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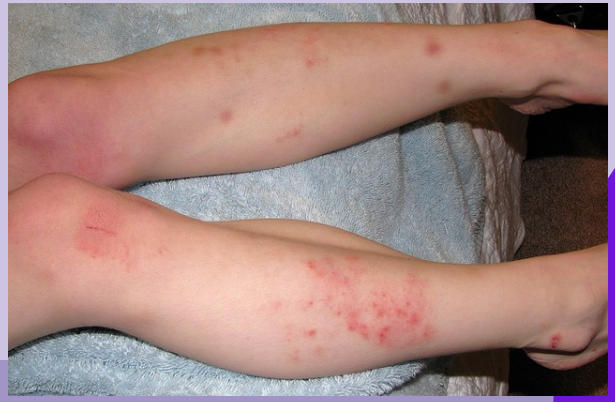
Guy's and St Thomas' NHS
NHS Foundation Trust

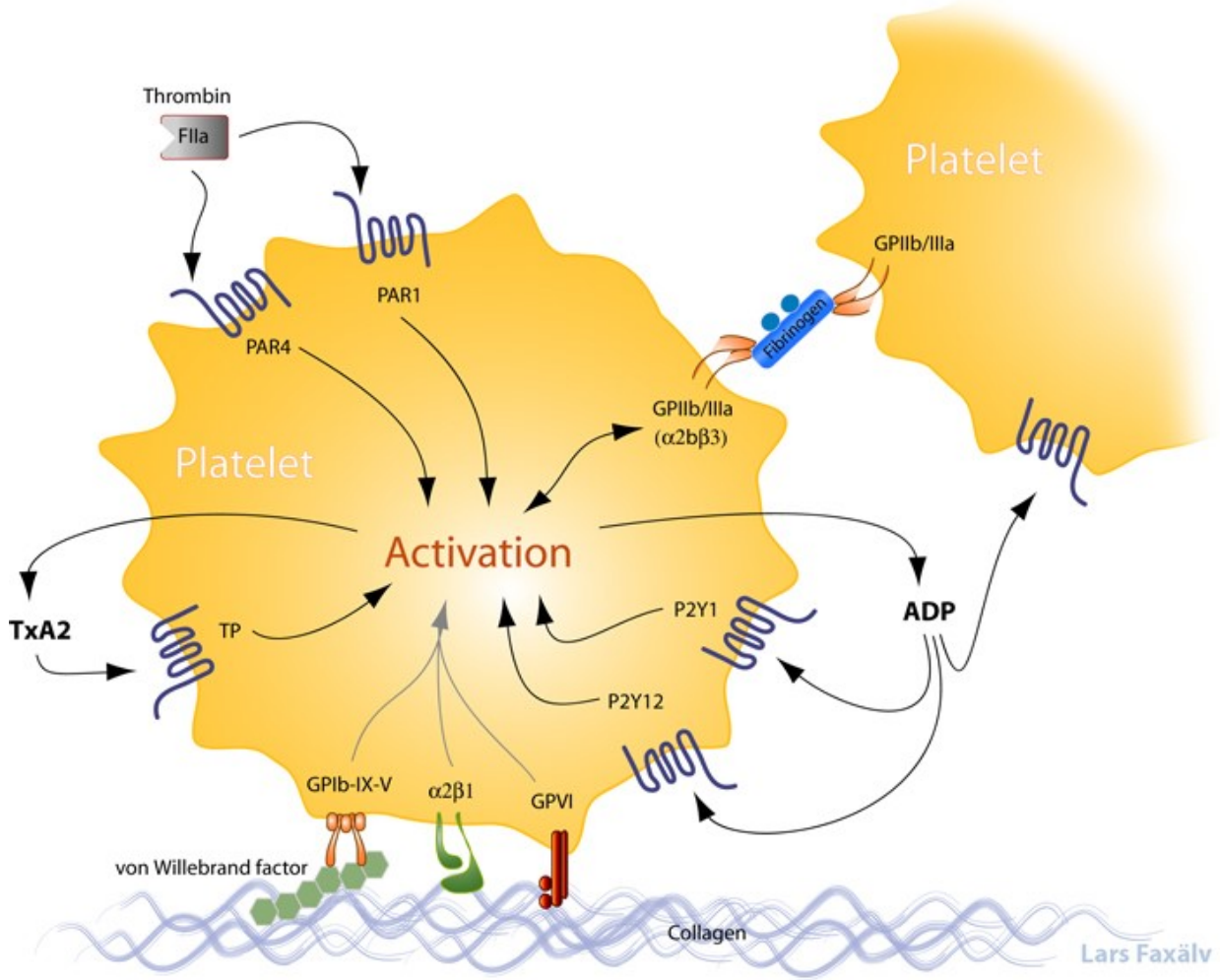
Platelet function testing: the view from the laboratory.



Dr. Áine McCormick

Friday 9th November 2018

Overview of platelet activation



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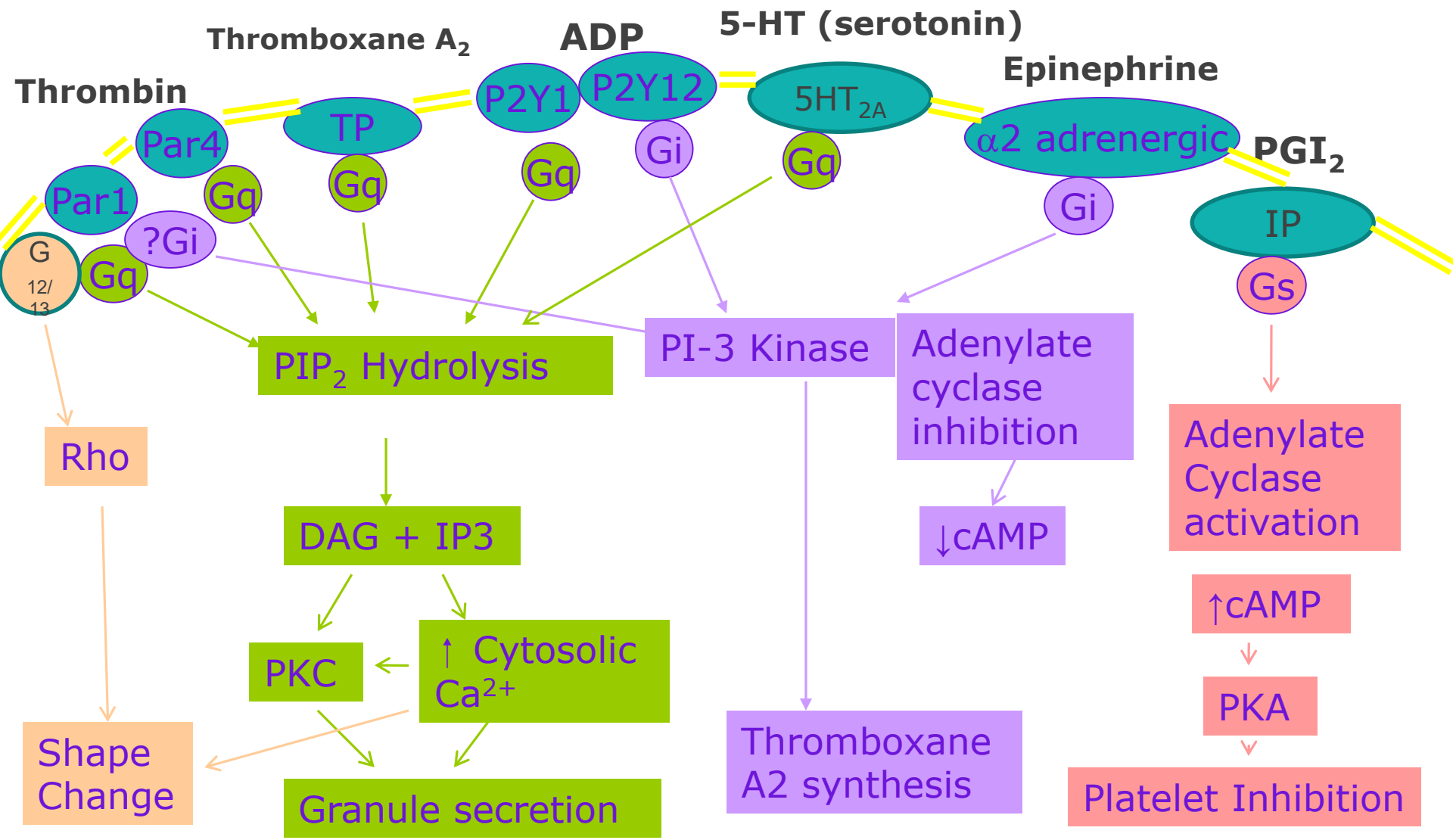
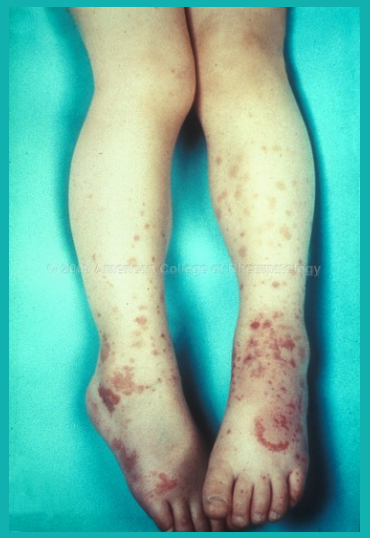
Platelet G protein coupled receptors



Platelet disorders

Classical symptoms of a platelet related disorder:



Mucocutaneous bleeds of varying severity:

nosebleeds
heavy menstrual bleeding
excessive bleeding after trauma/surgery

Severe disorders usually identified soon after birth: petechiae (<3mm) purpura (3-10mm)

Mild disorders may be undetected for years until haemostatically challenged.

Acquired platelet disorders

Antiplatelet agents most common causes of acquired defects associated with excessive bleeding

- aspirin
- clopidogrel
- ticlopidine

Considerations for platelet testing

- Clinical history
- VWD
- Medications being taken. Pre-analytical questionnaire.
- Efficiency of process
- Phlebotomy. Collection in vacutainer or syringe?
- Preparation of platelet rich plasma. Platelet count adjustment?
- Post-analytical results assessment. LTRA criteria to report?
- Discussions with clinical colleagues prior to results release.

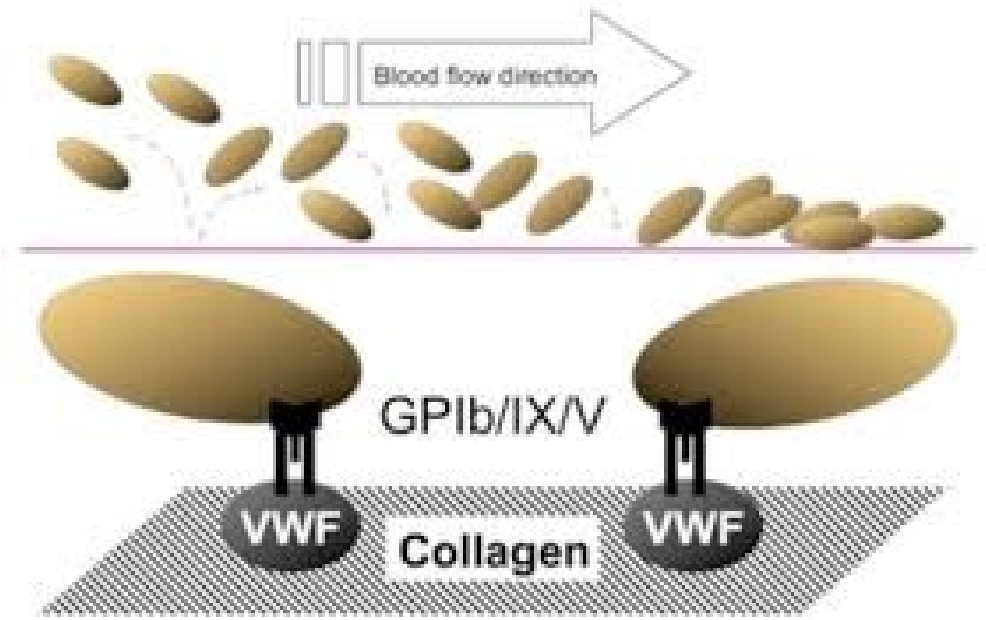
Investigating for platelet disorders

Clinical history: personal & FH
severity, frequency & type of bleeding
prescription & over the counter drugs
diet
lifestyle (smoking, exercise)

Laboratory testing:

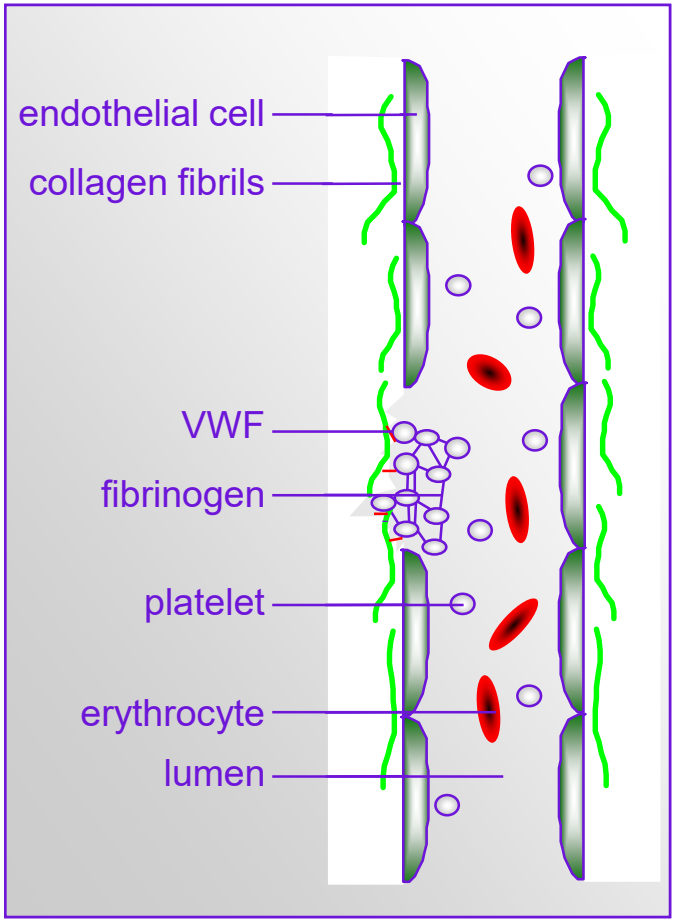
- PFA-100
- Light transmission aggregometry
- Lumiaggregometry
- Platelet nucleotide assay
- Flow cytometry
- Genetic testing
- Verify Now
- Platelet works
- Serotonin release assay
- VASP assay
- Platelet mapping using TEG
- Cone & plate assay

Platelet function analysis / VWF testing

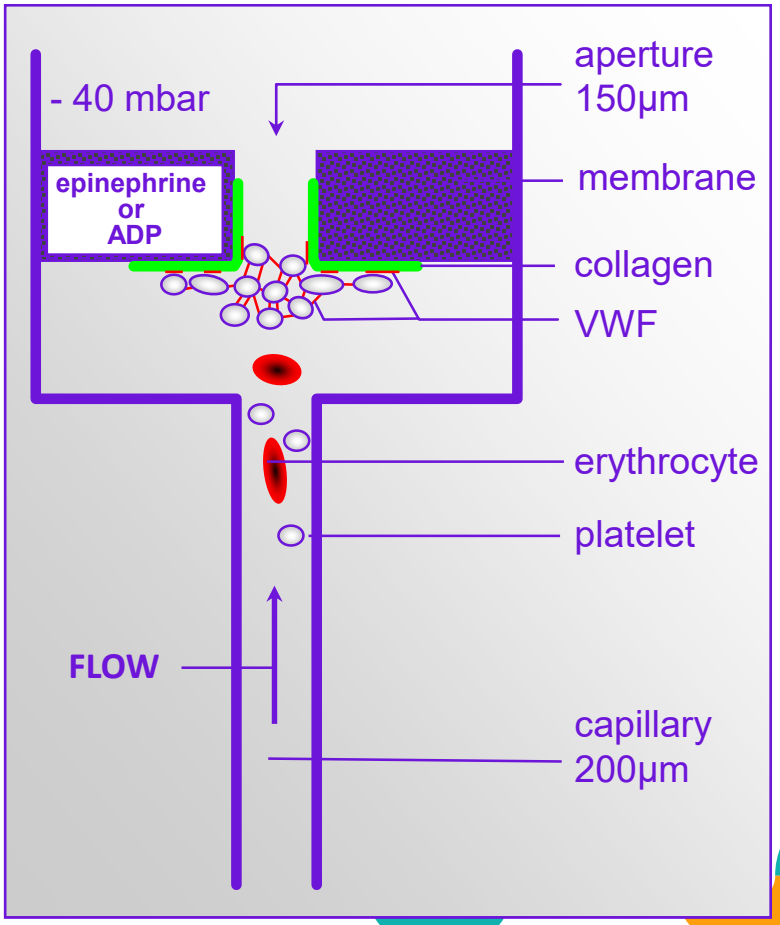


PFA-100® test principle

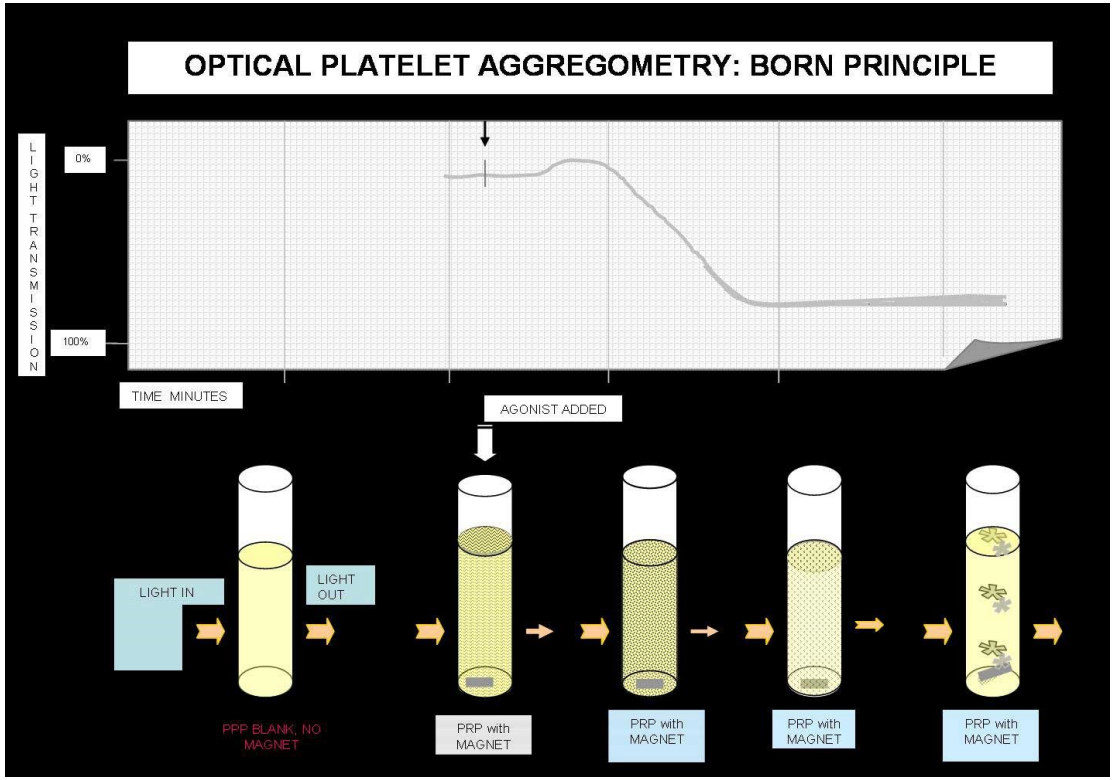
In vivo haemostasis



PFA-100®



Light transmission aggregometry



- Platelet rich plasma is produced using gentle centrifugation
- Aliquots are stirred in a cuvette between a light source and a photocell
- Agonist addition induces platelet shape change and aggregation
- Aggregation viewed as an increase in light transmission

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Quality control of platelet function testing

- Adherence to British Committee for standards in Haematology (BCSH) guidelines and compliance with United Kingdom Accreditation Service (UKAS) ISO 15189 standards.
- A particular challenge for platelet function testing!
- Definition of ‘normal control’.
- Incorporation of ‘abnormal control’.
- Also required standardisation of and validation of preparation techniques and reporting guidelines.

How to approach enhanced QC requirements?

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Appendix 1: Platelet Function Test – Patient / Control Questionnaire

Pre-analytical Questionnaire

Have you taken any of the following medicines within the times indicated?	
Aspirin (Acetylsalicylic acid) within the last **14 days**	**YES / NO**
Ibuprofen (eg Nurofen®), **Naproxen** (eg Feminax Ultra) or other **non-steroidal anti-inflammatory medicines*** within the last **48 hours**	**YES / NO**

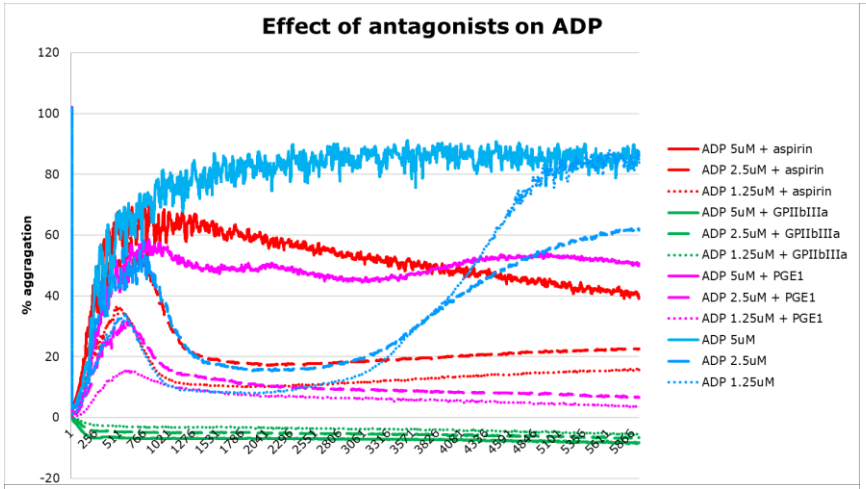
***Non-steroidal anti-inflammatory medicines** include aceclofenac, celecoxib, dexibuprofen, dexketoprofen, diclofenac, etodolac, etoricoxib, fenoprofen, flurbiprofen, ibuprofen, indometacin, ketoprofen, mefenamic acid, meloxicam, nabumetone, naproxen, piroxicam, sulindac, tenoxicam, tolfenamic acid

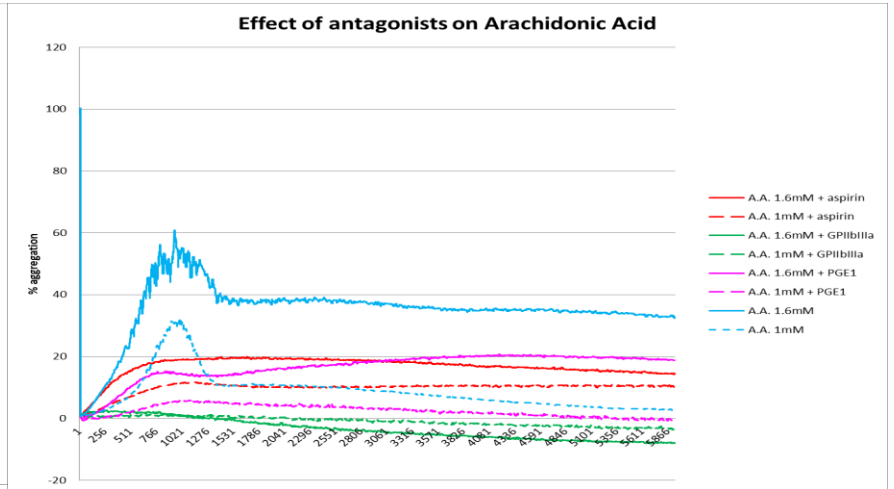
If the answer to either of the above questions is **YES** then platelet function testing will have to be rescheduled. Please contact the centre for Haemostasis & Thrombosis.

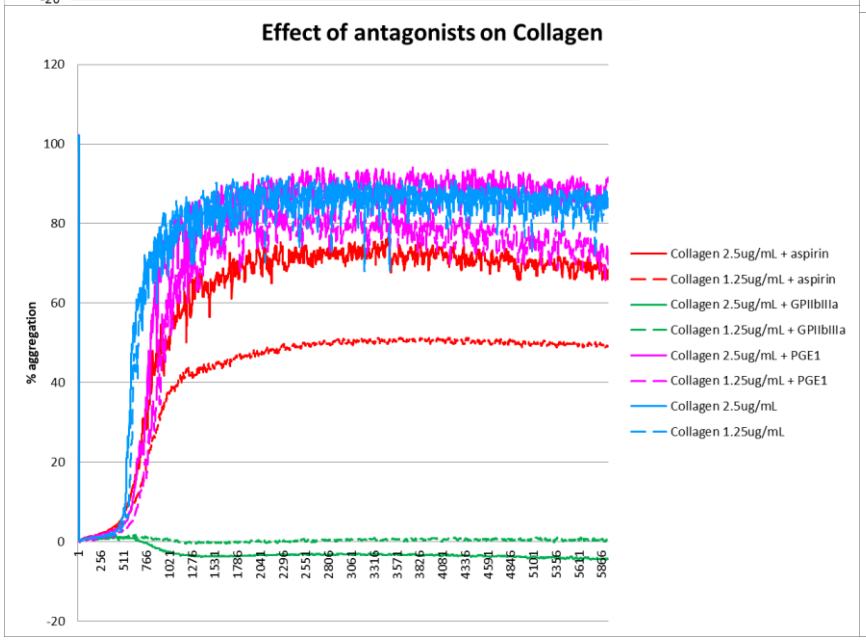
Please list below <u>all</u> the medicines you have taken within the last 7days.	
Prescribed Medicines: (including tablets/capsules, liquids, eye/ear/nose drops/sprays, inhalers, patches, medicated creams)	
Medicines bought in a Pharmacy or shop Please note: many well known brands have various products with different ingredients. Please specify the exact product.	
Medicines obtained from a friend or other source eg internet	
Vitamins	
Herbal remedies and other alternative medicines	
Diet supplements	
Do you drink alcohol?	**YES / NO**
Do you smoke?	**YES / NO**
(***For nursing staff only***)	
Was this a clean draw?	**YES / NO**

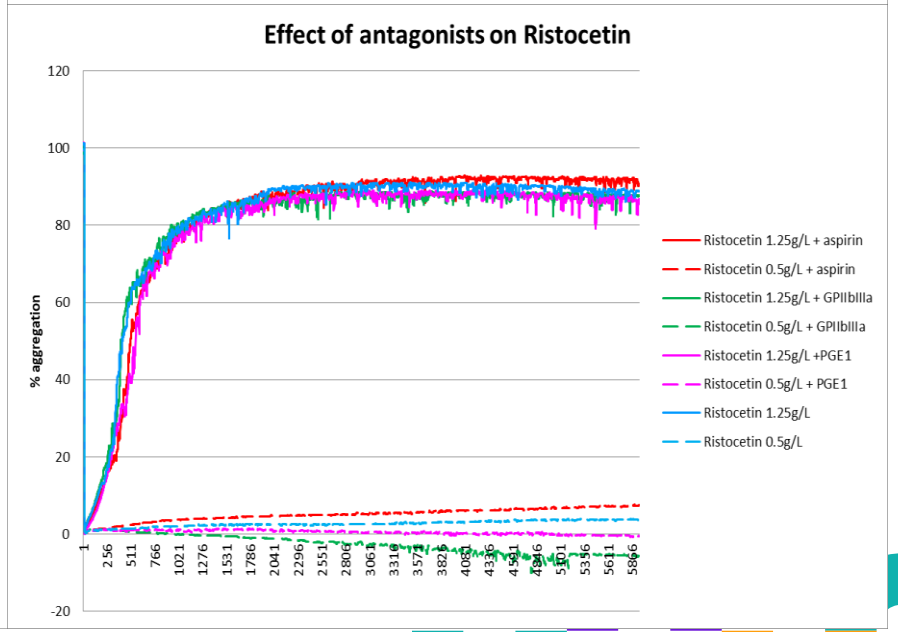
Please affix patient bar code to this form, or for donor controls – please sign and date.

Effect of antagonist addition to donor PRP response to agonists: Aspirin, GPIIbIIIa and PGE1









Batch acceptance testing for ristocetin

New batches of ristocetin are tested prior to use be performing platelet aggregometry testing using donor platelet rich plasma mixed with varying concentrations of donor platelet poor plasma / plasma from a genetically confirmed type III VWD patient.

PRP Plt count platelets /L	Max Agg. (%) VWF def PPP: Donor PRP 0:1	Max Agg. (%) VWF def PPP : Donor PRP 1:1	Max Agg. (%) VWF def PPP: Donor PRP 1:3	Max Agg. (%) VWF def PPP: Donor PRP 1:7	Max. Agg. (%) Donor PRP Nt	Max. Agg. (%) Donor PPP 1:1	Max. Agg. (%) Donor PPP1:3	Max. Agg. (%) Donor PP1:7
239	**87**	**47**	**65**	**75**	**89**	**75**	**88**	**79**

QC summary

The ‘Normal Control’: volunteers with no previous bleeding history and no interfering medications.

Pre-analytical questionnaires are filled out by patients and volunteer controls.

Centrifugation follows a defined protocol with whole blood, platelet rich and platelet poor plasma counts recorded for interpretation.

EDTA added to donor PRP routinely to simulate ‘abnormal control’

Batch acceptance testing is performed for all agonists prior to routine use.

Cross-site testing whereby a sample is processed by two laboratories simultaneously is performed biannually.

Interpretation is tested through NASCOLA case studies.

PFA-100 is shortly due to join an EQA scheme provided by the Royal College of Pathologists in Australasia (RCPAQAP).

Ristocetin Induced Platelet Aggregation (RIPA)

Type IIB VWD & pseudo VWD (platelet-type VWD) are hyper-responsive to ristocetin

Type IIB VWD HMW multimers have increased affinity for GPIb plasma defect

Pseudo VWD GPIb has increased affinity for VWF platelet defect

RIPA assay Wash platelets from patient and control

Re-suspended in the opposite PPP and exposed to reducing ristocetin concentrations

1.2 mg/mL

0.5 mg/mL

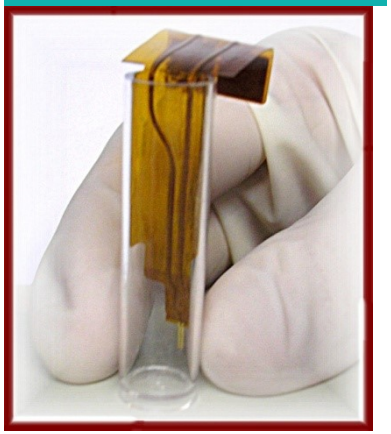
Platelets from a Type IIB VWD individual re-suspended in normal plasma will not aggregate at 0.5 mg/mL

Platelets from a pseudo-VWD individual re-suspended in normal plasma will aggregate at 0.5 mg/mL

Lumiaggregometry

- Lumiaggregometers are capable of simultaneously measuring aggregation and ATP release
- Can be in PRP or whole blood
- Whole blood diluted 1:1 in saline

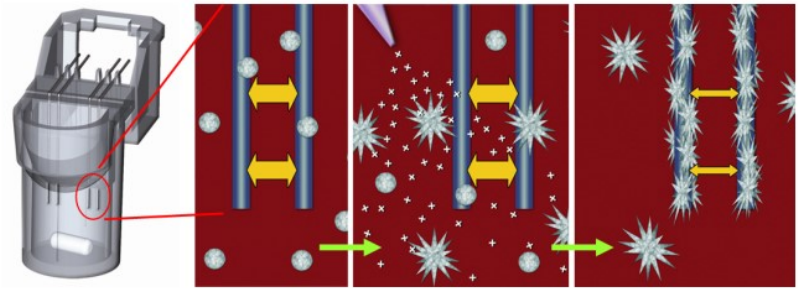
Electrode Lowered into suspension



Platelets form monoloayer on electrode

Agonist added & timer started

Current slowed by platelet aggregate



Resistance measured in ohms (Ω)

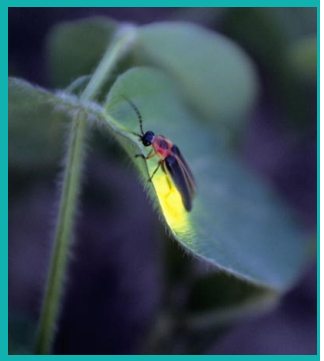
0 Ω = no aggregation

Aggregation proportional to Ω

Platelet nucleotide assay

- Measurement of ATP & ADP content is a valuable tool for the diagnosis of storage pool disorders. Up to 25% of patients with storage pool disorder may have normal platelet aggregometry.

- Platelet lysate prepared:
  - EDTA prevents Ca^{++} dependent reactions
  - Triton-X disrupts platelet membranes releasing cytosol
  - Ethanol precipitates membrane proteins & stabilises nucleotides
  - Centrifuge & analyse supernatant



Photinus pyralis

ATP + Luciferin $\xrightarrow{\text{Luciferase + } Mg^{++}}$ Adenyl Luciferin + Light

- ADP is unstable and cannot be measured directly
- Converted to ADP in a separate aliquot and ATP measured as above

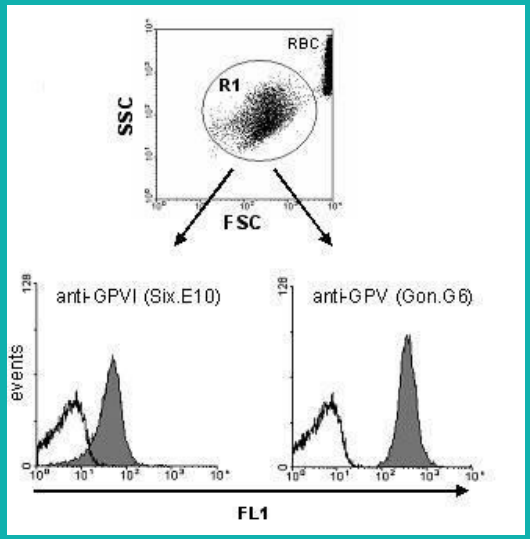
Phosphoenolpyruvate $\xrightarrow{\text{Pyruvate kinase}}$ Pyruvate

ADP → ATP

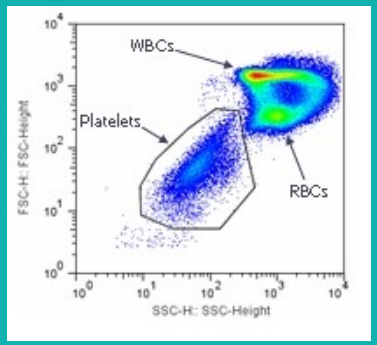
Platelet glycoprotein analysis

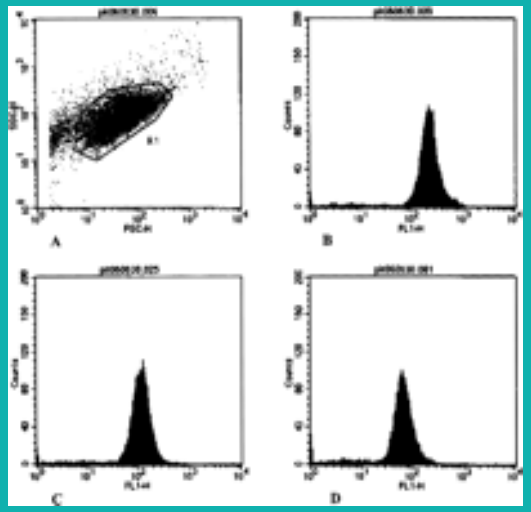
FITC or PE labelled antibodies against receptor subunits:

GPIX	CD42a
GPIbα	CD42b
GPIbβ	CD42c
GPIIa	CD29
GPIIb	CD41
GPIIIa	CD61
GPIIbIIIa	CD41b
P selectin	CD62P (activation marker)

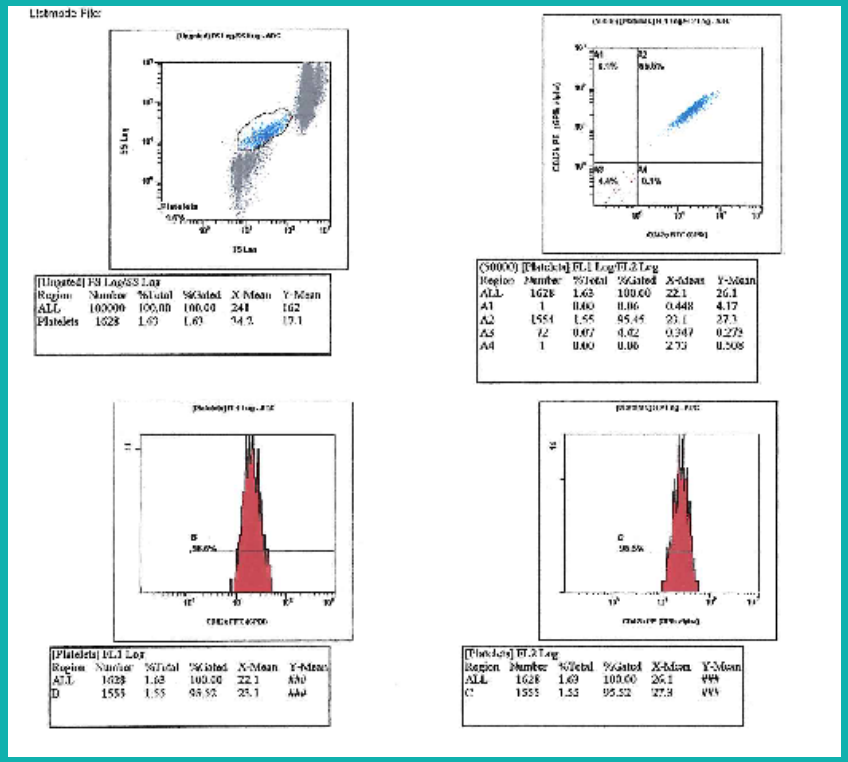


Positive antibody binding is detected by flow cytometry



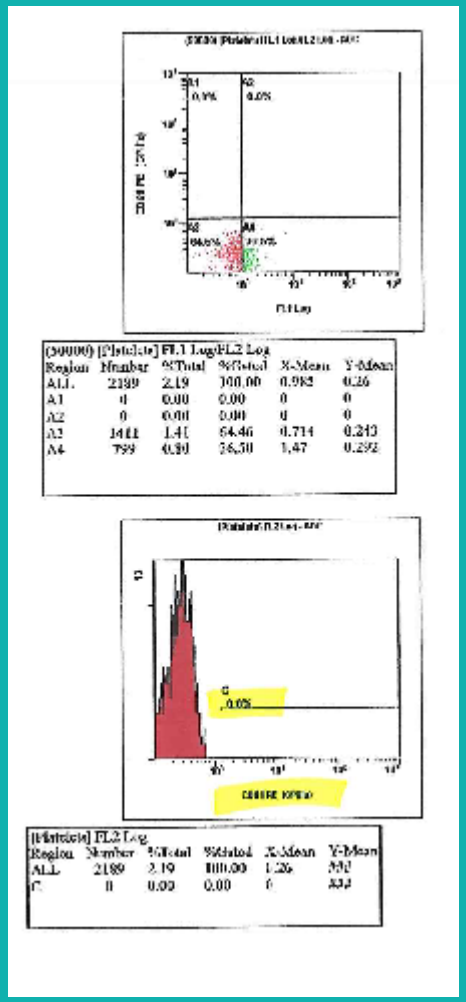


Platelet Glycoproteins – Glanzmann's Thrombasthenia

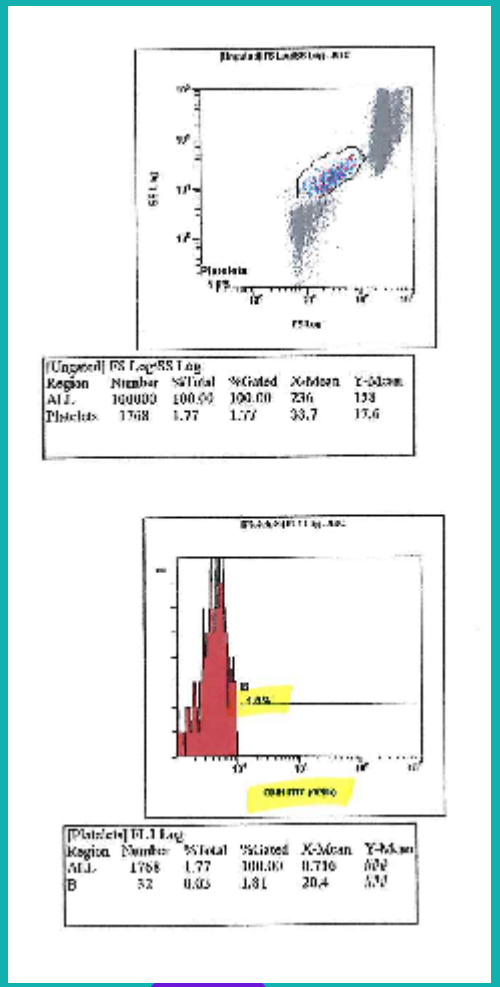


CD42a (GPIX)

CD42b (GPIb alpha)



CD61 (GPIIIa)



CD41 (GPIIb)

Automation of LTRA

PAP-8E

- Can run 8 tubes simultaneously
- Agonists must be added manually
- Aggregation profiles are viewed in real time
- Can run up to 3 patients and 1 control in one assay
- Preparation and running take approx. 3-4 hours.

Sysmex CS2400i

- Runs one sample at a time
- Agonists must be prepared manually but once onboard are added to the PRP automatically.
- No extra manual effort to do extra agonist concentrations once onboard – but does take extra time.
- Cannot view profiles in real time.
- Once set up can 'walk away'.

Preliminary results from our Multicentre study

rpth research and practice in thrombosis and haemostasis™

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A multicenter study to evaluate automated platelet aggregometry on Sysmex CS-series coagulation analyzers—preliminary findings

Sean Platton, MSc, [1] Áine McCormick, BSc (Hons), MMedSc PhD, [2] Musfira Bukht, BSc, MSc, [1] David Gurney, MSc, DBMS, [3] Ian Holding, BSc, (Hons), [4] and Gary W. Moore, BSc, DBMS [2]

Res Pract Thromb Haemost. 2018 Oct; 2(4): 778–789.

Final agonist panel

Platelet agonist concentrations employed for semi-automated aggregometry.
% aggregation ranges from Platton et. al.,2018.

Platelet agonist	Concentration	% Max. aggregation Mean (2.5 – 97.5 percentile)	Expected waveform	Target receptor / pathway
ADP	5 µM	84 (48-98)	Single wave	$P2Y_1$ / $P2Y_{12}$
ADP	2.5 µM	74 (22-100)	Single wave	$P2Y_1$ / $P2Y_{12}$
ADP	1 µM	56 (18-96)	Primary wave with reversal	$P2Y_1$ / $P2Y_{12}$
Collagen	2.5 µg/mL	97 (78-111)	Single wave	α2β1 / GPVI
Collagen	1.25 µg/mL	89 (54-100)	Single wave	α2β1 / GPVI
Ristocetin	1.25 g/L	92 (78-99)	Single wave	Promotes GP1b binding to VWF resulting in agglutination
Ristocetin	0.5 g/L	3 (0-15)	No aggregation	Response to low dose indicative of Type IIB VWD or pseudo VWD
Epinephrine	5 µM	85 (3-104)	Single wave/ primary wave with reversal	α2adrenergic receptor. Potentiates ADP and TXA_2 activation
Arachidonic Acid	1.5 mM	88 (59-103)	Single wave	Precursor to TXA_2 via COX-1
Arachidonic Acid	1.0 mM	86 (70-105)	Single wave	Precursor to TXA_2 via COX-1
TRAP (thrombin receptor activator peptide)	5 uM	90 (51-100)	Single wave	PAR-1
U46619 (thromboxane TXA_2 analog)	1 µM	86 (60-104)	Single wave	TXA_2
Saline	0.9 %	Test for spontaneous aggregation	None	None

Direct comparison of ADP results from a patient with confirmed storage pool disorder on both platforms

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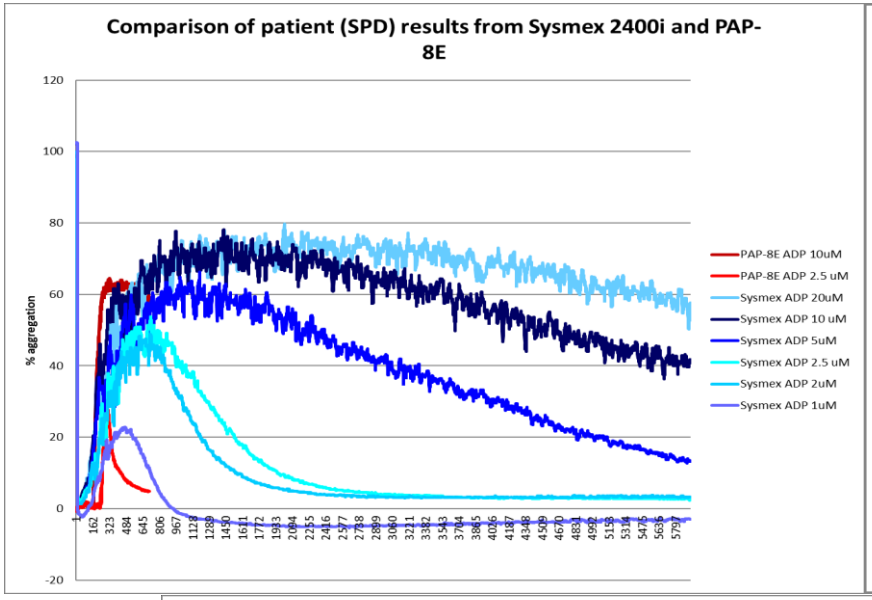
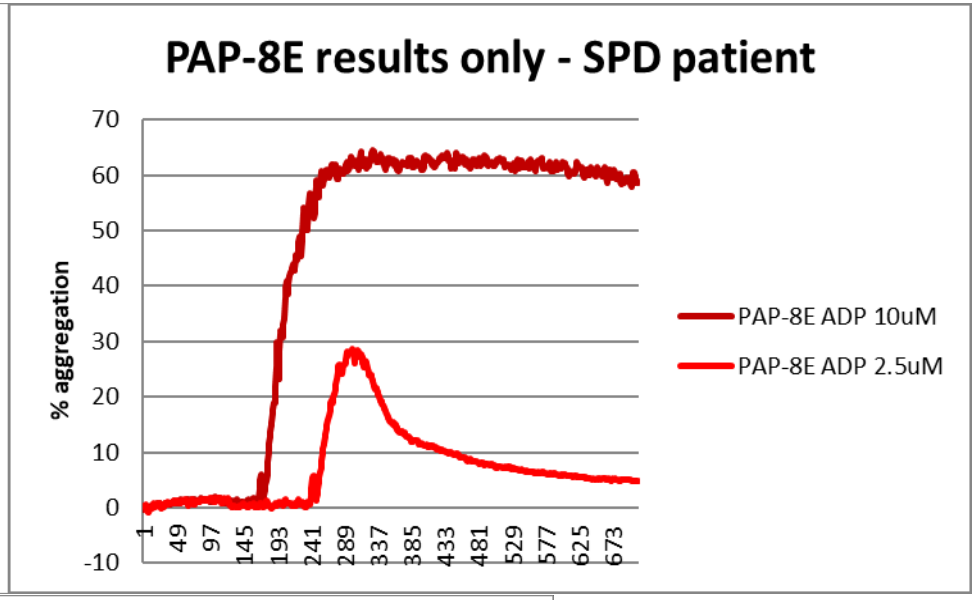
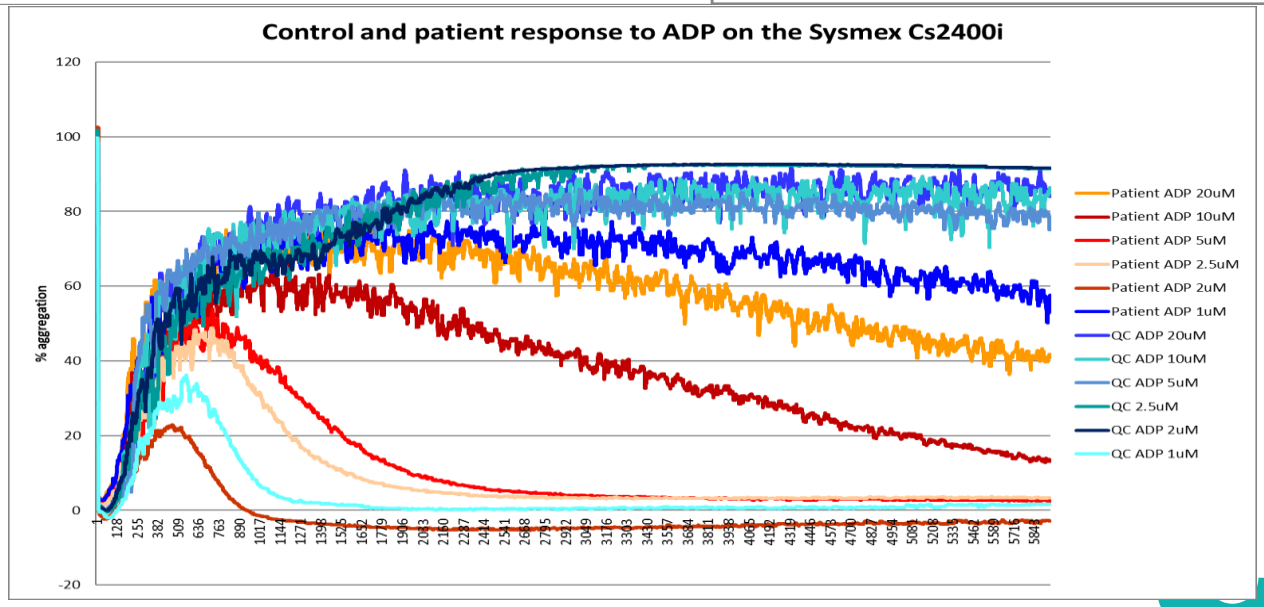
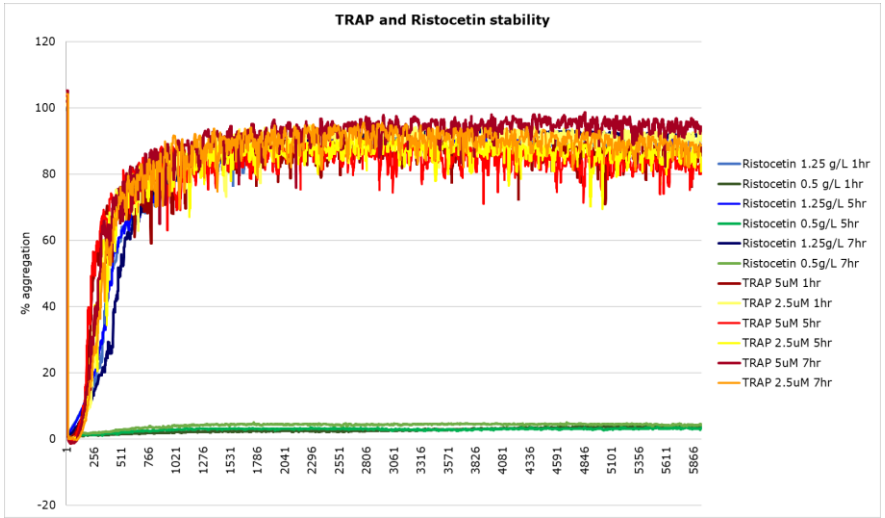


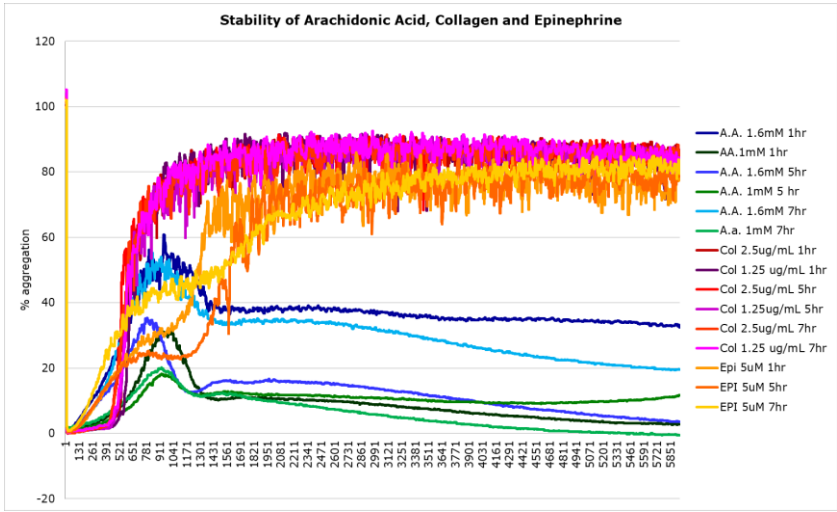


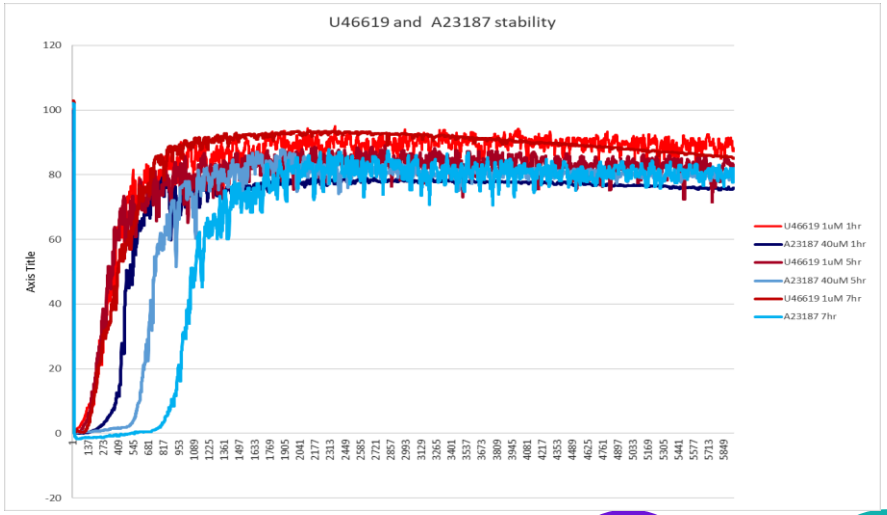


Reagent stability study

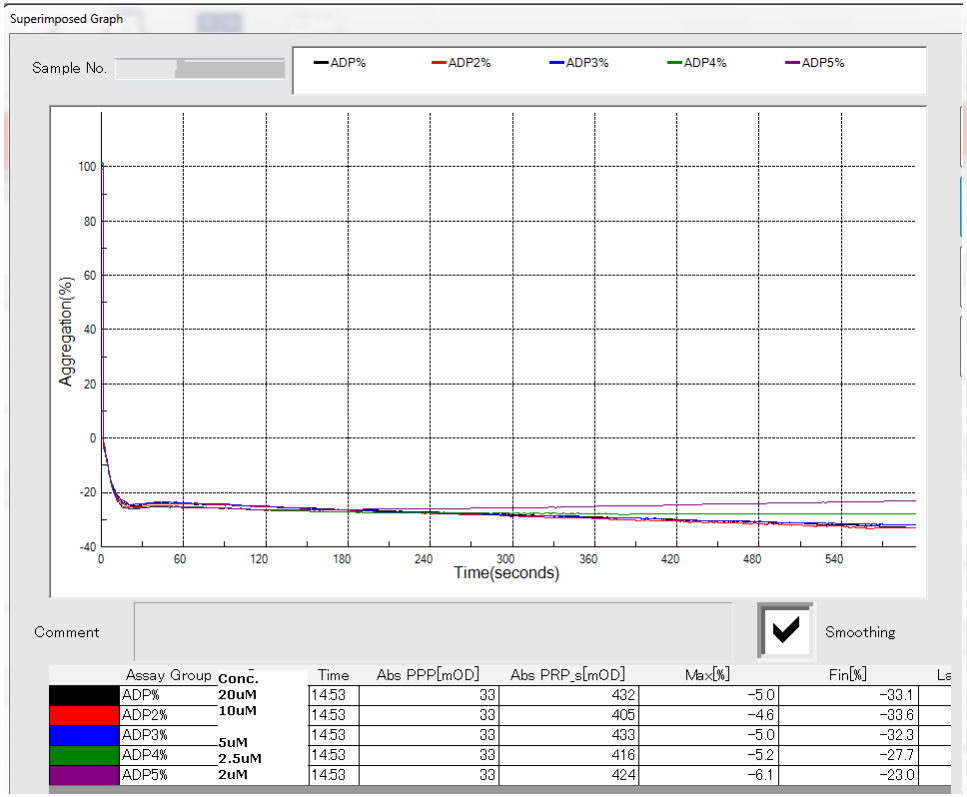
Reagents made up and run with fresh donor PRP.
Donor re-bled after 4 hrs and run using same reagents.



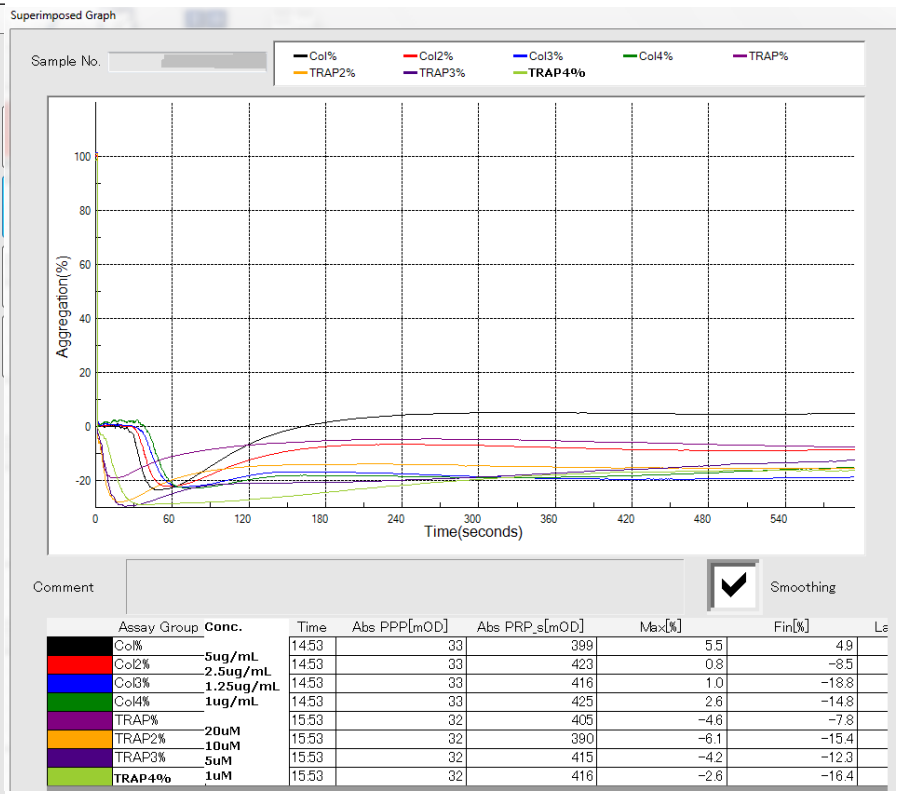




GT patient ADP/Coll/TRAP

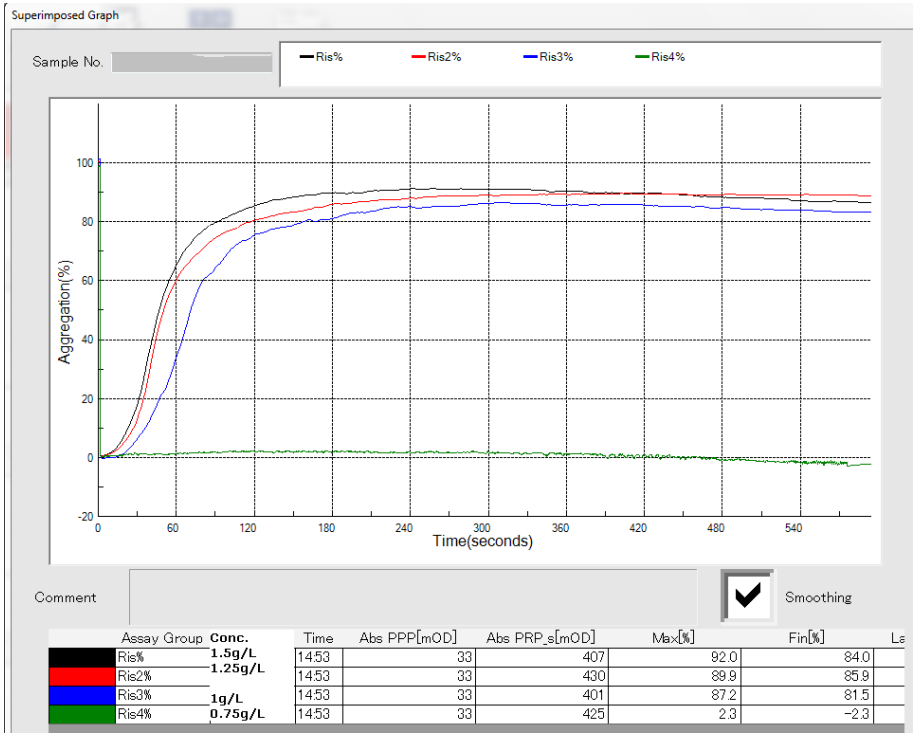


Assay Group	Conc.	Time	Abs PPP[mOD]	Abs PRP_s[mOD]	Max[%]	Fin[%]	La
ADP%	20uM	14:53	33	432	-5.0	-33.1	
ADP2%	10uM	14:53	33	405	-4.6	-33.6	
ADP3%	5uM	14:53	33	433	-5.0	-32.3	
ADP4%	2.5uM	14:53	33	416	-5.2	-27.7	
ADP5%	2uM	14:53	33	424	-6.1	-23.0	

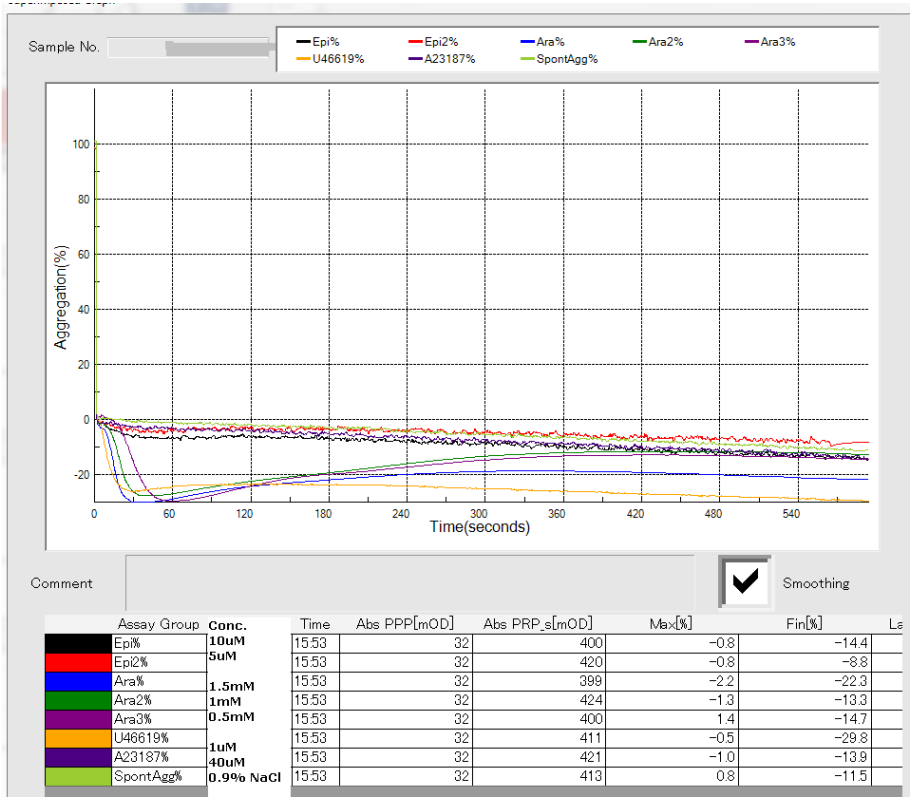


Assay Group	Conc.	Time	Abs PPP[mOD]	Abs PRP_s[mOD]	Max[%]	Fin[%]	La
Col%	5ug/mL	14:53	33	399	5.5	4.9	
Col2%	2.5ug/mL	14:53	33	423	0.8	-8.5	
Col3%	1.25ug/mL	14:53	33	416	1.0	-18.8	
Col4%	1ug/mL	14:53	33	425	2.6	-14.8	
TRAP%	20uM	15:53	32	405	-4.6	-7.8	
TRAP2%	10uM	15:53	32	390	-6.1	-15.4	
TRAP3%	5uM	15:53	32	415	-4.2	-12.3	
TRAP4%	1uM	15:53	32	416	-2.6	-16.4	

GT Risto/Epi/AAU46619/A23187/Spont. Agg



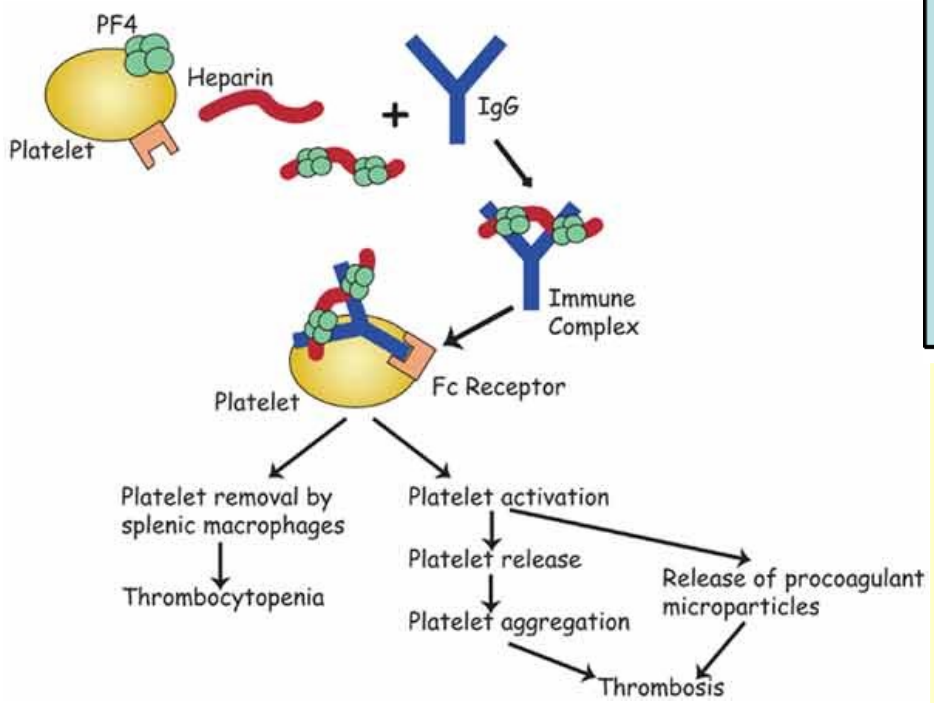
Assay Group	Conc.	Time	Abs PPP[mOD]	Abs PRP_s[mOD]	Max[%]	Fin[%]	La
Ris%	1.5g/L	14:53	33	407	92.0	84.0	
Ris2%	1.25g/L	14:53	33	430	89.9	85.9	
Ris3%	1g/L	14:53	33	401	87.2	81.5	
Ris4%	0.75g/L	14:53	33	425	2.3	-2.3	



Assay Group	Conc.	Time	Abs PPP[mOD]	Abs PRP_s[mOD]	Max[%]	Fin[%]	La
Epi%	10uM	15:53	32	400	-0.8	-14.4	
Epi2%	5uM	15:53	32	420	-0.8	-8.8	
Ara%		15:53	32	399	-2.2	-22.3	
Ara2%	1.5mM	15:53	32	424	-1.3	-13.3	
Ara3%	1mM	15:53	32	400	1.4	-14.7	
U46619%	0.5mM	15:53	32	411	-0.5	-29.8	
A23187%	1uM	15:53	32	421	-1.0	-13.9	
SpontAgg%	40uM	15:53	32	413	0.8	-11.5	
	0.9% NaCl						

HIT mechanism

Caused by formation of IgG antibodies against heparin : PF4 complex
Immune complexes can bind platelet FC receptors leading to activation and consequent thrombocytopenia and thrombosis



OD [Units]	Interpretation
<0.40	0
0.40 – <1.00	<5%
1.00 – <1.40	~20%
1.40 – <2.00	~50%
>2.00	>90%

Platelet activation	ELISA	Interpretation
+	+	HIT II confirmed
–	–	HIT II unlikely[a]
+	–	HIT II likely
–	+	HIT II unlikely[a,b]

HIT II = heparin-induced thrombocytopenia type II
[a] Look for other causes of thrombocytopenia.
[b] Consider repeat platelet activation assay if clinically warranted.

Light transmission aggregometry – HIT

Normal PRP is mixed with patient PPP at low & high concentration of heparin (0.5 & 100 U/mL)

HIT antibodies present will bind and activate donor platelets at low dose heparin resulting in aggregation

HIT antibodies swamped at high dose heparin

PRP donor selection is critical. HITA sensitivity varies from 35-85%.

HIT – flow cytometry

Test serum + donor platelets incubated at low & high heparin concentration

Measure platelet surface alteration binding of Annexin V to activated surface (-ve charge) P-selectin expression

Concluding remarks

- Platelet function testing currently preserve of specialist laboratories
- ? Underdiagnosed
- Streamlining of process combined with improved productivity through automation.
- In future – genetic based diagnosis?

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Dr Áine McCormick

Diagnostic Haemostasis
Viapath Analytics
4th Floor North Wing
St. Thomas' Hospital
London
SE1 7EH

Email
aine.mccormick@viapath.co.uk