Platelet Functional Disorders and Testing

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Disclosures for Catherine P. M. Hayward

Research Support/PI	No support from industry Supported by: Heart and Stroke Foundation of Ontario Canadian Institutes for Health Research Canada Research Chair
Employee	N/A
Consultant	N/A
Major Stockholder	N/A
Speaker's Bureau	N/A
Scientific Board Advisory	N/A

N/A = Not applicable (no conflicts)

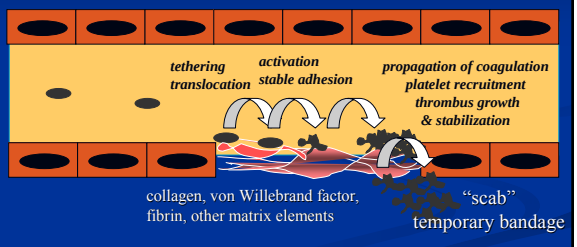
Session Objectives

- Important tests for evaluating platelet functional disorders
- Common forms of platelet functional disorders
- Why platelet function testing is difficult to standardize and issues important to test quality
- Understand, through case-based examples, the importance of diagnostic testing for platelet functional disorders

Perspectives on Platelet Functional Disorders in 2007

- Disorders - common and important
- Uncertainties about best test practices for evaluating these conditions and about test sensitivity, specificity, positive predictive value, negative predictive value
- Also lack tools for standardizing the clinical part of the diagnostic assessment

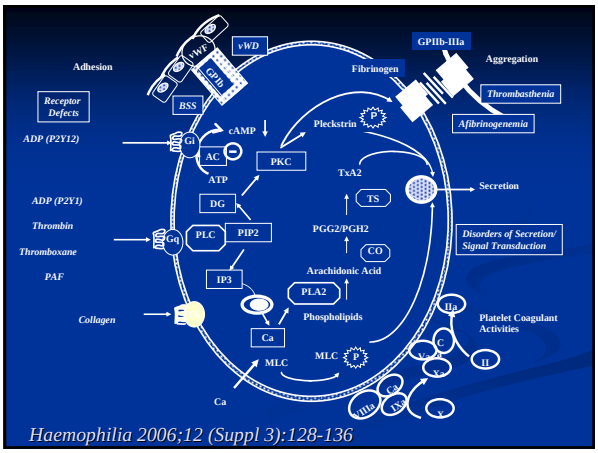
Current Concepts on Platelet Functions Implications for Types of Potential Defects



Disorders with Impaired Platelet Function

- Congenital or acquired defects in
  - Receptors for:
    - Adhesive proteins
  - Signaling/Activation Problems
    - Receptors for important agonists*
    - Signaling/secretion pathways that enhance activation (including release of dense granule contents)*
    - Platelet procoagulant activity
- Some conditions that affect platelet numbers also impair platelet function

* common



Haemophilia 2006;12 (Suppl 3):128-136

Acquired Qualitative Defects

- Drugs – antiplatelet agents are the most common
- Uremia
- Liver disease
- Cushing's Syndrome
- Cardiopulmonary bypass
- Inhibitory antibodies
- Bone marrow disorders
  - Diverse – e.g. storage pool defects, membrane glycoprotein deficiencies

Platelet Disorders

Lack data from population surveys

Secretion defects are more common than dense granule deficiency

Dense granule deficiency is almost as common as von Willebrand disease

about 3-5% of referred patients at our center

Screening Tests for Platelet Functional Disorders

- Bleeding time
  - Sensitivity limited, performance issues
  - Use – predicting response to DDAVP therapy?
- Closure Time measured by PFA-100™
  - Rapid, simple, test of shear-dependent platelet adhesion
  - Sensitivity
    - not perfect for screening
    - 24% to >90% sensitivity for congenital platelet disorders
      - Poorer for studies prospectively evaluating platelet disorders
      - Poorer for common 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.

PFA-100® Closure Times in Congenital Platelet Disorders

JTH 2006; 4: 312-319.

** - common disorders* *‡ - associated with thrombocytopenia*

	CADP CT	CEPI CT
Glanzmann Thrombasthenia	↑↑	↑↑
Aspirin-Like Defect	N	↑
ADP Receptor/Signaling Defect	↑-N	N
Dense Granule Deficiency*	N or ↑	N or ↑
Hemansky Pudlak Syndrome	N to ↑	N to ↑
Primary Secretion Defects*	N	N or ↑
Platelet Procoagulant Defect	N	N
Bernard-Soulier Syndrome‡	↑↑	↑↑
Platelet-type von Willebrand Disease ‡	↑↑	↑↑
Gray Platelet Syndrome ‡	↑	↑
Hereditary Macrothrombocytopenia Associated with Nonmuscle Myosin Heavy Chain IIa Syndromes ‡	N	N or ↑
Undefined Autosomal Dominant Thrombocytopenia ‡	N	N

CT in Diagnostic Testing for Platelet Disorders

JTH 2006; 4: 312-319

JTH 2005: 3;1309-11

- Potential advantages
  - Early clues about a defect if abnormal
  - Abnormal results may trigger a referral
- More evidence is needed on its most appropriate use in clinical practice related to platelet disorders
  - Sensitivity is better for VWD than for platelet disorders
  - Diagnostic Screening: FURTHER TESTING NEEDED, REGARDLESS
  - Role in drug monitoring – needs further evaluation

CT with anti-platelet drugs

JTH 2006; 4: 312-319

Drug	CADP CT	CEPI CT
inhibitors of $\alpha_{IIb}\beta_3$ abciximab, tirofiban, eptifibatide	P	P
COX-1 inhibitors (aspirin and other NSAIDs)	N	N or P
Thienopyridines: Ticlopidine or clopidogrel	N or P	N or P
Ticlopidine or clopidogrel plus aspirin	N or P	P

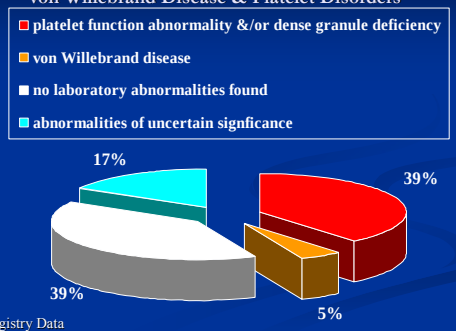
Tests for Drug Resistance Assay	Advantages	Disadvantages *All need more data on relationship to outcomes, sensitivity and specificity for resistance*
Aggregation ‡	"Gold standard"	Tech challenging, time consuming, not standardized between labs, some variability, choice of procedures (light transmission, electrical impedance; use of platelet rich plasma, whole blood)
VerifyNow® Aspirin & P2Y12 ‡ (mod. aggreg. with fibrinogen coated beads; RPFA)	Point-of-Care, simple, rapid, semi-automated	
PlateletWorks® (count platelets post-activation)	Simple, rapid, uses platelet counter	relationship to outcomes?
PFA-100® ‡ (high shear adhesion test with activation: CEPI or CADP)	Simple, Rapid, Semi-automated	influenced by other variables (hematocrit, platelet count, von Willebrand factor level)
Thromboxane Assays Serum (ex vivo generation) or urine (in vivo generation) ‡	With both: samples can be stored for batch analyses More complex than Point-of-Care	methods currently not in widespread use possible problems re specificity
Impact (Cone and Plate device)	Simple, Rapid, Semi-automated	relationship to outcomes?
Research Assays of Platelet Activation (e.g. flow cytometry)	modifiable	Not in wide use, complex, time consuming
VASP (vasodilator stimulated phosphoprotein – phosphorylation influenced by P2Y12)	Endpoint for P2Y12 function	Flow cytometry-based test; not in wide use

Complexity of Diagnostic Testing for Platelet Disorders

Include an assessment of/ for:

- Platelet number and size, platelet and leukocyte morphology
  - ~17% of referrals for testing are thrombocytopenic
    - Option – immunostaining for some conditions – e.g. MHY9 related disorders
- Platelet function, evaluated by aggregation tests
- Platelet dense granule deficiency
  - Aggregation, BT, PFA-100™ CT - *may be normal*
- Platelet secretion, evaluated by release of dense granule contents
  - ? More sensitive endpoint for defective function
- Adhesion testing
  - apart from tests such as the PFA-100™ CT, this remains in research domain
- Optional
  - Tests for procoagulant defects (appear rare, testing rarely done - ?Serum protein consumption to screen)
  - Others
    - transmission electron microscopy, glycoprotein analysis, thromboelastography (platelets contribute to properties of clots), etc

What do we find with our standardized testing?
Data for 391 Unselected Patients Prospectively Evaluated for von Willebrand Disease & Platelet Disorders



Hamilton Registry Data
March 2002

Quality Assurance and Platelet Tests

Hayward CPM, Eikelboom J. Platelet function testing: Quality assurance. STH

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
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 as a result of undergoing the test
Monitoring of Antiplatelet Therapy	Specific	Measures the effect of the drug on its target
	Clinically relevant	Results independently correlated with clinical outcome
	Modifiable	Altering antiplatelet treatment based on the results of the test improves clinical outcome
	Cost effective	Benefits of testing outweigh the direct and indirect costs of testing and follow up

All Labs are Not the Same…..

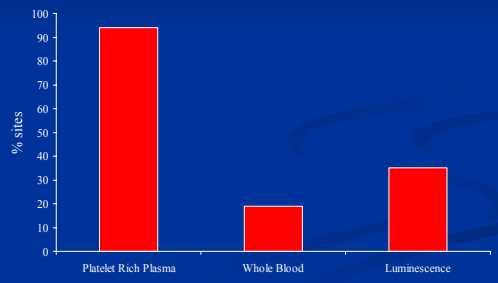
Variability Between Clinical Laboratories in Diagnostic Testing for Disorders of Platelet Function

Moffat et al, Thromb Haemost. 2005;93:549-53

- Goals
  - identify common practices and problems in the testing for disorders of platelet function
  - Enthusiastic participation!
    - 47 participating labs

Aggregation methodologies

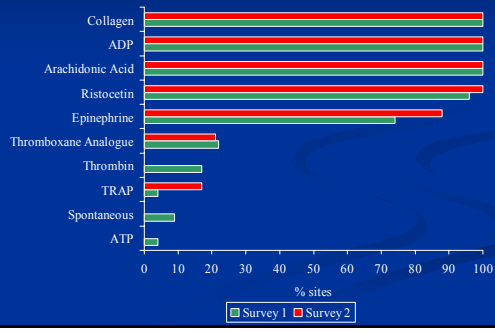
37% of NASCOLA sites used >1 method



NASCOLA Survey Data

agonists used for clinical aggregation

minority (15%) compared arachidonic acid & thromboxane analogue responses



Final Agonist Concentration for Testing Platelet Function by Aggregation

	Survey 1		Survey 2	
	Range	*Median*	*Range*	*Median*
Collagen	0.19 – 125 µg/mL	5 µg/mL	0.62 – 190 µg/mL	2.5 µg/mL
ADP	0.5 – 1000 µM	5 µM	1 – 20 µM	5 µM
Epinephrine	0.1 – 100 000 µM	18 µM	0.1 – 1000 µM	10 µM
Arachidonic Acid	0.0005 – 1.7 mM	0.5 mM	0.0016 – 2.5 mM	1.6 mM
Ristocetin	0.25 – 1.5 mg/mL	*Low Dose*: 0.5 mg/mL *High Dose*: 1.2 mg/mL	0.0012 – 2.0 mg/mL	*Low dose*: 0.5 mg/mL *High dose*: 1.25 mg/mL

Sources of reference intervals

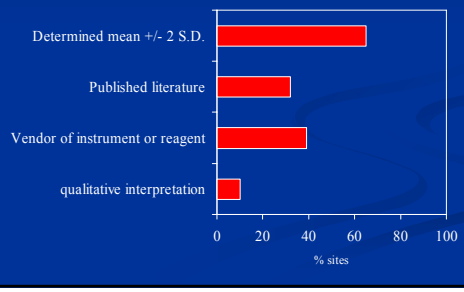
26% of sites used >1 method

29% if sites had not determined their own reference range (12-250 tests/yr)

Only 1 site did qualitative, without quantitative, interpretations

Only 1 site formally evaluated data for normality in distribution

See Moffat abstract this meeting



Concerns Raised About Platelet Aggregation Testing

there were many.....

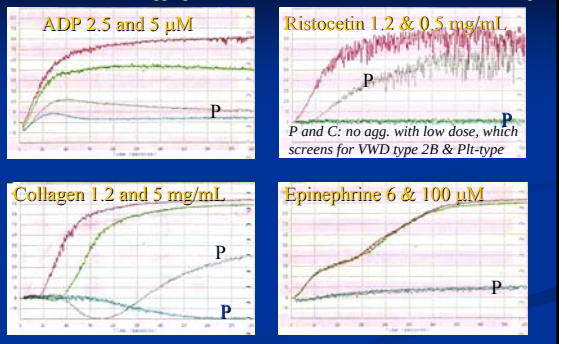
- Labor intensive
- Lack of evidence-based guidelines
- Uncertainties – how to:
  - evaluate thrombocytopenic patients
    - Tam Abstract - this meeting
  - interpret epinephrine aggregation
- Challenging to obtain reliable drug histories, uncertainties about the effects of different drugs
- Influence of pre-analytical errors
  - proper sample procurement & transport

Aggregation Testing – What is Best?

- Agonist Concentrations
  - Medians – some conformity
    - Are these appropriate concentrations?
    - Review of published literature
      - *The medians are probably good concentrations for testing*
  - Useful strategies
    - e.g. comparing arachidonic acid/thromboxane responses
      - NASCOLA Study: 15% of labs used this comparison to sort out possible ASA/NSAID-like defects at the time of this survey

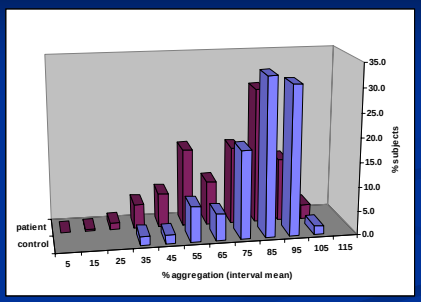
Control (red, green) vs. Patient (P) with Secretion Defect

P: also reduced aggregation with arachidonic acid and thromboxane analogue



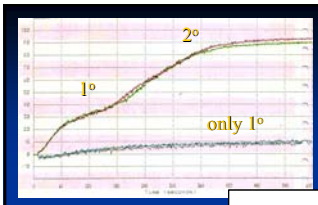
higher concentration of agonist: shown in red (C) & black (P)

Illustration of Aggregation Findings
% aggregation with 4 µM ADP

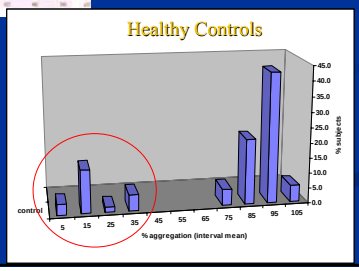


data is bimodal in distribution

→ likely due to an influence of P2Y12 polymorphisms



Epinephrine Aggregation



Variability in Aggregation Tests?

data from repeat tests done on 115 patients in Hamilton

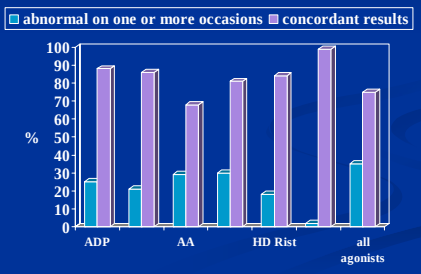
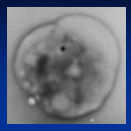


Illustration: Usefulness of Aggregation Tests

	Reference Interval % aggregn	Glanzmann Thrombasthenia	Secretion Defect	Dense Granule Deficiency (1/3 have normal results)	Thromboxane Generation Defect
ADP 5 uM	50-109	0	56	71	71
Collagen 5 ug/mL	85-101	0	83	70	62
Collagen 1.25 ug/mL	68-108	0	43	12	7
Epinephrine 6 uM	5-37 70-105	0	15	41	36
Arachidonic Acid 1.6 mM	72-108	0	84	47	6
Thromboxane analogue 1 uM	72-108	0	21	60	94
Ristocetin 0.5 mg/Ml	0-7	0	3	8	4
Ristocetin 1.25 mg/mL	76-104	47	62	85	90

Secretion absent or reduced with these agonists but normal with thrombin

δ-granules: ~ 2-5/platelet



Diagnostic Evaluation of Platelet "Luggage" Defects

α-granules: ~ 80/platelet

Types of Luggage

alpha (α) – protein storage container

delta (δ)– electron dense*

δ-granule deficiency:

Fairly common

~ 4% prevalence in our patients

Aggregation, BT, CT may be normal

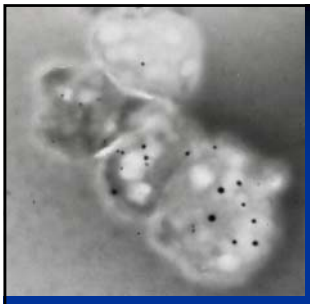
α-granule deficiency:

GRAY platelets - *rare*

Clue – from evaluation of blood film

combined αδ deficiency:

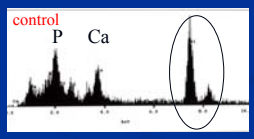
Rarer than δ-granule deficiency

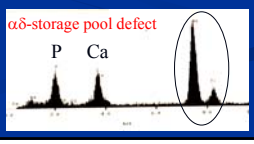


Whole Mount
Most popular method for assessing dense granule deficiency in North America
Controls: average of 4 or more electron dense granules per platelet

EDS
Electron Dispersion Spectral Analysis

analysis of the different dense granule constituents



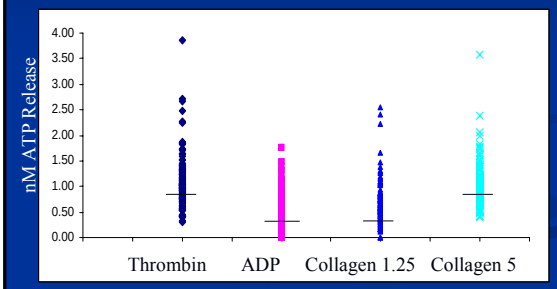


Diagnostic Evaluation of Platelet Secretion

- Secretion Defects
  - *Paradox or knowledge translation gap*
    - most common form of platelet disorder, yet secretion testing isn't commonly done
- Potential implications of NOT evaluating secretion?
  - Diagnostic label issue
  - Reduced detection of some platelet disorders?
- Methods to evaluate secretion
  - Radioactive: e.g. serotonin release
  - Nonradioactive: e.g. luminescence, other assays for nucleotides

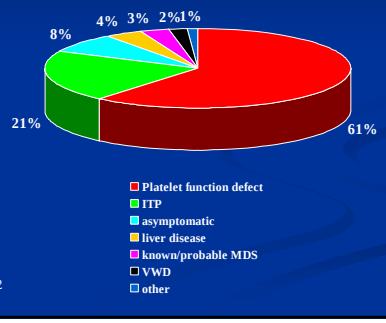
Hamilton Platelet Secretion Testing
Second Line Investigation - Luminescence Procedure

~56% of patients are abnormal - half of these have normal aggregation studies
bars - lower limit of reference range (determined using 48 controls)
local testing done with 8 parameters, 6 agonists



Testing Platelet Function in Thrombocytopenic Patients

17% of patients tested in Hamilton

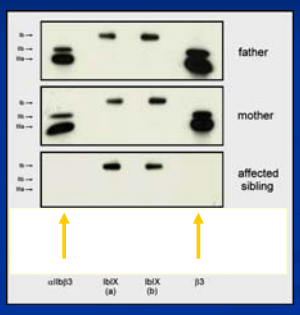


Data from 2002

	Reference Interval for samples with platelet count of 250 X 10^9/L % aggregation	Bernard Soulier Syndrome PRP: 29 X 10^9/L (less than 5% GP IbIXV by flow)	Control tested at same platelet count (PRP: 29 X 10^9/L)
ADP 5 uM	50-109	13	37
Collagen 5 ug/mL	85-101	21	72
Collagen 1.25 ug/mL	68-108	22	8
Epinephrine 6 uM	5-37 70-105	20	21
Arachidonic Acid 1.6 mM	72-108	17	62
Thromboxane analog. 1 uM	72-108	18	41
Ristocetin 0.5 mg/Ml	0-7	0	3
Ristocetin 1.25 mg/mL	76-104	0	79

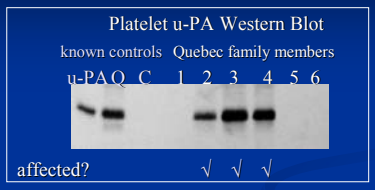
Special Diagnostic Evaluations

Illustration of Glycoprotein Analysis for Glanzmann Thrombasthenia

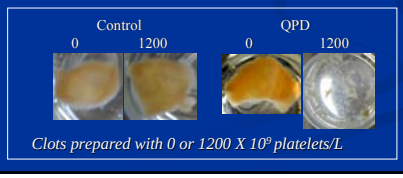


Testing for Rare Disorders - Quebec Platelet Disorder

clues: family history, delayed bleeding responsive only to fibrinolytic inhibitors, absent epinephrine aggregation, reduced to low normal platelet counts



J Thromb Haemost 2006;4:1086-94

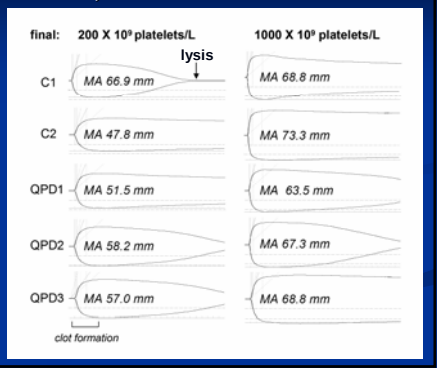


Clots prepared with 0 or 1200 X 10^9 platelets/L

Thromboelastography

platelets contribute to clot strength

blood samples recalcified, added TF & low concentration of t-PA



J Thromb Haemost 2006;4:1086-94

Mystery Case – VWD screen

55 year old male, severe bleeding after renal biopsy

- First sample (referred in)
  - FVIIIC 2.43 U/mL (243 U/dL)
  - VWF:Ag 1.31 U/mL (131 U/dL)
  - VWF:RCo 0.29 U/mL (29 U/dL)
  - Interpretative comment: *The von Willebrand factor ristocetin cofactor activity is significantly reduced. The discrepancy between this value and the normal VWF antigen suggest a form of type 2 von Willebrand disease. An analyses of von Willebrand factor multimers would be helpful to further evaluate. Is there a family history of von Willebrand disease or a bleeding history that suggests acquired von Willebrand disease? Repeat testing, including ristocetin-induced platelet aggregation would be helpful to confirm and further evaluate the von Willebrand factor abnormalities.*

Further Investigations

	RI for samples with 250 X 10^9platelets/L % aggregation	Patient	Control tested same day at same platelet count
ADP 5 uM	50-109	18	73
Collagen 5 ug/mL	85-101	45	91
Collagen 1.25 ug/mL	68-108	4	88
Epinephrine 6 uM	5-37 70-105	12	86
Arachidonic Acid 1.6 mM	72-108	34	90
Thromboxane analog. 1 uM	72-108	32	89
Ristocetin 0.5 mg/Ml	0-7	0	3
Ristocetin 1.25 mg/mL	76-104	3	91

Patient CBC: Plt 64 X 109/L, MPV 8.5 fL

Within RI for sample platelet count

Additional Investigations

- VWD screen done on day of aggregation testing
  - FVIIIC 1.34 U/mL
  - VWF:Ag 2.39 U/mL
  - VWF:RCo neat - 0.26; 1/2 - 0.63; 1/8 – 0.85 U/mL
  - Multimers Normal
- Further RIPA testing (1.25 mg/mL) done after a 30 minute incubation of patient or control PPP, with control PRP (PPP added to adjust platelets from 440 down to 250 X 10^9/L)
  - Patient Mixture: 2% aggregation
  - Control Mixture: 86% aggregation
- Diagnosis? Further tests that you would do?

Acknowledgments:

Colleagues and Collaborators

Clinical and Research Lab Staff

NASCOLA Platelet Study
K. Moffat, M. Ledford-Kraemer, W. L. Nichols

ISTH Platelet Physiology SSC PFA-100 Working Group