and final aggregation (FA). (Biodata, distributed by Alpha Labs, Hants, UK).

verify the function of the thromboxane receptors. In the investigation of collagen binding, separating which receptor is at fault can be useful. Convulxin is a fraction isolated from the venom of the South American or Neotropical rattlesnake (*Crotalus durissus terrificus*) activates GPVI specifically. An artificial peptide has been developed with the same properties ((collagen related peptide [CRP]) which can be produced in the laboratory and is therefore safer to manufacture. Both agonists give information on the function of both GPVI and GPIa/IIa activity. A23187 is a calcium ionophore,[35] which causes a release of arachidonate from the platelet membrane due to activation of cytosolic phospholipase A_2 ($cPLA_2$) via an increase in [Ca^{2+}] concentration and can therefore be used in calcium and arachidonate release studies.[3] Due to the complexity of platelet responses and the semi-qualitative nature of the assays, interpretation of data output of these tests is a highly skilled job and should be carried out by laboratory staff with sufficient regular experience in the field.[36] If platelet aggregation does not yield definitive results (or the results are negative) and there is a strong clinical suspicion of a bleeding disorder further testing should be carried out.

Specialised platelet analysis

Further testing focuses on two major aspects of the platelet, the external receptors and the internal contents. Analysis of the platelet membrane receptors and contents requires equipment that is more advanced. Referral of these analyses should be to a specialist centre with expert clinicians and scientists.

Analysis of the external receptors

Flow cytometers were developed from the automated cell counter that was patented in 1953 by Wallace

Table 2. The wide variation of disorders and trace possibilities (modified from [3]).

Condition	Platelets		Agonist							Further investigation
	Count	Size	ADP	Coll	Ri	AA	Epi	TRAP	U46619	
Glanzmanns thrombasthenia	N	N	0	0	1	0	0	N	N	Flow cytometry for GPIIb/IIIa/ Genetic testing
Bernard-Soulier syndrome	Low	Large	N	N	0	N	N	N	N	Flow cytometry for GPIb/Genetic testing
COX Deficiency	N	N	1/N	R	N	R	N	N	N	Enzyme pathway analysis
Drug induced (aspirin shown)	N	N	1	R	N	R/0	N	N	N	Stop aspirin (clinical review)
Thromboxane deficiencies	N	N	1/N	R	N	R/0	N	N	N	Nucleotide analysis
Thromboxane receptor defects	N	N	1/N	R	N	R/0	N	N	N	Receptor analysis
$P2Y_{12}$ receptor defects	N	N	R/0	N	N	N	N	N	N	Receptor analysis
Ehlers Danlos syndrome	N	N	N	N	N	N	N	N	N	Genetic testing
VWD	N	N	N	N	0/R/N	N	N	N	N	VWF screening/Genetic testing
Storage Pool defects										
Grey platelet syndrome	Low	N	R/1	N/R	N	N	N	R	N	Electron microscopy
Chediak-Higashi syndrome	N	N	R/1	R	N	N	R	N	N	Nucleotide analysis/Genetic testing
Hermansky Pudlak syndrome	N	N	R/1	R	N	N	R	N	N	Nucleotide analysi/Genetic testing
Wiskott–Aldrich syndrome	Low	Small	R/1	R	N	N	R	N	N	Genetic analysis
Membrane anomalies										
Scott syndrome	N	N	N	*N*	N	N	N	N	N	Flow cytometry Annexin A5 analysis/Genetic testing
Paris-trousseau syndrome	Low	Large	N/R	*N*	N	N	N	R/1	N	Electron microscopy/Genetic testing
Stormorken syndrome	Low	Large	N	R	N	N	N	N	N	Flow cytometry Annexin A5 analysis/Genetic testing

Note. Coll – collagen, RI – Ristocetin, AA – arachidonic acid, Epi – epinephrine, N – normal, R – reduced, 1 – first phase only, 0 – absent.

Coulter. These cell counters used a stream of fluid passing through a charged gate. Each time a cell passed within the gate, an electrical impulse would be created. The size of the impulse denoted the size of the cell. Using size-based guidance, leucocytes being the largest, then erythrocytes and finally platelets, the smallest of these cells could be classed into groups and automated blood cell count could be performed. As technology developed, the fluid dynamics of the system became more controlled and the underlying electronics improved with micro-circuitry, enabling the development of advanced analysers. A further development was the use of lasers to interrogate cells passing through the gate. The laser was used to evaluate the transparency and shape of the cell. Monoclonal antibodies were discovered during the same period and the laser was implemented further to excite fluorescent dye markers, which were attached to antibodies directed at the receptors of interest.

As some platelet disorders are caused by the reduction or mutation in receptors on the platelet surface, these assay methodologies can be used in the diagnosis of these disorders.[37] Receptors such as the GPIIb/IIIa (CD41/61) and GPIb (CD42b) on the platelet surface can have had monoclonal antibodies raised against them. When these antibodies are conjugated with a fluorescent molecule, it allows rapid diagnosis of Glanzmann's thrombasthenia (GT) [38,39] and Bernard Soulier (BSS),[40,41] respectively. Further targets have been sought for platelet analysis [42] including P-selectin (CD62P) and the commercial antibody PAC-1 (BD Biosciences, Oxford, UK) are only expressed on activated platelets and are used as markers for this. Platelets can exhibit receptor proteins on their surface, which are non-functioning, such as Glanzmanns Type III, so diagnostic data should be considered holistically rather than isolated test results.

Thienopyridines (such as clopidogrel and prasugrel, commercial $P2Y_{12}$ blockade agents) activity can be measured using the vasodilator-stimulated phosphoprotein (VASP) assay.[43] VASP is an intracellular platelet protein that is non-phosphorylated at rest. Regulation of the VASP phosphorylation state is by the cyclic adenosine monophosphate (cAMP) pathway, which is activated by prostaglandin E1 (PGE1) and de-phosphorylated by ADP. Mediation of the ADP de-phosphorylation is by the $P2Y_{12}$ receptor. Measuring the phosphorylation state of the VASP molecule gives indirect information on the state of the $P2Y_{12}$ receptor. The assay involves the analysis of VASP phosphorylation after incubating PRP with PGE_1 and ADP, compared to PGE1 alone, using an antibody against the phosphorylation site of VASP. When a thienopyridine molecule inhibits the ADP binding to $P2Y_{12}$, VASP phosphorylation decreases. If the phosphorylation does not decrease, the assumption is that there is no drug present. Flow cytometry on PRP can also probe Annexin A5 to analyse phosphatidylserine on the platelet surface. This is a diagnostic parameter for Scott syndrome.[44] Flow cytometry methods are also available for microparticle analysis [45]; now that the technology available in both the instruments and software means the ability to gate smaller particles that are previously possible. For microparticles, the fluorescent antibody targets depend on which cell they originated from, for platelet microparticles the target of choice are the membrane glycoproteins. Heparin induced thrombocytopenia (HIT) can also be diagnosed as the patients plasma is incubated with heparin and donor control PRP.

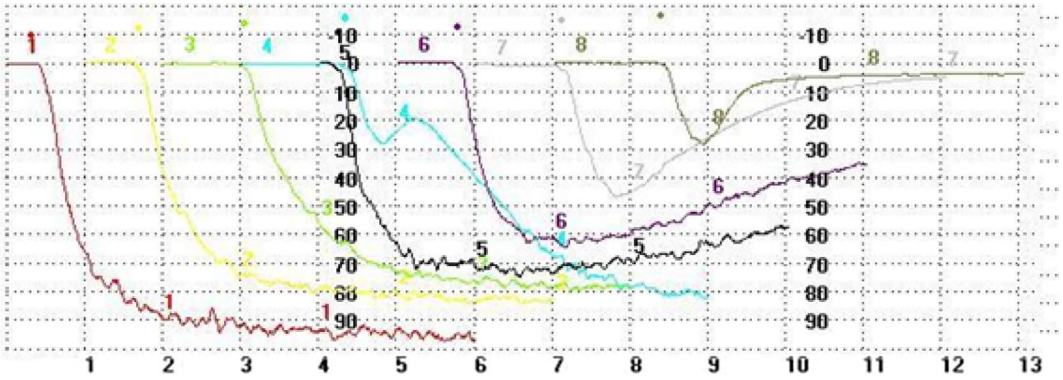

Figure 6. ADP Traces from a control and a patient with storage pool disorder run on a PAP-8E, note reversal of aggregation even at high ADP concentrations. (1) ADP 10 µM Control, (2) ADP 5 µM Control, (3) ADP 2 µM Control, (4) ADP 1 µM Control, (5) ADP 10 µM Patient, (6) ADP 5 µM Patient, (7) ADP 2 µM Patient and (8) ADP 1 µM Patient.

Subsequent detection of the activation marker Annexin A5 indicates the patients plasma has the potential to activate platelets in the presence to heparin, therefore denoting HIT positive.[46]

Initial analysis of the contents of the platelet

The most common defect of platelet function is a storage pool disorder, where the contents of the alpha or delta granules are either absent or unable to be released.[47] The most common of these is the dense, or delta storage pool disorders.[48] These are characterised by a biphasic response to ADP at high concentrations, first phase with reversal at lower concentrations in LTA (see Figure 6) and weak release and response in WBA/MEA. Therefore, the first line of investigations undertaken at this stage will be analysis of the vesicle contents. The most abundant of these are ADP and adenosine triphosphate (ATP), collectively known as nucleotides. They also contain calcium ions, magnesium ions, CD62P, CD63, lysosome associated membrane protein (LAMP-2), histamine, GPIIb/IIIa and GPIb.

Platelet nucleotides were first measured using preparations prepared from the Common Eastern firefly (*Photinus pyralis*), commonly known as Big Dipper after the star constellation. These extracts produce luminescence in proportion to the ATP concentration. More recently the active ingredient, luciferase, has been produced artificially and therefore is readily available in kit form. ATP Luciferin-luciferase reagent is a stable light emitter with decay rate of ATP and light emission around 0.5%/min. This stability of emission makes it possible to use simple single tube luminometers. It is also suitable for ATP measurements down to 10–15 mol. The Chronolog 700 series whole blood analysers (LabMedics, Stockport, Cheshire) are able to measure platelet aggregation and luminescence on a combined platform, visualising nucleotide release during aggregation (see Figure 7). However, the Chronolog system only confirms the lack of release during the aggregation process. The PRP can be processed into a lysate, which is a sample preparation were the membrane components of the platelet (both external and organelle) are broken down and removed. The lysate is then composed of just the cytosolic component. Measurement of the luminescence of the resulting lysate sample is done in a luminometer. A standard curve is prepared with a known standard ATP solution and the lysate result compared. The addition of phosphoenol pyruvate to the reaction converts the ATP into a measurable, stable form in the ATP tube (see Figure 8). Excess pyruvate kinase is added to the reaction causing all of the ADP to be converted into ATP. Subtraction of the pre-conversion ATP value from post-conversion value gives the residual ADP value. This will record the total nucleotide content of the platelet regardless of release mechanism. ADP:ATP ratio is a diagnostic parameter when investigating release defects, due to large proportion of ADP residing in the dense granules; however, up to 30% of ATP is found in the cytoplasm powering cellular reactions.

With the combination of the Chronolog system and the analysis technique described above, enzymatic disturbances within the platelet can be diagnosed. An example would be a protein kinase defect in which the

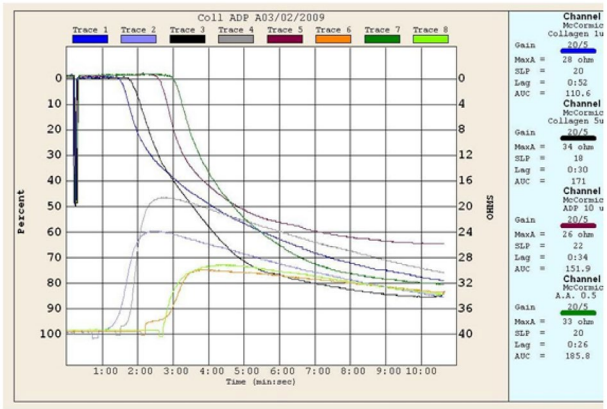

Figure 7. Chronolog 700 whole blood lumi-aggregometer tracing showing both platelet aggregation traces (top) and nucleotide release (bottom) showing Channel information Maximum aggregation (MaxA), Secondary lag phase (SLP), Lag phase (Lag) and Area under the curve (AUC).

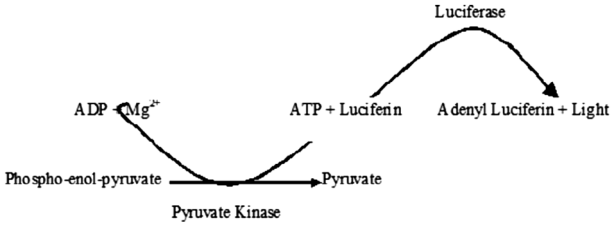

Figure 8. Graphic showing the reaction principle of the ATP Luciferin–luciferase reaction.

patients' platelets have normal nucleotide levels but internal enzymatic signalling is defective;[49] thus the granules are present but are not being activated and moving to the surface to release their contents.

Storage pool disorders are not limited to the lack of the dense/delta granules. Grey platelet syndrome [50] is the lack of content in the alpha granules. It causes them to appear pale on a Romanowsky stained blood film, leading to the disorders name.

If further confirmation is required or there still is no diagnostic evidence, it becomes the territory of supra-regional laboratories and university research establishments. The facilities can provide electron microscope suites for the further classification [51] and identification [52] of platelet disorders and novel testing such as flow chamber experiments.[53] Research is being produced into alternatives so that more laboratories can access diagnostic information without the need for such specialised equipment.[54]

Genetic analysis

The first platelet disorders to be genetically resolved are the most common, with the genes encoding for platelet glycoproteins being elucidated in the early 1990s [55,56] and the first GT and BSS mutations discovered in the late 1990s.[57]

With the advances in genetic analysis [58] and the development of whole genome sequencing the genetic causes of many diseases are being elucidated. The NHS has started the 100,000 genomes project [59] to discover the underlying genetic mechanisms for rare disorders. The 100,000 genomes project will be taking place in 13 nominated specialist regional laboratories. This project, and its precursors, the Genotyping and Platelet Phenotyping (GAPP) [60] and BRIDGE-BPD [61] study have already started to bear fruit in platelet diseases with a number of novel mutations being discovered.[62–64]

Future developments

As knowledge of the role of the platelet expands beyond that of the clot,[65] platelet testing will expand along with it. Analysis of the platelet, and its role in immunity,[66] inflammation [67,68] and cancer [66,69] will bring with it a raft of new prognostic testing modalities.

Acknowledgments

The author would like to thank the editor for requesting this review. Dr Gary Moore for continued mentoring, support and friendship. Tom Phillips and Martin Nkau for proof reading and eternal optimism.

Funding

The author has organised meetings that have received sponsorship funding from Alpha Laboratories, Bayer, Hart Biologicals, Helena, LabMedics, Pathway Diagnostics, Roche, Stago, Sysmex and Werfen.

References

[1] Carter. Report of the Review of NHS Pathology Services in England [Internet]. 2006. Available from: http://www.dh.gov.uk/en/Publicationsandstatistics/Publications/PublicationsPolicyAndGuidance/DH_4137606

[2] Duncan EM, Bonar R, Rodgers SE, et al. Methodology and outcomes of platelet aggregation testing in Australia, New Zealand and the Asia-Pacific region: results of a survey from the Royal College of Pathologists of Australasia Haematology Quality Assurance Program. Int. J. Lab. Hematol. [Internet]. 2009 [cited 2010 Oct 19];31:398–406. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18371058

[3] Moore GW, Gurney DA. Bleeding disorders and their laboratory investigation. In: MooreG, Gavin Knight AB, editors. Haematology (Fundamentals of Biomedical Science [Internet]. 1st ed. Oxford: OUP Oxford; 2010. p. 473–525. Available from: http://www.amazon.co.uk/Haematology-Fundamentals-Biomedical-Science-Moore/dp/0199568839

[4] Gresele P, Harrison P, Bury L, et al. Diagnosis of suspected inherited platelet function disorders: results of a worldwide survey. J. Thromb. Haemost. [Internet]. 2014 [cited 2014 Sep 27];12:1562–1569. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24976115

[5] Konkle B. Acquired disorders of platelet function. Hematology[Internet]. 2011 [cited 2014 Oct 19];2011:391–396. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22160063

[6] Kerimi A, Williamson G. The cardiovascular benefits of dark chocolate. Vascul. Pharmacol. [Internet]. 2015;71:11–15. Available from: http://linkinghub.elsevier.com/retrieve/pii/S1537189115001135

[7] Bolton-Maggs PHB, Chalmers EA, Collins PW, et al. A review of inherited platelet disorders with guidelines for their management on behalf of the UKHCDO. Br. J. Haematol. [Internet]. 2006 [cited 2014 Oct 19];135:603–633. Available from: http://www.ingentaconnect.com/content/bsc/bjh/2006/00000135/00000005/art00005

[8] Gresele P. Diagnosis of inherited platelet function disorders: guidance from the SSC of the ISTH. J. Thromb. Haemost. [Internet]. 2015 [cited 2014 Nov 19];13:314–322. Available from: http://doi.wiley.com/10.1111/jth.12792
[9] Harrison P, Briggs C, Machin SJ. Platelet counting. Methods Mol. Biol. Clift. NJ [Internet]. 2004;272:29–46. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15226532
[10] Briggs C, Harrison P, Machin SJ. Continuing developments with the automated platelet count. Int. J. Lab. Hematol. [Internet]. 2007 [cited 2014 Oct 19];29:77–91. Available from: http://www.ncbi.nlm.nih.gov/pubmed/17474881
[11] Briggs C, Kunka S, Hart D, et al. Assessment of an immature platelet fraction (IPF) in peripheral thrombocytopenia. Br. J. Haematol. [Internet]. 2004;126:93–99. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15198738
[12] Maluf CB, Barreto SM, Vidigal PG. Standardization and reference intervals of platelet volume indices: Insight from the Brazilian longitudinal study of adult health (ELSA-BRASIL). Platelets [Internet]. 2014 [cited 2014 Aug 10];i:1–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25101826
[13] Harrison P, Horton A, Grant D, et al. Immunoplatelet counting: a proposed new reference procedure. Br. J. Haematol. [Internet]. 2000;108:228–235. Available from: http://www.ncbi.nlm.nih.gov/pubmed/10691847
[14] Masters A, Harrison P. Platelet counting with the BD Accuri(TM) C6 flow cytometer. Platelets [Internet]. 2013 [cited 2013 Sep 20];7104:1–6. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23772865
[15] Harrison P, Ault KA, Chapman S, et al. An interlaboratory study of a candidate reference method for platelet counting. Am. J. Clin. Pathol. [Internet]. 2001 [cited 2014 Nov 29];115:448–459. Available from: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=1124280210.1309/91PR-E4G6-XBAF-N8DY
[16] Meess A. Platelet transfusion in chemotherapy patients: comparison of the effect of intravenous infusion pumps versus gravity transfusion. Br. J. Biomed. Sci. [Internet] 2015;72:111–114. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26510266
[17] Kundu S, Heilmann E, Sio R, et al. Description of an in vitro platelet function analyzer – PFA-100. Semin. Thromb. Hemost. 1995;21:106–112
[18] Favaloro EJ, Bonar R. External quality assurance for the PFA-100®. J. Thromb. Haemost. [Internet]. 2011 [cited 2011 Feb 15];9. Available from: http://doi.wiley.com/10.1111/j.1538-7836.2011.04231.x
[19] Favaloro EJ. Clinical Utility of the PFA-100. Semin. Thromb. Hemost. [Internet] 2008 Nov [cited 2011 Aug 10];34:709–733. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19214910
[20] Harrison P, Mackie I, Mumford A, et al. Guidelines for the laboratory investigation of heritable disorders of platelet function. Br. J. Haematol. [Internet]. 2011 [cited 2014 Sep 27];155:30–44. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21790527
[21] Karger R, Donner-Banzhoff N, Müller H-H, et al. Diagnostic performance of the platelet function analyzer (PFA-100®) for the detection of disorders of primary haemostasis in patients with a bleeding history – a systematic review and meta-analysis. Platelets. 2007 [cited 2011 Aug 10];18:249–260. Available from: http://www.ncbi.nlm.nih.gov/pubmed/17538845
[22] McGlasson DL, Fritsma GA. Comparison of four laboratory methods to assess aspirin sensitivity. Blood Coagul. Fibrinolysis. [Internet]. 2008;19:120–123. Available from: http://www.bloodcoagulation.com/pt/re/bcf/abstract.00001721-200803000-00002.htm;jsessionid=H3SGQhv2WzyQwTbrPhySSGbk0fFW3x7BLvgWWSLpfms03pbQpyXN!-383192544!181195628!8091!-1
[23] Hans GA, Besser MW. The place of viscoelastic testing in clinical practice. Br. J. Haematol. [Internet]. 2016. Available from: http://doi.wiley.com/10.1111/bjh.13930
[24] Jandrey KE. Assessment of platelet function. J. Vet. Emerg. Crit. Care (San Antonio) [Internet]. 2012 [cited 2014 Oct 30];22:81–98. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23016745
[25] Kenet G, Lubetsky A, Shenkman B, et al. Cone and platelet analyser (CPA): a new test for the prediction of bleeding among thrombocytopenic patients. Br. J. Haematol. [Internet]. 1998;101:255–259. Available from: http://www.ncbi.nlm.nih.gov/pubmed/9609519
[26] Li CKN, Hoffmann TJ, Hsieh PY, et al. The Xylum clot signature analyzer®: A dynamic flow system that simulates vascular injury. Thromb. Res. [Internet]. 1998 [cited 2011 Aug 7];92:S67–S77. Available from: http://www.sciencedirect.com/science/article/pii/S0049384898001637
[27] Görög P, Ahmed A. Haemostatometer: a new in vitro technique for assessing haemostatic activity of blood. Thromb. Res. [Internet]. 1984 [cited 2012 Aug 19];34:341–357. Available from: http://www.ncbi.nlm.nih.gov/pubmed/6740567
[28] Born GVR. Aggregation of blood platelets by adenosine diphosphate and its reversal. Nature [Internet]. 1962 [cited 2012 Jan 24];194:927–929. Available from: http://www.nature.com/doifinder/10.1038/194927b0
[29] Cattaneo M, Cerletti C, Harrison P, et al. Recommendations for the standardization of light transmission aggregometry: a consensus of the working party from the platelet physiology subcommittee of SSC/ISTH. J. Thromb. Haemost. [Internet]. 2013 [cited 2014 Jul 13];1183–1189. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23574625
[30] Davidson C, Colvin B, Barrowcliffe TW, et al. Guidelines on platelet function testing. J. Clin. Pathol. [Internet]. 1988 [cited 2011 Dec 12];41:1322–1330. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1141768&tool=pmcentrez&rendertype=abstract
[31] Surin WR, Barthwal MK, Dikshit M. Platelet collagen receptors, signaling and antagonism: emerging approaches for the prevention of intravascular thrombosis. Thromb. Res. [Internet]. Forthcoming. Available from: http://www.sciencedirect.com/science/article/B6T1C-4R7CYM4-1/2/e7a1a12d3cb1e27905179ef3e1db22fc
[32] Bigalke B, Stellos K, Weig H-J, et al. Regulation of platelet glycoprotein VI (GPVI) surface expression and of soluble GPVI in patients with atrial fibrillation (AF) and acute coronary syndrome (ACS). Basic Res. Cardiol. [Internet]. 2009 [cited 2011 Aug 8];104:352–357. Available from: http://www.springerlink.com/content/g274m12vx404t612
[33] Bigalke B, Stellos K, Stakos D, et al. Influence of platelet count on the expression of platelet collagen receptor glycoprotein VI (GPVI) in patients with acute coronary syndrome. Thromb. Haemost. [Internet]. 2009 [cited 2011 Aug 8];101:911–915. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19404545
[34] Nuyttens BP, Thijs T, Deckmyn H, et al. Platelet adhesion to collagen. Thromb. Res. [Internet]. 2011 [cited 2011 Jul 7];127 Suppl:S26–S29. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21193111

[35] White JG, Rao GH, Gerrard JM. Effects of the Ionophore A23187 on blood platelets I. Influence on aggregation and secretion. Am. J. Pathol. [Internet]. 1974;77:135–149. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1910910&tool=pmcentrez&rendertype=abstract

[36] Hayward C, Moffat K, Spitzer E, et al. Results of an external proficiency testing exercise on platelet dense-granule deficiency testing by whole mount electron microscopy. Am. J. Clin. Pathol. [Internet] 2009 [cited 2011 Aug 10];131:671–675. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19369626

[37] Schmitz G, Rothe G, Ruf A, et al. European working group on clinical cell analysis: consensus protocol for the flow cytometric characterisation of platelet function. Thromb. Haemost. [Internet]. 1998 [cited 2012 Sep 13];79:885–896. Available from: http://www.ncbi.nlm.nih.gov/pubmed/9609215

[38] Franchini M, Favaloro EJ, Lippi G. Glanzmann thrombasthenia: an update. Clin. Chim. Acta. [Internet]. 2010 [cited 2012 Oct 6];411:1–6. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19854165

[39] Kannan M, Saxena R. Glanzmann's thrombasthenia: an overview. Clin. Appl. Thromb. Hemost. [Internet]. 2009 [cited 2011 Aug 10];15:152–165. Available from: http://cat.sagepub.com/cgi/content/abstract/15/2/152?rss=1

[40] Bernard J, Soulier J. Sur une nouvelle variété de dystrophie thrombocytaire hémorragipare congénitale [On a new variety of thrombocytopaenic bleeding disorder]. Sem. des Hôpitaux Paris. 1948;24:3217–3223.

[41] Pham A, Wang J. Bernard-Soulier syndrome: an inherited platelet disorder. Arch. Pathol. Lab. Med. 2007;131:1834–1836.

[42] van Velzen JF, Laros-van Gorkom BA, Pop GA, et al. Multicolor flow cytometry for evaluation of platelet surface antigens and activation markers. Thromb. Res. [Internet]. 2012 [cited 2012 Jun 5];130:92–98. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22424855

[43] Jakubowski J, Payne C, Li YG, et al. A comparison of the antiplatelet effects of prasugrel and high-dose clopidogrel as assessed by VASP-phosphorylation and light transmission aggregometry. Thromb. Haemost. [Internet]. 2008 [cited 2011 Aug 10];99:215–222. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18217157

[44] Flores-Nascimento MC, Orsi FLA, Yokoyama AP, et al. Diagnosis of Scott syndrome in patient with bleeding disorder of unknown cause. Blood Coagul. Fibrinolysis. [Internet]. 2012 [cited 2011 Nov 29];23:75–77. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22027759

[45] Shantsila E, Montoro-García S, Gallego P, et al. Circulating microparticles: challenges and perspectives of flow cytometric assessment. Thromb. Haemost. [Internet]. 2014 [cited 2014 Feb 22];111:2–7. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24553954

[46] Garritsen HS, Probst-Kepper M, Legath N, et al. High sensitivity and specificity of a new functional flow cytometry assay for clinically significant heparin-induced thrombocytopenia antibodies. Int. J. Lab. Hematol. [Internet]. 2013 [cited 2014 Mar 16];00:135–143. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23981347

[47] Mumford AD, Frelinger AL, Gachet C, et al. A review of platelet secretion assays for the diagnosis of inherited platelet secretion disorders. Thromb. Haemost. 2015;114:14–25.

[48] Masliah-Planchon J, Darnige L, Bellucci S. Molecular determinants of platelet delta storage pool deficiencies: an update. Br. J. Haematol. [Internet]. 2012 [cited 2012 Oct 6];160:5–11. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23025459

[49] Patel YM, Patel K, Rahman S, et al. Evidence for a role for G i1 in mediating weak agonist-induced platelet aggregation in human platelets: reduced G i1 expression and defective Gi signaling in the platelets of a patient with a chronic bleeding disorder. Blood. [Internet]. 2003;101:4828–4835. Available from: http://www.ncbi.nlm.nih.gov/pubmed/12609843

[50] Bain BJ, Bhavnani M. Gray platelet syndrome. Am. J. Hematol. [Internet]. 2011 [cited 2011 Aug 11];86:1027. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21800352

[51] Clauser S, Cramer-Bordé E. Role of platelet electron microscopy in the diagnosis of platelet disorders. Semin. Thromb. Hemost. 2009;35:213–223.

[52] Islam MS, Alamelu J. Morphological and electron microscopic characteristics of grey platelet syndrome. Br. J. Haematol. [Internet]. 2010 [cited 2010 Oct 21]:1. Available from: http://doi.wiley.com/10.1111/j.1365-2141.2010.08405.x

[53] Van Kruchten R, Cosemans JMEM, Heemskerk JWM. Measurement of whole blood thrombus formation using parallel-plate flow chambers – a practical guide. Platelets. [Internet]. 2012 [cited 2012 Aug 20];23:229–242. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22502645

[54] Westmoreland D, Shaw M, Grimes W, et al. Super-resolution microscopy as a potential approach to platelet granule disorder diagnosis. J. Thromb. Haemost. [Internet]. 2016. Available from: http://doi.wiley.com/10.1111/jth.13269

[55] Solum NO, Clemetson KJ. The discovery and characterization of platelet GPIb. J. Thromb. Haemost. [Internet]. 2005;3:1125–1132. Available from: http://www.blackwell-synergy.com/doi/abs/10.1111/j.1538-7836.2005.01072.x

[56] George JN, Caen JP, Nurden A. Glanzmann's thrombasthenia: the spectrum of clinical disease. Blood. 1990;75:1383–1395

[57] Noris P, Arbustini E, Spedini P, et al. A new variant of Bernard-Soulier syndrome characterized by dysfunctional glycoprotein (GP) Ib and severely reduced amounts of GPIX and GPV. Br. J. Haematol. [Internet]. 1998;103:1004–1013. Available from: http://www.ncbi.nlm.nih.gov/pubmed/9886312

[58] Jones ML, Murden SL, Bem D, et al. Rapid genetic diagnosis of heritable platelet function disorders using next generation sequencing: proof-of-principle with Hermansky–Pudlak syndrome. J. Thromb. Haemost. [Internet]. 2011 [cited 2011 Nov 24];10:306–309. Available from: http://doi.wiley.com/10.1111/j.1538-7836.2011.04569.x

[59] http://www.genomicsengland.co.uk/the-100000-genomes-project/

[60] Watson SP, Lowe GC, Lordkipanidzé M, et al. Genotyping and phenotyping of platelet function disorders. J. Thromb. Haemost. [Internet]. 2013 [cited 2013 Mar 26];11:351–363. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23516995

[61] https://bridgestudy.medschl.cam.ac.uk/whatis.shtml

[62] Albers CA, Cvejic A, Favier R, et al. Exome sequencing identifies NBEAL2 as the causative gene for gray platelet syndrome. Nat. Genet. [Internet]. 2011 [cited 2012 Oct 6];43:735–737. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21765411

[63] Westbury SK, Turro E, Greene D, et al. Human phenotype ontology annotation and cluster analysis to unravel genetic defects in 707 cases with unexplained bleeding and platelet disorders. Genome Med. [Internet]. 2015;7:30. Available from: http://genomemedicine.com/content/7/1/36

[64] Favier R, Raslova H. Progress in understanding the diagnosis and molecular genetics of macrothrombocytopenias. Br. J. Haematol. [Internet]. 2015;170:626–639. Available from: http://doi.wiley.com/10.1111/bjh.13478

[65] McFadyen JD, Kaplan ZS. Platelets are not just for clots. Transfus. Med. Rev. [Internet]. 2015;29:110–119. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0887796315000164

[66] Golebiewska EM, Poole AW. Platelet secretion: From haemostasis to wound healing and beyond. Blood Rev. [Internet]. 2015;29:153–162. doi:http://dx.doi.org/10.1016/j.blre.2014.10.003

[67] Jenne CN, Urrutia R, Kubes P. Platelets: bridging hemostasis, inflammation, and immunity. Int. J. Lab. Hematol. [Internet]. 2013 [cited 2013 Apr 19];35:254–261. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23590652

[68] Herter JM, Rossaint J, Zarbock A, et al. Platelets in inflammation and immunity. J. Thromb. Haemost. 2014;12:1764–1775.

[69] Kwaan HC, Vicuna B. Thrombosis and bleeding in cancer patients. Oncol. Rev. [Internet]. 2007 [cited 2012 Sep 8];1:14–27. Available from: http://www.springerlink.com/index/10.1007/s12156-007-0003-7