Platelet Function Testing: Laboratory Tests and Quality Control

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DISCLOSURES

Relevant Financial Relationship(s)

None

Off Label Usage

None

The session objectives are to:

- Discuss and provide examples of the importance of laboratory testing for diagnosing hereditary and acquired platelet function disorders
  - Include some recent research on the common and the rare

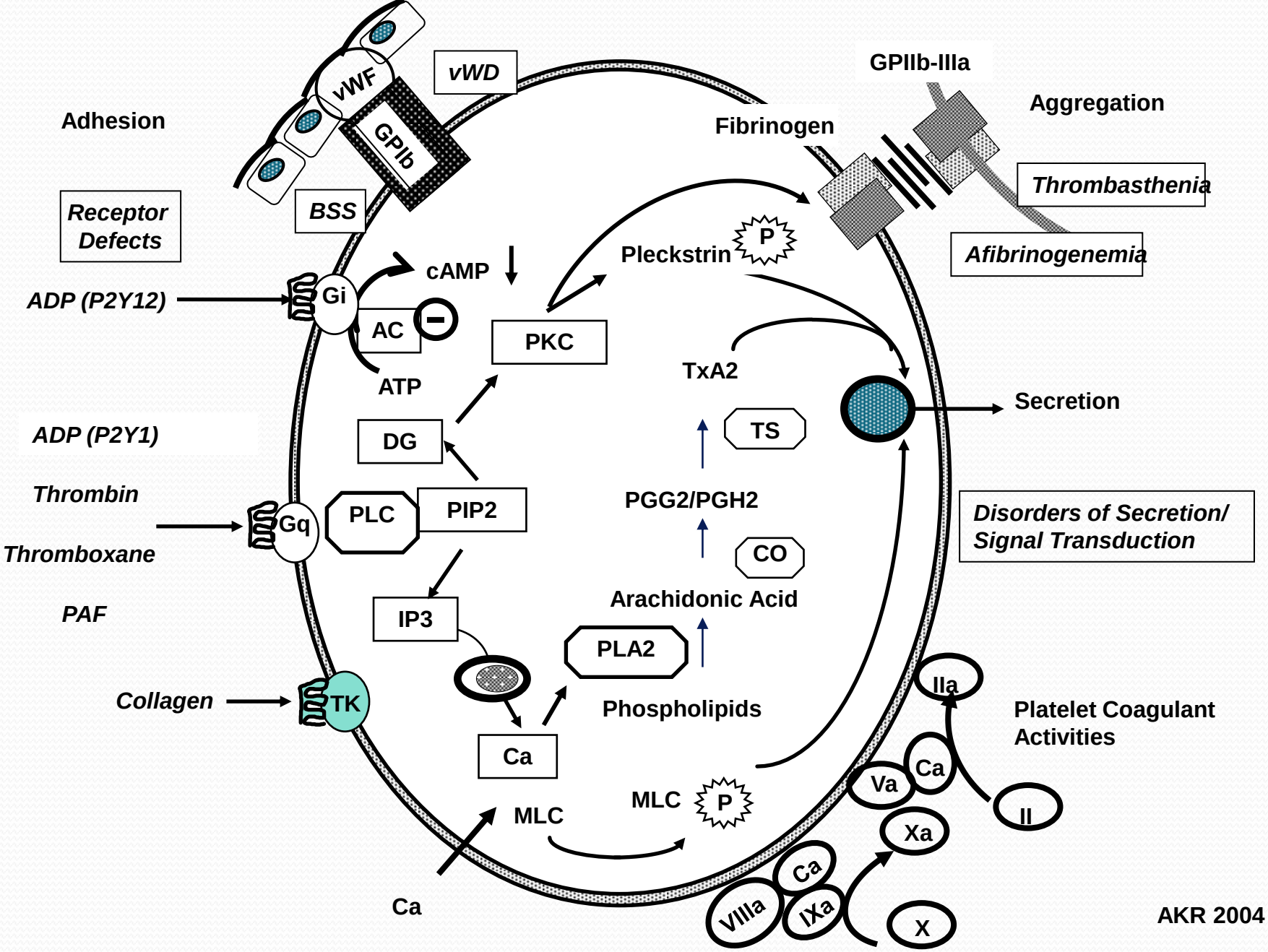
- Discuss standardization initiatives, quality practices and external quality initiatives for platelet function testing

Why is a functional testing for platelet disorders important?

- Platelet function tests are essential for the diagnosis of many platelet disorders
- Inherited and acquired platelet function disorders are common
  - possibly more prevalent than von Willebrand disease
- Diagnostic information that the tests provide helps to guide and plan appropriate therapy

Unpublished Data CHAT Study: Platelet Disorder Prevalence Among Individuals Referred for Bleeding Assessments

- Inherited and acquired platelet disorders are very common
- In our prospective cohort study, inherited and acquired platelet disorders were more common than von Willebrand disease among referred patients



Hayward et al, Haemophilia 2006;12 (Suppl 3):128-136

Current Situation

- Much more is known about a few rare disorders than about common platelet function disorders
- Few research studies have addressed key, practical issues on the diagnostic utility of functional tests for platelet function disorders
- Significant gaps exist in knowledge translation
  - e.g. Secretion defects are known to be common but few labs offer tests for assessing platelet secretion or dense granule deficiency which may not be detected by aggregation tests, bleeding times or closure times
- Guidelines are only beginning to emerge

What are the challenges to the laboratory diagnostic evaluation for a congenital platelet disorder?

- NO simple kits, standards
- NO widely accepted guidelines for doing the tests that are available, or for interpreting findings
  - Practices vary considerably
- Testing is expensive, time consuming, requires fresh blood samples, rapid testing
- Knowledge on the clinical/diagnostic utility of different assays and procedures for detecting congenital platelet disorders is limited
  - Needed for guidance on best/optimal practices
- Quality controls & proficiency testing – limited scope

In an Ideal World
Desirable Diagnostic Tests for Platelet Function Disorders

Modified from Hayward & Eikelboom, Semin Thromb Haemost, 2007

Setting	Characteristic	Additional comments
General	Convenient	Simple (no operator expertise required), rapid, inexpensive
	Accurate & Precise	The test measures what it is supposed to measure. Reproducible, different observers agree on interpretation
	Standardized	Test procedure is well described, standards are available, existing quality control program
	Affordable	Financial considerations are not a deterrent
Diagnosis of Platelet Dysfunction	Sensitive	Negative test rules out disease
	Specific	Positive test rules in disease
	Population norms to guide interpretation	Test has been evaluated in full range of subjects (mild & severe, treated and untreated disease) and in subjects with other conditions that fall within the differential diagnosis
	Proven utility	Patients are better off after undergoing the test

Complexities in the Diagnostic Evaluation for Platelet Disorders

need an assessment of many things, including:

- Platelet number and size, platelet and leukocyte morphology
  - ~17% of referrals are thrombocytopenic
- Platelet function by aggregometry (often by light transmission – LTA)
- ± Tests for platelet dense granule deficiency
  - Why? Aggregation, BT, PFA-100™ CT - *may all be normal*
- ± Tests of platelet secretion - release of dense granule contents
  - Why? More sensitive to some common function defects?
- Optional tests for specific individuals
  - tests for procoagulant defects (appear rare but testing rarely done)
  - transmission electron microscopy (EM), glycoprotein analysis, genetic testing, etc
    - e.g. Western blots or ELISA for uPA for Quebec Platelet Disorder
    - e.g. EM for gray platelet syndrome

Value of Screening Tests

- Closure Time measured by PFA-100™
  - Rapid, simple, test of shear-dependent platelet adhesion

"The test should be considered optional as current evidence indicates that although the PFA-100 CT is abnormal in some forms of platelet disorders, the test does not have sufficient sensitivity or specificity to be used as a screening tool for platelet disorders."

Hayward, Harrison, Cattaneo, Ortel and Rao; the Platelet Physiology SSC of ISTH. Platelet function analyzer (PFA)-100 closure time in the evaluation of platelet disorders and platelet function. JTH 2006; 4: 312-9.

- Bleeding Time
  - Sensitivity limited, performance issues
  - Jennings presentation at ISTH SSC 2007
    - Still done by many laboratories!

Availability of Functional Assays for Platelet Disorders

- A problem even in affluent countries:
  - Screening tests >>> Aggregation assays >>Specialized tests
    e.g. – dense granule tests done by <10% of specialized labs in North American Specialized Coagulation Laboratory Association
- Tests not standardized
  - might not be an issue for detecting the very rare severe problems like Glanzmann thrombasthenia BUT……

Importance of Aggregation Assays

still the most useful test for diagnosing platelet disorders

- Essential to diagnose some disorders, including:
  - Glanzmann thrombasthenia
    - αIIbβ3 or IIbIIIa deficiency or dysfunction
    - absent aggregation with all agonists except ristocetin
  - Bernard Soulier Syndrome
    - IbIXV deficiency or dysfunction
    - absent aggregation with ristocetin
  - Platelet type von Willebrand disease
    - Abnormal IbIXV function with increased aggregation with low concentration ristocetin, like type 2B VWD
  - Secretion defects (most common, quite heterogenous)
- Can help exclude drug induced dysfunction

Reality: Aggregation Tests Are Helpful

illustrative "real-life" cases evaluated by a standardized LTA method

	Reference interval % maximal aggregation	Glanzmann Thrombasthenia	Secretion Defect	Dense Granule Deficiency*	Thromboxane Generation Defect
ADP 5 µM	43-97	0	24	71	71
Collagen 5 µg/mL	85-104	0	83	70	62
Collagen 1.25 µg/mL	51-96	0	43	12	7
Epinephrine 6 µM	9-100	0	15 No secondary wave	41 No secondary wave	36 No secondary wave
Arachidonic Acid 1.6 mM	77-99	0	84	47	6
Thromboxane analogue U46619 1 µM	70-99	0	21	60	94
Ristocetin 0.5 mg/mL	0-7	0	3	7	4
Ristocetin 1.25 mg/mL	75-100	47	62	85	90

*** results can be normal in this type of platelet disorder**

Range of Initiatives to Address Standardization

- ISTH, CLSI
- Collection of data to define current testing practices
  - NASCOLA, RCPA, UK-NEQUAS - published
  - ISTH - not yet published
- Consensus and standardization :
  - opinions and practices
  - reviews of published evidence
  - expert opinions

Platelet Physiology Scientific Subcommittee (SSC) of the International Society of Thrombosis and Haemostasis

- Working groups to address topics including:
  - the use of platelet function testing for evaluating aspirin resistance
    - A. D. Michelson, M. Cattaneo, J. W. Eikelboom, P. Gurbel, K. Kottke-Marchant, T. J. Kunicki, F. M. Pulcinelli, C. Cerletti, A. K. Rao. J Thromb Haemost 2005;3:1309-1311.
  - the assessment of platelet function using the Platelet Function Analyzer, PFA-100®
    - *Hayward CP, Harrison P, Cattaneo M, Ortel TL, Rao AK. J Thromb Haemost 2006;4:312–9.*
  - the assessment of platelet function by light transmission platelet aggregation (LTA)

CLSI H58-P Guideline

- **Requirements/recommendations for:**

  specimen collection, pre-examination considerations, patient preparation, sample processing, testing, and quality control in relation to platelet function testing by aggregometry

- **Covers:**

  anticoagulants, specimen storage and transport temperatures, sample selection for various methodologies, establishment of reference intervals, result reporting, assay validation, and troubleshooting

- **Intended use by:**

  clinicians, hospital and reference laboratorians, manufacturers, and regulatory agencies

- ***Not for guiding:***

  global hemostasis tests, platelet counting, flow cytometry, point-of-care, test interpretation or therapy

Some Issues of Controversy

e.g. to dilute or not dilute PRP for testing LTA

- World practices are divided
- Recent publications
  - MA different if PRP is tested diluted in some LTA assays
  - Dilution with PPP may introduce an artifact (reduced aggregation response to some agonists) compared to adding buffer
- Is it acceptable to use "diluted PRP" for testing?
  - Our own data:
    - minimal differences (0.5-7.6%) in mean MA for native and adjusted PRP samples from healthy controls
  - Further prospective evaluation underway
    - patients versus control findings

Application of Proficiency Challenges for Platelet Function Testing by External Quality Programs

- CAP and RCPA Initiatives
  - Proficiency testing challenges with inhibitory additives
    - Test PFA-100® etc.
    - Description of findings to participants – data: wide scatter

- NASCOLA Platelet EQA
  - Challenges for LTA case interpretation
    - NASCOLA - completed; ECAT – survey still open
  - Completed: pilot proficiency testing challenge on testing for platelet dense granule deficiency
    - real clinical samples (normal + abnormal)

NASCOLA EQA Survey
Abnormal Sample Donor
Abnormal aggregation tests, absent to reduced ATP release, confirmed dense granule deficiency

- 38 year old woman, nickname **"Bruises"**
- Lifelong easy bruising, NO pigmentation abnormalities
  - Mistaken for a victim of abuse many times in life – by physicians
  - Permanent discoloration from some injuries
- Menorrhagia, since menarche
  - Interfering with lifestyle, requiring therapy
  - Complicated by iron deficiency anemia
- Hemostatic challenges
  - No serious bleeding problems so far with surgeries
  - Family physician concerned about her risks for bleeding

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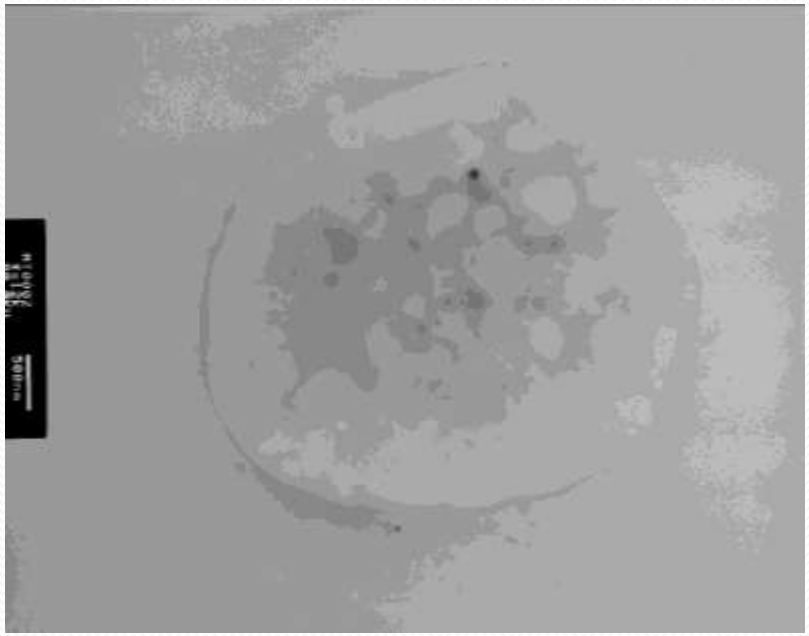
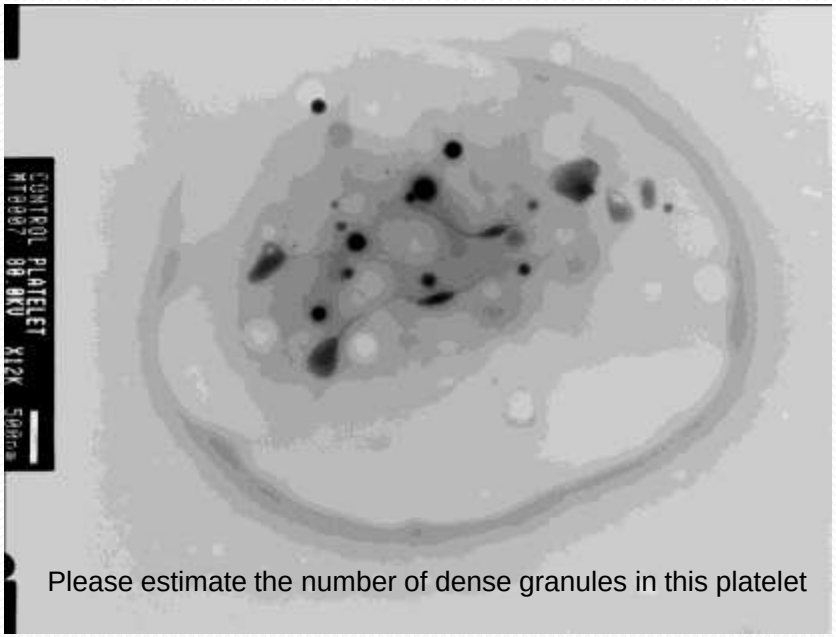
EQA Challenge

Abnormal Platelet Sample



Normal Platelet Sample - Image



Please estimate the number of dense granules in this platelet

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Findings of Proficiency Challenge

8 sites participated – 6 "experienced"

- Grid Challenges
  - Participants correctly recognized:
    - Dense granule deficient sample as abnormal
    - Healthy control sample as normal
- Image challenges (*What to count, or not count):*

  Agreement: >82% overall, higher for "experienced"
  - Reference interval implications
- Important proof of feasibility
- Peer comparisons helpful to less experienced

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LTA Interpretation EQA

- NASCOLA results
  - Point out an important need for such EQA exercise
- Positive feedback from participant for future exercises

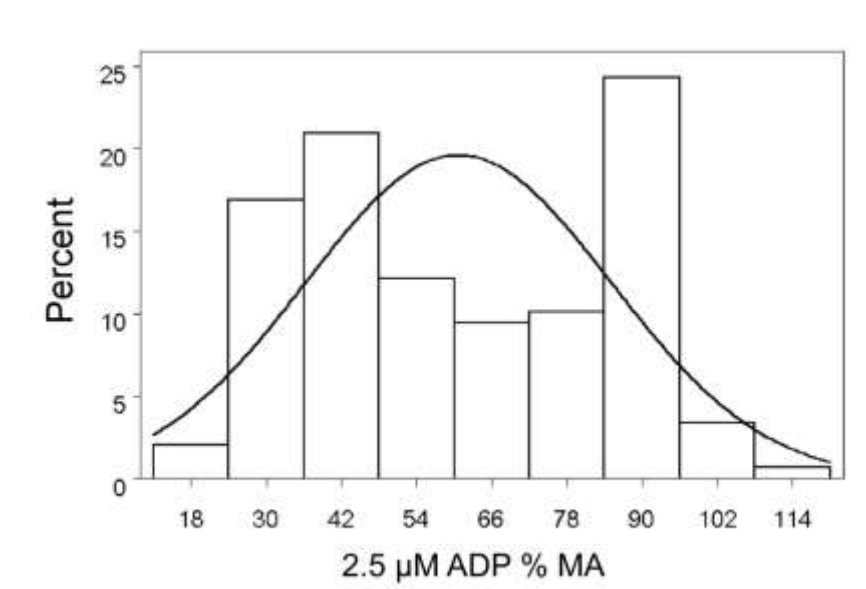
Just How Useful is LTA?

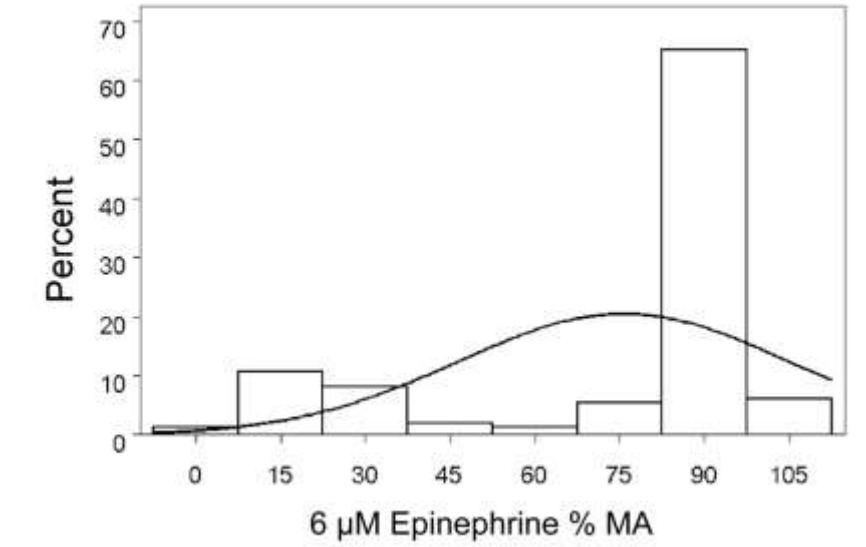
- Need to consider
  - Variability in practices……
  - False positives
    - ↑ risk as testing with multiple agonists
    - Were RI for test set appropriately?
  - False negatives
    - Were RI for test set appropriately?
    - Some disorders not always detected
      - Secretion defects , including dense granule deficiency
    - True negatives: Scott Syndrome

Challenges in evaluating platelet function

Normal variability in aggregation responses

TH 2008;100:134-145





Nonparametric analysis for determining aggregation RI

reduces false positives & false negatives, & allows inclusion of data for subjects tested multiple times

Hayward et al, TH 2008;100:134-145

	Reference intervals for % maximal aggregation (% controls abnormal)			
Agonist (total # samples/# of unique controls)	Nonparametric *All tests*	Nonparametric *First test*	Mean±2SD *First test*	Mean±2SD after log transforming *First test*
2.5 mM ADP (454/148)	24-96 (2.0)	24-96 (2.0)	13-108 (0.0)	24-132 (2.0)
5.0 mM ADP (440/145)	43-97 (3.4)	41-97 (2.1)	53-109 (5.5)	52-120 (5.5)
1.25 mg/mL Collagen (259/106)	51-96 (1.9)	48-101 (1.9)	61-111 (3.8)	34-200 (0.9)
5.0 mg/mL Collagen (450/149)	85-104 (2.7)	84-105 (2.0)	85-102 (2.7)	85-102 (2.7)
6 mM Epinephrine (453/149)	9-100 (2.0)	9-101 (2.0)	19-133 (10)	16-272 (6.7)
100 mM Epinephrine (440/148)	11-101 (2.7)	9-102 (2.0)	29-130 (11)	20-257 (6.8)
1.6 mM Arachidonic Acid (426/145)	77-99 (2.1)	77-99 (2.1)	81-101 (3.5)	81-102 (3.5)
1 mM Thromboxane Analogue U46619 (416/141)	70-99 (2.1)	68-99 (2.1)	71-108 (2.1)	64-123 (2.1)
0.5 mg/mL Ristocetin (453/150)	0-6 (2.7)	0-7 (1.3)	1-6 (2.7)	1-7* (1.3)
1.25 mg/mL Ristocetin (455/150)	75-100 (2.0)	75-100 (2.0)	78-103 (4.0)	78-104 (4.0)

Limits far above highest value (112) ever reported by instrument!

Quality Control Monitoring & Platelet Function Tests

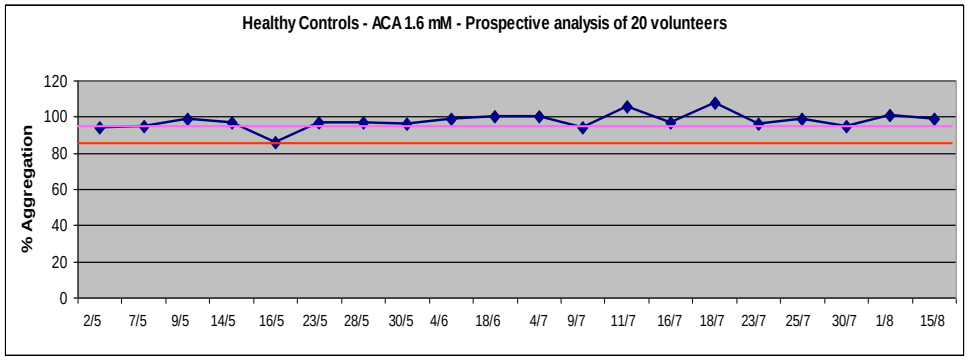
- Levey Jennings Charting
  - Feasible to do, even though data is for different controls
  - Useful also to monitor changeovers in reagents and instruments

PROCESS CONTROL CHART - MULTIPLE CHARACTERISTICS

% Aggregation in Healthy Controls

No.		% Aggregation																				row total	freq.	%
1	Individual Volunteer	95	92	96	95	100	94	91	85	93	94	99	95	96	94	91	94	93	96	99	94	1886	1.00	100
2																						0	0.00	0
	Date	8/1	10/1	15/1	29/1	5/2	7/2	19/2	28/02	5/3	13/3	14/3	21/3	28/3	2/4	4/4	11/4	16/4	18/4	25/4	30/4	**1886**	**1**	**100**
	Sample size	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	Sample total:	**20**	
	Column totals	95	92	96	95	100	94	91	85	93	94	99	95	96	94	91	94	93	96	99	94	Year:		
	% aggregation	95	92	96	95	100	94	91	85	93	94	99	95	96	94	91	94	93	96	99	94	Hospital:		
	Moving Range		3	4	1	5	6	3	6	8	1	5	4	1	2	3	3	1	3	3	5	Ward/Dept:		
	Mean aggregation* =	**94**				**Mean range =**				**4**				**LCL * =**			**85**							

(* Value of Centre line [mean p], LCL to use for next chart)



LCL for this chart =	85		Mean % aggregation for this chart =	94

Hamilton Data on LTA and BT: CHAT Study

Hayward et al, submitted

Study Design

- Prospective cohort: 331 patients referred for bleeding assessment
- Consented to participate in study
- Most tested by a panel of investigations that included LTA; testing deferred if subjects on NSAID, other function inhibitors

Diagnoses

- 2 physician review of charts, cross check with lab data, adjudication
- Final group: CBC platelets ≥ 150 X 10^9 L^{-1}, no evidence of VWD
- 229 patients (71% all subjects) ages 5-88,
- 48% with diagnosed bleeding disorders;
- Secretion defects: most common inherited platelet disorder (75%)

LTA & BT Analysis

- Sensitivity, specificity
- Likelihood: Odds Ratio estimates
  - LTA compare subjects with platelet disorders to 105 healthy controls (used first test with full panel)
  - BT compare subjects with platelet disorders to no bleeding disorder

BT Findings for CHAT Study

subjects without thrombocytopenia or VWD

- Poor sensitivity:
- Poor specificity:
  - Significant false + among subjects with “no bleeding disorder”
- Result doesn’t correlate with other findings
  - not predictive of abnormal LTA associated with a platelet disorder

BT and Symptoms Among CHAT Study Subjects with Inherited Platelet Disorders

- Essentially NO relationship with many of the major symptoms
- Action: omitted BT from bleeding disorder assessments

LTA – Is it Better than BT
Results for CHAT Study Subjects

- There is a much higher likelihood of detecting impaired platelet function by LTA than by BT
- No increase likelihood of abnormal LTA in subjects with “no bleeding disorder” than healthy controls
  - Therefore, healthy controls provide a good group to compare with patient test findings

LTA - Subjects without Thrombocytopenia or VWD

False Positives and False Negatives

- Testing with a panel
  - When all results are considered together, the number of false positives is greater than for single agonists but less than anticipated by chance as agonist responses show a relationship to each other
  - Need other tests (e.g. ATP release, dense granule tests) to adequately detect platelet disorders
  - LTA diagnostic utility is higher for inherited platelet function disorders compared to acquired platelet disorders
    - many acquired disorders are due to drugs or bone marrow disorders

Odds Ratio analysis of LTA findings

- Likelihood of detecting a platelet disorder varies by agonist
- In general, lower agonist concentrations are more helpful
- Two agonist abnormalities are more predictive of an inherited or acquired platelet function defect than single agonist abnormality, which can be a false positive abnormality

Receiver Operator Curve Analysis

Evaluation of %MA Finding - Inherited Platelet Disorders

- Illustrates that LTA has high specificity, moderate sensitivity
- Agonist responses show relationships with each other
- Multivariate analysis for inherited disorders:
  - most information on common inherited platelet function defects is provided by:
    - 1.25 μg mL^{-1} Horm collagen
    - 6 μM epinephrine
    - 1.6 mM arachidonic acid
    - 1.0 μM thromboxane analogue

Implications of Findings

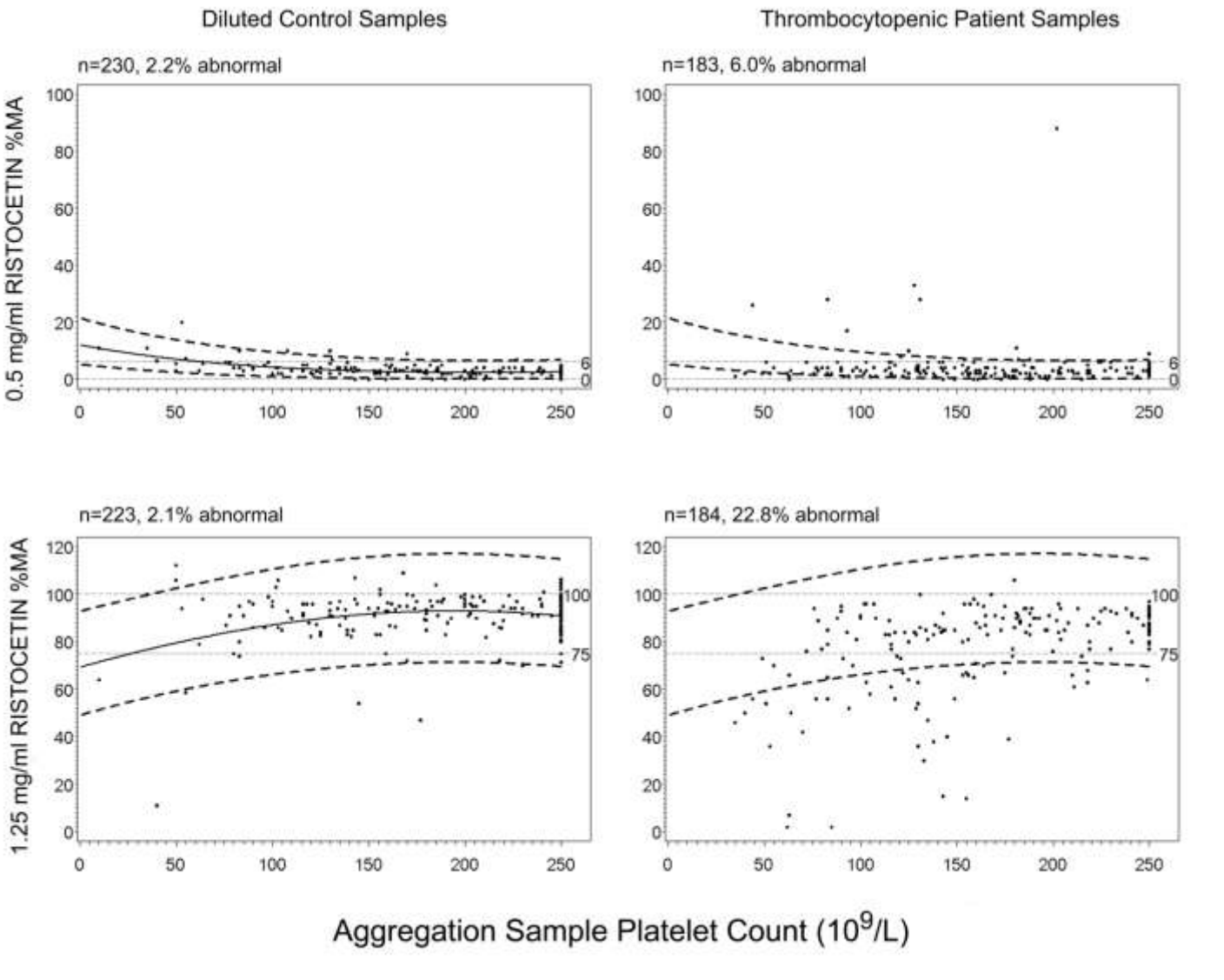
- LTA has important diagnostic utility
- Need strategies to distinguish “true” from “false” positives when testing with a panel
- Might be able to simplify test panels
  - i.e. to use single, concentrations of ADP, collagen, and epinephrine that are sensitive to common disorders
  - Do not want to cut back too much → false negatives
  - Still need agonists like ristocetin….

Challenge of Doing LTA on Thrombocytopenic Patients

Hayward et al, TH 2008;100:134-145

- Requests are not rare: ~17% of referrals for diagnostic platelet function testing in Hamilton
- Many labs (~1/3 from ISTH SSC survey) refuse to test platelet function if platelet count is low!

Hayward et al Thrombosis Hemostasis 2008;100:134-145



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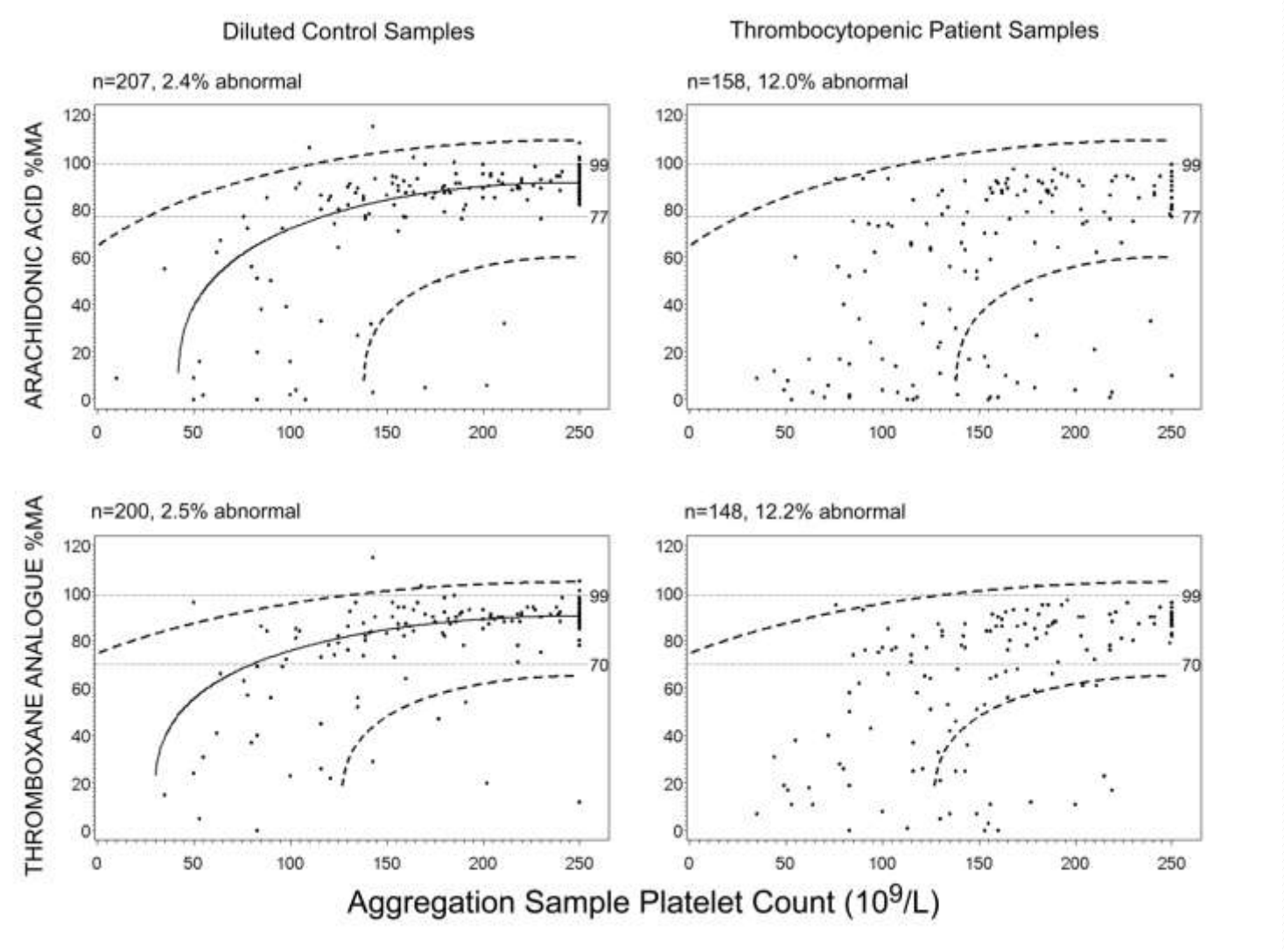


Hayward et al Thrombosis Hemostasis 2008;100:134-145

	PRP Platelet Count (10^9/L)			
Agonist	**≤80**	**>80–≤100**	**>100 - ≤140**	**>140–250**
Ristocetin 0.5 mg/mL	Use derived limits for samples with low platelet counts If aggregation is increased, evaluate for possible type 2B or platelet-type von Willebrand disease			
Ristocetin 1.25 mg/mL	Test all samples If maximal aggregation is reduced, exclude Bernard Soulier Syndrome and von Willebrand disease, if clinically indicated			
Collagen 1.25 μg/mL	Omit for all samples with low platelet counts or limit testing to samples with platelet counts >140 X 10^9/L, using derived limits for samples with low counts			
Collagen 5 μg/mL	Interpret with caution	Use derived limits for samples with low platelet counts		
ADP 2.5 μM	Omit	Use derived limits for samples with low platelet counts		
ADP 5.0 μM	Omit or consider using RI for samples with low platelet counts	Use derived limits for samples with low platelet counts		
U46619 1 μM	Omit			Use derived limits for samples with low platelet counts
Arachidonic acid 1.6 mM	Omit			Use derived limits for samples with low platelet counts
Epinephrine 6 & 100 μM	Omit			Evaluate for absent primary and/or secondary aggregation, if suspect Quebec Platelet Disorder (better to test for ↑ platelet uPA)

How Much Testing and What Kind of Testing is Enough?

	Current RI (% MA) PRP 250x10^9/L	Daughter Native PRP 255x10^9/L	Son Native PRP 255x10^9/L
ADP 2.5 μM	24-96	57	33
ADP 5.0 μM	43-97	69	63
Collagen 1.25 μg/mL	51-96	4	2
Collagen 5.0 μg/mL	85-104	86	80
Epinephrine 6 μM	9-100	74	29
Epinephrine 100 μM	11-101	74	41
Arach. Acid 1.6 mM	77-99	82	84
U46619 1 μM	70-99	16	20
Risto 0.5 mg/mL	0 – 7	6	3
Risto 1.25 mg/mL	75-100	84	90

Platelet Secretion Studies – Children Summarized

	Current RI (nM ATP release)	**Daughter**	**Son**
Thrombin 1U/mL	0.80 – 2.64	0.60	0.63
ADP 5.0 μM	0.35 – 1.58	0.18	0.24
Collagen 1.25 μg/mL	0.35 – 1.59	0.14	0.15
Collagen 5.0 μg/mL	0.81 – 1.83	0.49	0.33
Epinephrine 6 μM	0.37 – 1.53	0.32	0.00
Epinephrine 100 μM	0.28 – 1.72	0.18	0.00
Arach. Acid 1.6 mM	0.31 – 2.42	0.28	0.00
U46619 1 μM	0.20 – 1.04	0.00	0.00

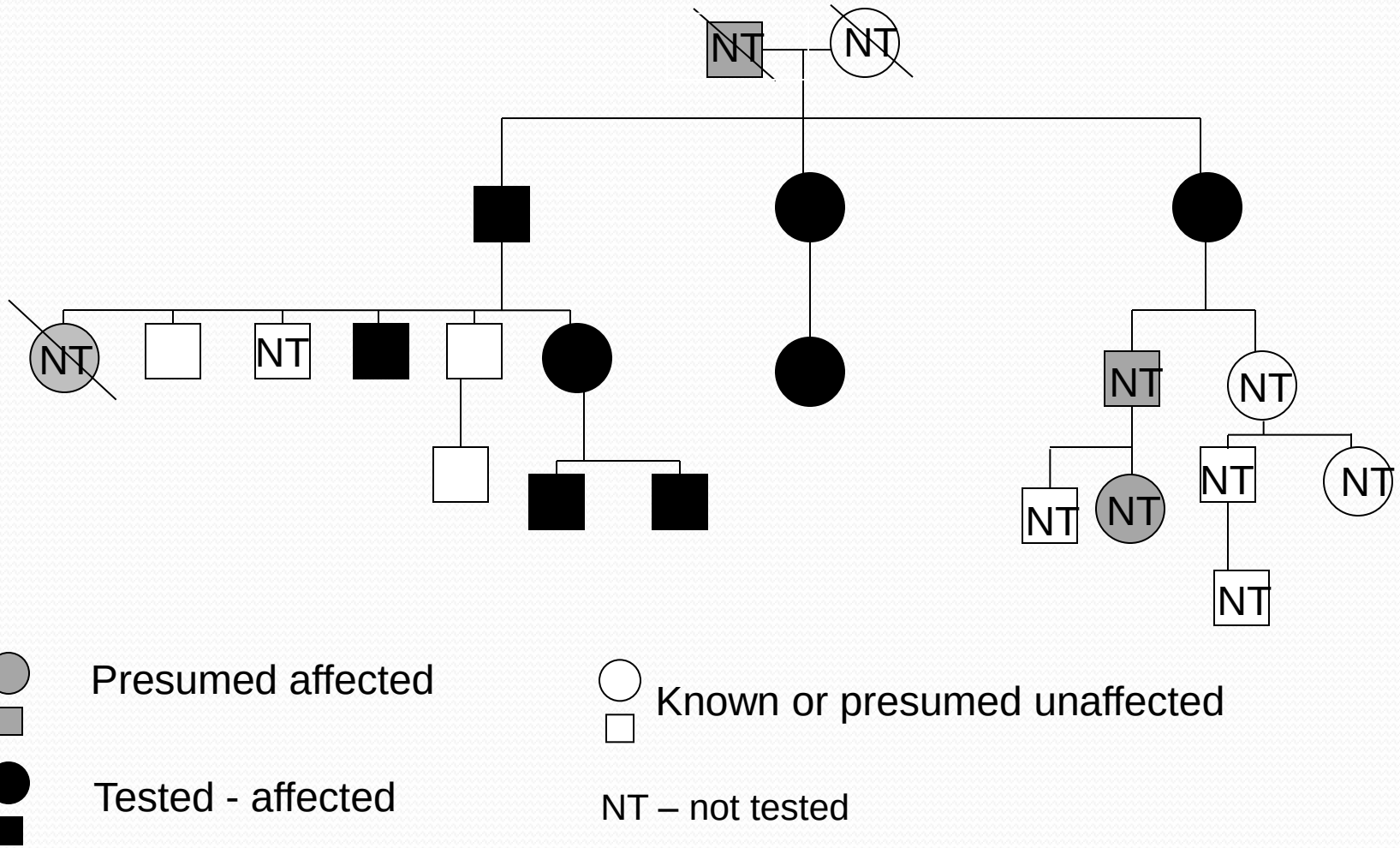
quality initiatives – SOP for test interpretation

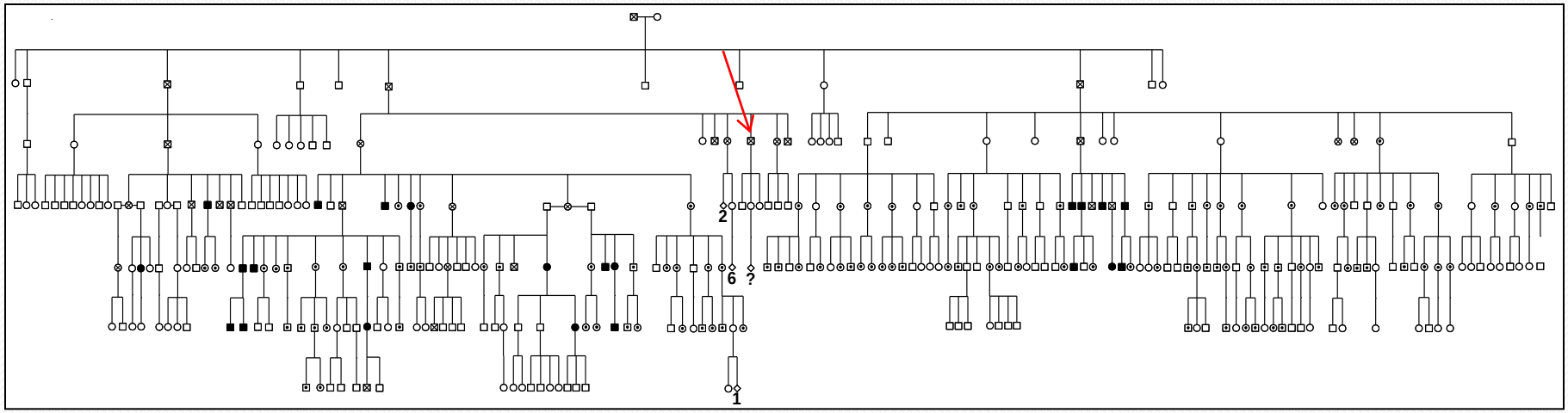
Hayward CPM. Transfusion and Apheresis Science 38 (2008): 65–76

Was the testing done using a sample with a normal platelet count?	**If NO, evaluate response to ristocetin and interpret findings for other agonists with caution as the reference ranges for samples with normal counts may not apply**
Are any of the aggregation responses abnormally reduced?	If NO: assess for any abnormal patterns of response (e.g. deaggregation). Consider further testing for disorders that may have normal aggregation findings (e.g. Scott Syndrome, δ-granule deficiency) If YES: consider confirmation on another sample. Do findings fit with: a) aspirin-like defects (aggregation is ↓ or absent with arachidonic acid, normal with thromboxane, ↓ with low dose collagen, and there is absent secondary aggregation with epinephrine); the drug history should be reviewed b) Glanzmann thrombasthenia (aggregation is present only with ristocetin) c) Bernard Soulier Syndrome (aggregation is absent with high concentrations of ristocetin; check that von Willebrand factor deficiency has been excluded) d) Type 2B or platelet-type von Willebrand disease (↑ aggregation with low concentrations of ristocetin; if type 2B, this abnormality may be present when test plasma is added to normal platelets; if platelet-type is suspected, check if aggregation occurs with added cryoprecipitate) e) another type of abnormality (e.g. ↓ aggregation with multiple agonists that could be more striking for weak agonists, such as ADP and epinephrine). Consider first the common causes of this kind of abnormality, including δ-granule deficiency and secretion defects. Note: ADP response should be normal in δ-granule deficiency. If there is markedly reduced aggregation with ADP, consider the possibility of a P2Y12 defect. Consider the QPD if there is a family or personal history of delayed bleeding and reduced aggregation with epinephrine, with or without ↓ aggregation with ADP and collagen)

Example of need for other diagnostic tests for platelet function defects

family with “bleeders”: low to normal platelet counts, normal VWF, no secondary wave with epinephrine (or no response), some with reduced aggregation with ADP and/or collagen, dense granule release found to be abnormal - ?secretion defect

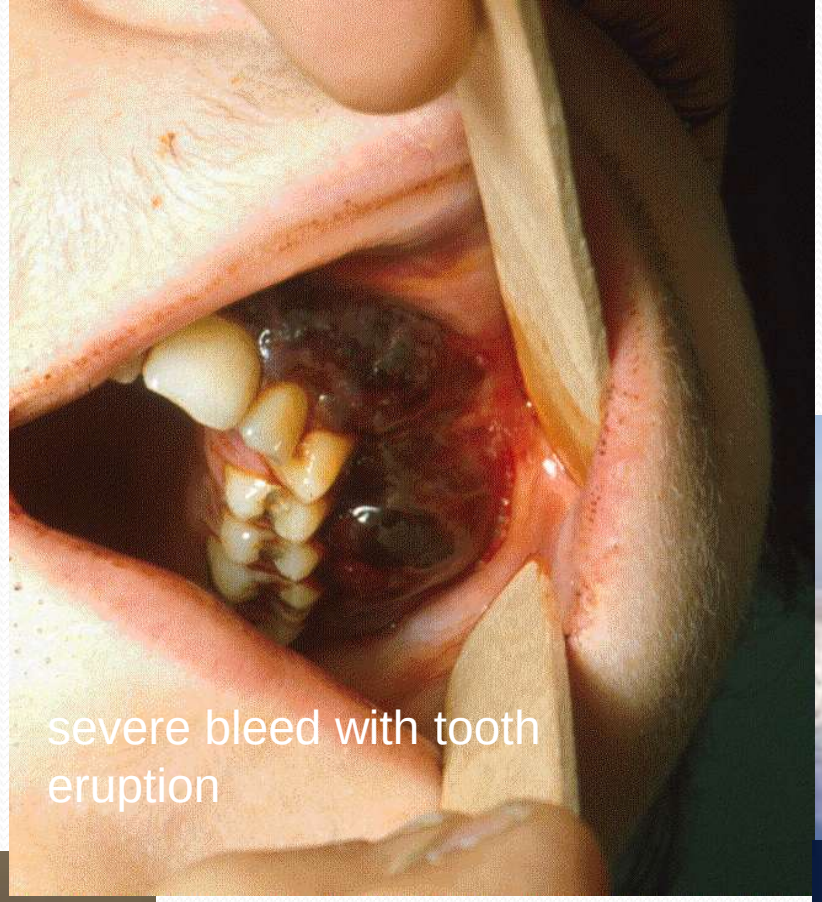


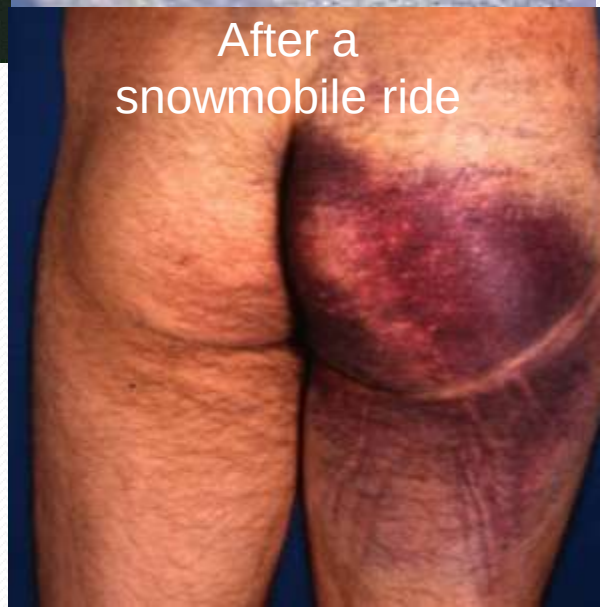


Part of a larger family – with a known bleeding disorder

Bleeding history – unusual……

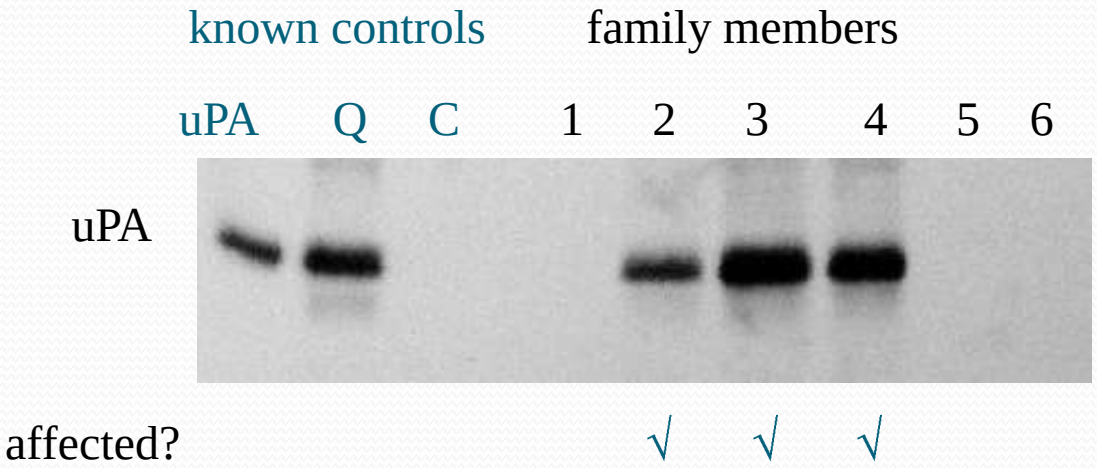
Illustrations of bleeding in a member of this family





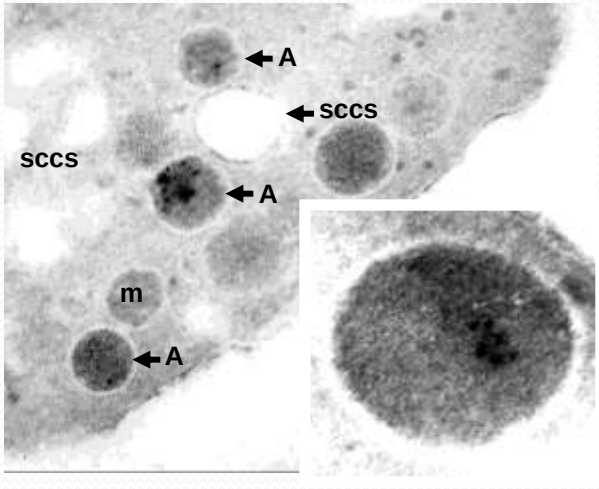
Diagnostic test performed.....for profibrinolytic platelets
Finding: increased platelet uPA (and also α-granule protein degradation)

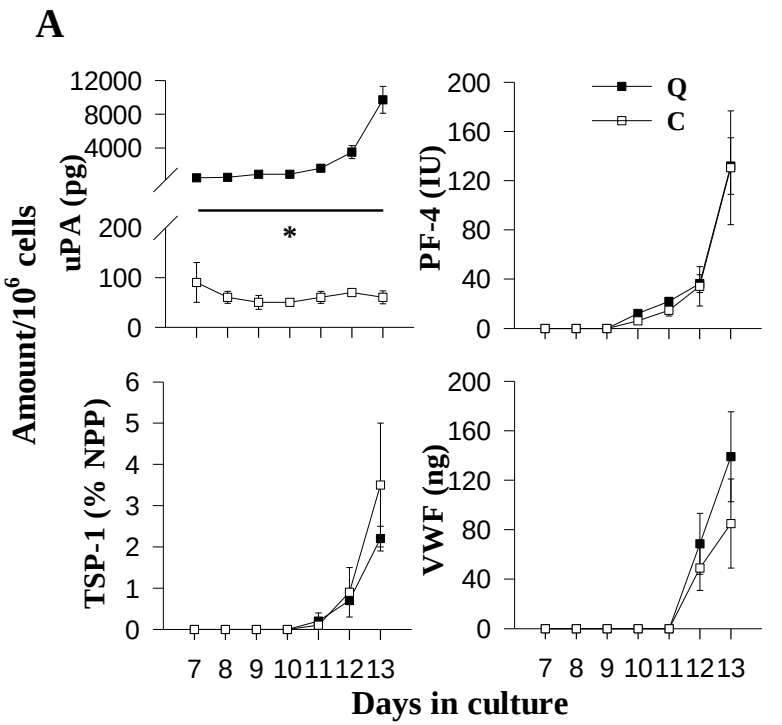
platelet uPA Western blot

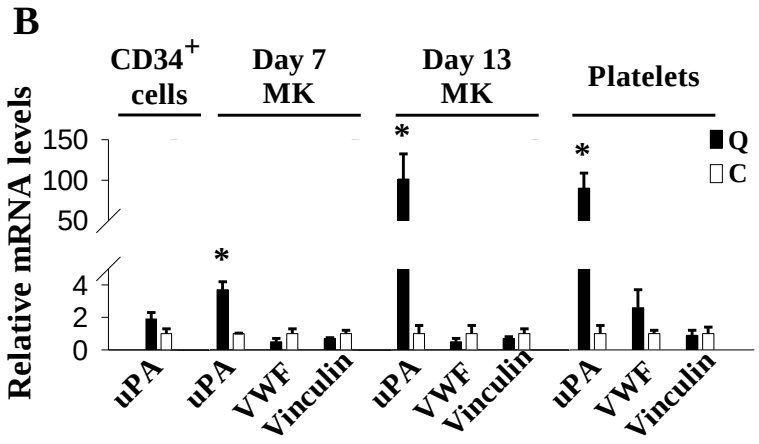


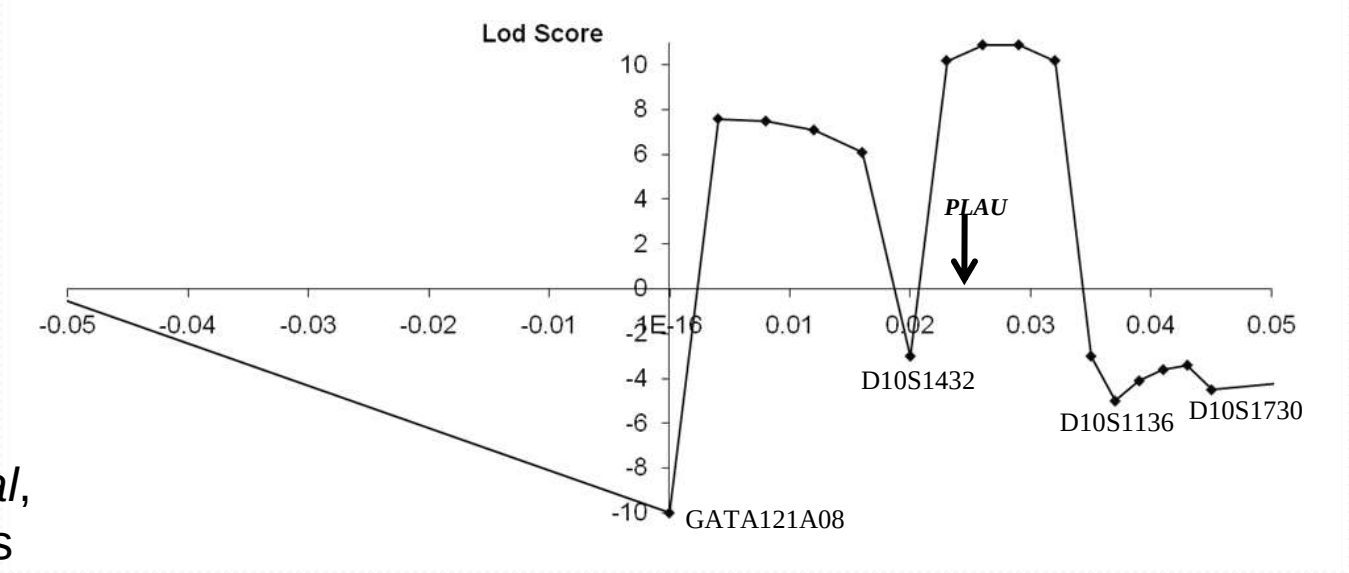
Disorder prevalence:
About 1 per Million in Canada
1:300,000 in Quebec
1:150,000 in another province of Canada

Veljkovic *et al*,
Blood, In Press









Diamandis *et al*,
Blood, In Press

QPD is mutation linked to inheritance of PLAU

To be presented at ASH:
due to a tissue-specific cis-regulatory defect in *PLAU* transcription, that does not affect transcription of the adjacent genes for vinculin and CAMK2G
that is not caused by mutations within PLAU or its characterized regulatory elements

Near Future: genetic tests specific for this disorder

Concluding thoughts

- Function testing for platelet disorders
  - If performed well, LTA is commonly helpful but other assays are needed to diagnose some platelet function disorders
  - All platelet function tests benefits from quality initiatives, standardization and performance assessments, even though this can be challenging
  - Need for more evidence to define appropriate diagnostic criteria for many platelet function disorders
    - Ideally based on both: bleeding history and laboratory findings
      - Has been done for QPD, need to do for more common disorders!

Acknowledgments

- Colleagues and collaborators
  - NASCOLA
  - ECAT
  - ISTH SSC
  - CHAT study
  - QPD studies
  - Research Team
  - HRLMP Special Coagulation