

Heparin Induced Thrombocytopenia

Diagnostic Principles and Testing Methods

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Overview and Objectives

- Provide background information about Heparin function and the use of Heparin derivatives
- Discuss the mechanisms of the formation of HIT antibodies
- List the clinical symptoms of HIT antibody formation
- Describe the test methods for detecting HIT antibodies
- Outline the application of the “4T” scoring system

Heparin

- A sulfated glycosaminoglycan containing a pentapeptide active site which binds to Antithrombin III (ATIII)
- The Heparin-ATIII complex binds Thrombin (Factor IIa), then the polysaccharide backbone wraps around the bound thrombin blocking its bioavailability
- Stored endogenously in the secretory granules of tissue Mast cells and circulating basophils
- Binds to 2500 different sites on the surface of platelets
- Heparin-Platelet Factor 4 (PF4) binding activates platelets and causes microparticle formation creating neoepitopes that can induce HIT antibody formation

Antithrombin III (ATIII, AT)

- Referred to as a serpin (serine proteinase inhibitor), it regulates coagulation *in vivo* by binding and neutralizing the serine protease factors: IIa (Thrombin), IXa, Xa, XIa, and XIIa, as well as kallikrein and plasmin
- Any heparin molecule of >17 saccharide units binds to ATIII and Thrombin simultaneously resulting in the inactivation of the bound Thrombin molecule
- Low molecular weight heparin binds to ATIII but displays preferential binding to Factor Xa over Thrombin
- Deficiencies of ATIII result in thrombotic episodes and reduced efficacy of heparin treatment; functional assays for ATIII are run as part of a thrombophilia panel

Unfractionated Heparin (UFH)

- A mixture of glycosaminoglycans extracted and purified from porcine gut mucosa
- Injectable medication used to treat patients with unstable angina and myocardial infarction (MI)
- Used to treat and prevent deep vein thrombosis (DVT), pulmonary embolism (PE), arterial thromboembolism
- Drives the thrombin-antithrombin reaction through a bridging mechanism which is 4x faster than the rate of the Factor Xa-antithrombin reaction
- UFH overdose is reversed using protamine sulfate
- UFH therapy is monitored using the aPTT and the chromogenic anti-Factor Xa heparin assay, but.....

Disadvantages of Using aPTT to Monitor Heparin Therapy

- No specificity, the aPTT can be prolonged in the absence of heparin under the following conditions:
 - Hypofibrinogenemia
 - Intrinsic pathway factor deficiencies (Hemophilias)
 - Intrinsic pathway factor inhibitors (Autoimmune)
 - Presence of Lupus Anticoagulant (Antiphospholipid Ab)
 - Presence of Fibrin Degradation Products (FDP's)
 - Presence of paraproteins (Monoclonal Gammopathy of Undetermined Specificity - MGUS, Multiple Myeloma)
- Inflammatory states in patients typically cause hyperfibrinogenemia, and Factor VIII activity of >150%, both of which shorten the aPTT and cause the aPTT to be less sensitive to the true level of the plasma heparin

Disadvantages of Using aPTT to Monitor Heparin Therapy

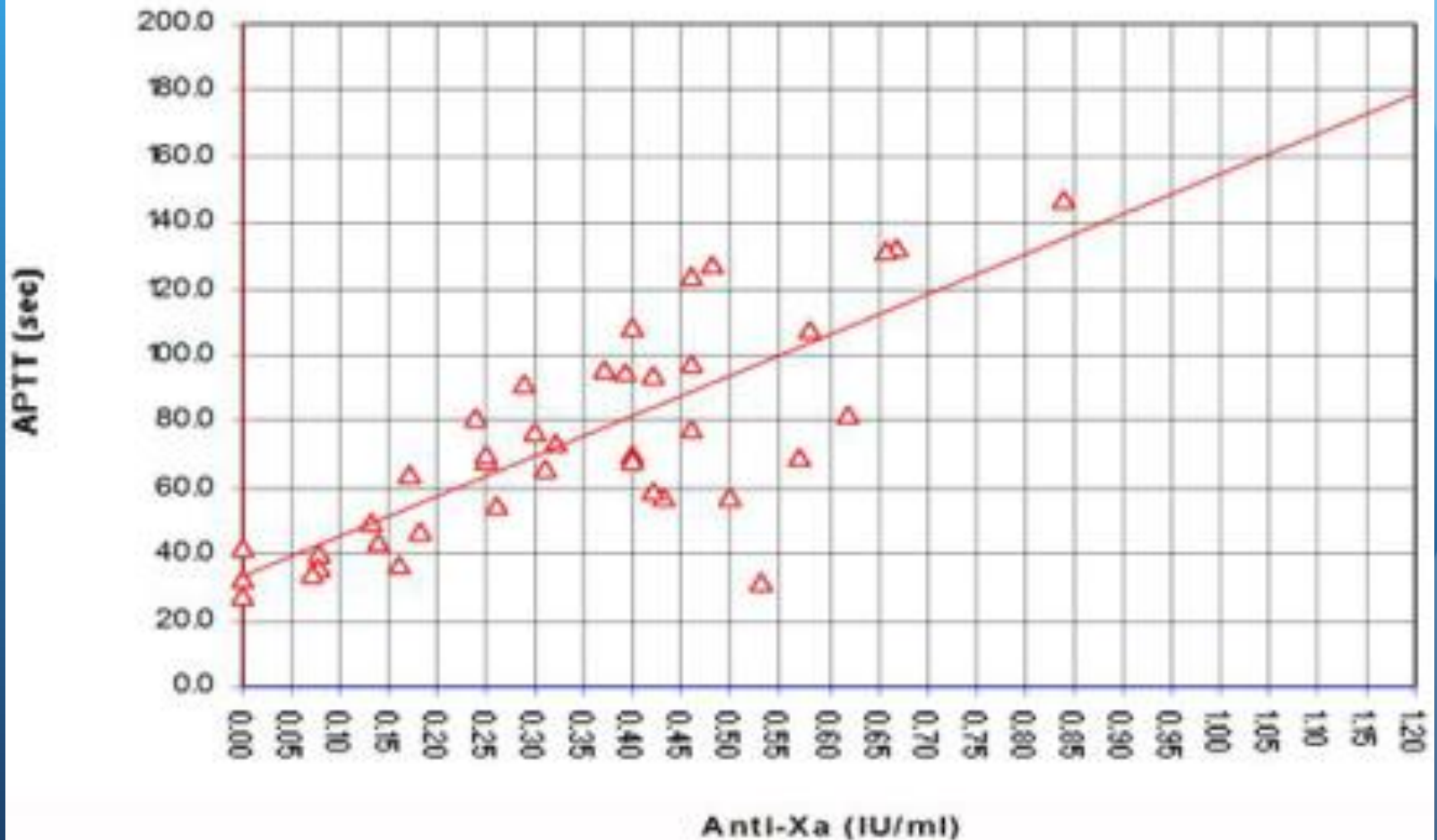
- Prolonged heparin therapy in patients can deplete the ATIII levels causing the aPTT values to be within the normal range even with high doses of heparin
- Platelets release Platelet Factor 4, which neutralizes heparin. In patients on heparin therapy, shortening of the aPTT begins after only 1 hour due to *in vitro* PF4 release, unless the blue-top specimen is immediately centrifuged and the plasma is removed from the cells
- There can be a great deal of variability in reagent responsiveness to Heparin due to differences in activator compounds, phospholipid composition and concentrations, lot-to-lot variation, etc.

Disadvantages of Using aPTT to Monitor Heparin Therapy

- CAP recommends that every laboratory establish and document its own Heparin therapeutic range for each new lot of aPTT reagent or with any change in coag instrumentation or reagent manufacturer by method comparison with the Anti-Xa assay:
 - 50-60 samples from patients with varying levels of UFH within the therapeutic range (0.3-0.7 IU/mL)
 - The PT and INR should be normal (INR <1.3)
 - Run PT, aPTT, and Anti-Xa UFH assays for all samples
 - Graph results with aPTT on the Y-axis and Anti Xa units on the X-axis (Brill-Edwards Technique)
 - Draw the best-fit line through the data points
 - CAP regulations HEM.23453 (Phase I) and HEM.23476

Graph of Brill-Edwards Technique

HEPARIN THERAPEUTIC RANGE



Disadvantages of Using aPTT to Monitor Heparin Therapy

- As an alternative to method comparison, CAP outlines a procedure for comparison testing of two different lots of the same reagent aPTT or two different aPTT reagent kits by using the cumulative summation method to detect drift:
 - Obtain at least 30 samples from different patients receiving heparin and run parallel comparison aPTT testing of both reagents or lots
 - Plot data with the old reagent on the x-axis and the new reagent on the y-axis and perform visual or regression analysis to identify discrepant or outlier results
 - Calculate the sum, mean, and standard deviation for each reagent and record the differences for historic compilation
 - Prepare a cumulative summation of all test comparison data, a difference of reagent means or cumulative change of more than 7 seconds is unacceptable and requires action

For More Information:

- Webinar On-Demand: Heparin Monitoring: aPTT versus Anti-Xa assay, Donna D. Castellone, New York Presbyterian Hospital - Columbia University Medical Center, Feb. 7, 2017, Stago-edvantage.com
- John D. Olson, MD, PhD, How to validate heparin sensitivity of the aPTT, October 2004, captodayonline.com

Chromogenic Anti-Factor Xa Heparin Assay

- Reagents are a known concentration of Factor Xa, and a substrate specific to Factor Xa
- Some assay kits add a known concentration of ATIII, others rely on the patient's plasma ATIII level
- Plasma heparin binds to ATIII, then the Heparin-ATIII complex binds to the reagent Factor Xa
- The excess free Factor Xa digests the reagent substrate which yields a chromogenic product whose intensity is inversely proportionate to the concentration of heparin
- Different standard curves are necessary for each heparin derivative (UFH, LMWH, Fondaparinux)

Anti-Factor Xa Assay Diagram

pNA = Paranitroanaline Chromophore

Plasma [*heparin*] + Antithrombin



[AT-Heparin-Xa] + Residual FXa



Synthetic Heparin Derivatives

- Low Molecular Weight Heparin (LMWH) is a shorter molecule than UFH with the same pentapeptide active site that binds ATIII, but preferentially binds FXa
- LMWH does not induce platelet aggregation as strongly as UFH and is not likely to form HIT antibodies
- Different LMWH compounds have different generic and Trade names: dalteparin (Fragmin), enoxaparin (Lovenox), tinzaparin (Innohep), et al.
- Fondaparinux (Arixtra) contains only the peptapeptide active site which binds ATIII and FXa and does not bind thrombin or activate platelets

Taken from journals.sagepub.com

Table 1 Comparison of the main characteristics of low-molecular-weight heparin (LMWH) and unfractionated heparin (UFH).

	LMWH	UFH
Mean molecular weight (range)	4500 (2000–10 000)	15 000 (4000–30 000)
Anti-Xa to anti-IIa activity ratio	2–5	1
Half-life of anti-Xa activity		
following IV application	2 h	1 h
following SQ application	4 h	2 h
Bioavailability	>90%	40%
Elimination	Kidney	Liver
Binding to PF ₄ and EC	(+)	++
Application		
prophylaxis	SQ (1 inj/day ^a)	SQ (2–3 inj/day)
therapy	SQ (1–2 inj/day)	Continuous IV infusion or SQ (2–3 inj/day)
Monitoring in prophylaxis	No	No
Monitoring in therapy	No	Yes (APTT)
Heparin-induced thrombocytopenia	+	++
Heparin-induced osteoporosis	?	+

^aIn the USA, enoxaparin is approved for prevention of DVT following total hip or knee arthroplasty at a dosage of 2 daily SQ injections of 30 mg (3000 IU).

IV, intravenous; SQ, subcutaneous; PF₄, platelet factor 4; EC, endothelial cell; inj, injection; APTT, activated partial thromboplastin time.

(+), very low importance or absent; +, low importance; ++, major importance.

Taken from

fac.org.ar/scvc/llave/coronary/fergu/fergui.htm

Limitations of UFH

Unpredictable anticoagulant effect

- Significant protein binding
- Saturable clearance mechanism
- Inactivation by platelet factor 4
- < 25% of patients in therapeutic range 12 hours after beginning Rx (TIMI 9B)
- Inaccessibility to clot-bound thrombin

Monitoring required

IV administration

Platelet activation

Risk of HIT

Advantages of LMWH

More predictable anticoagulant response

- Reduced protein binding
- Less inactivation by PF 4

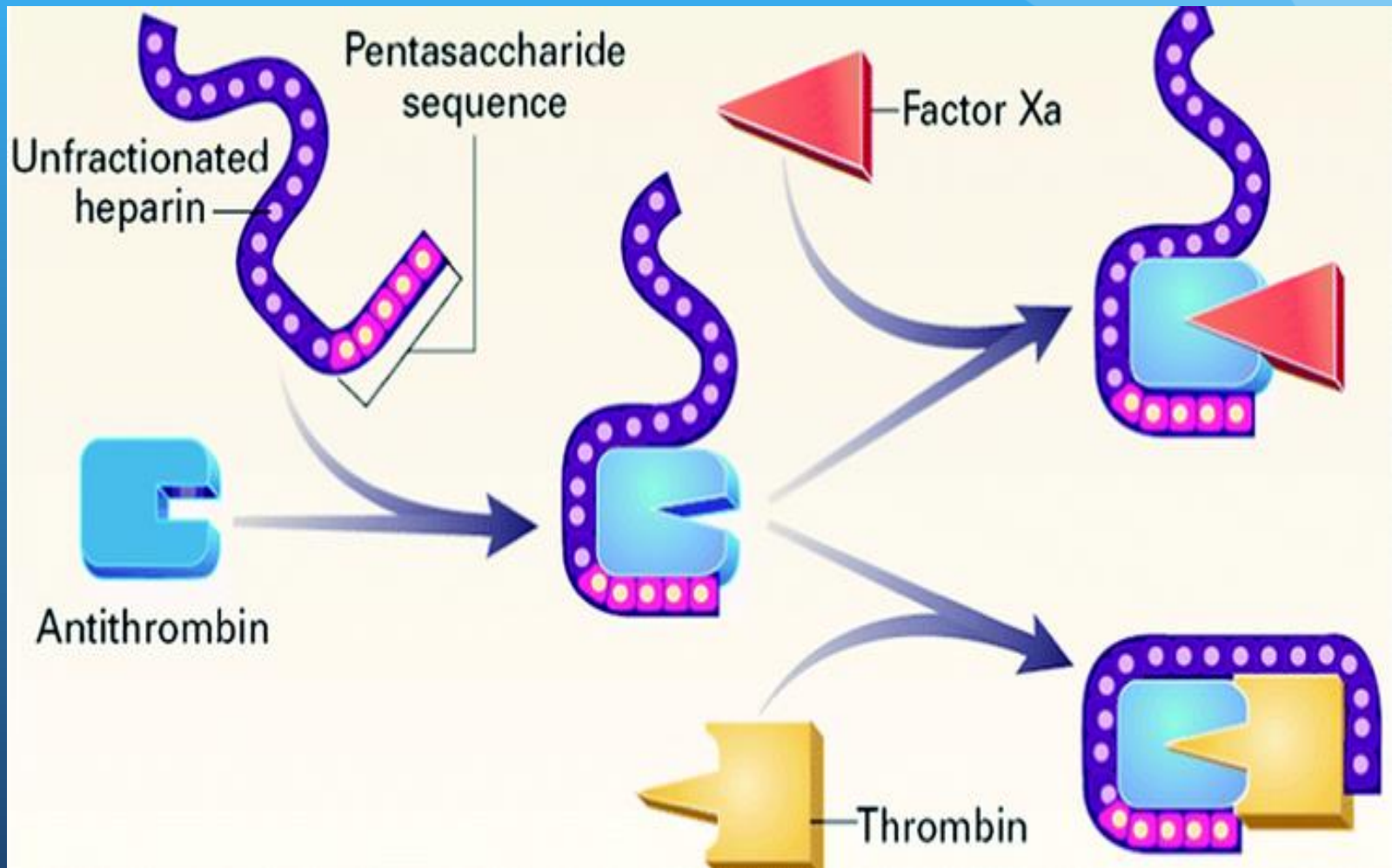
No monitoring required

SQ dosing

Reduced platelet activation

Less risk of HIT

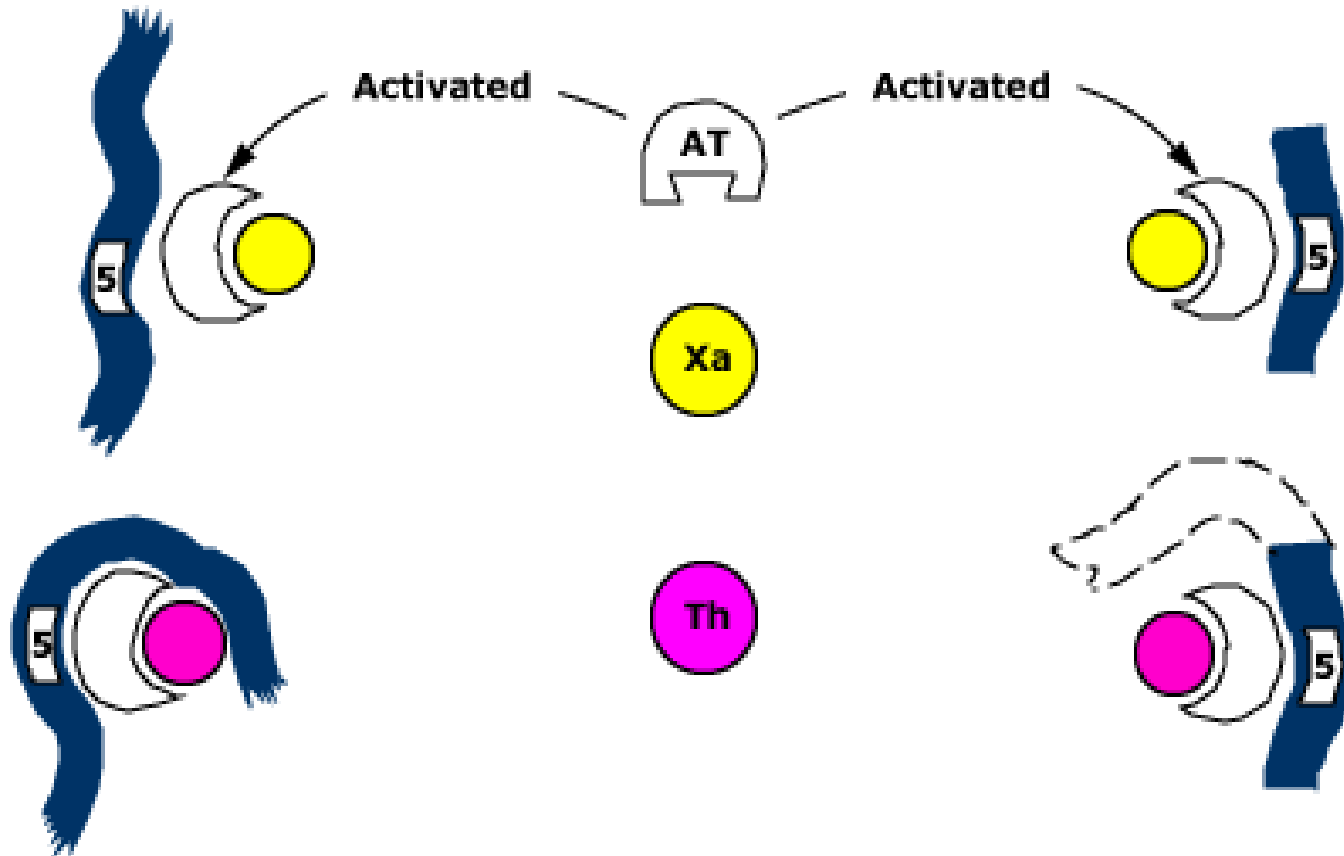
Taken from pubs.rsc.org



Taken from cursoenarm.net

Unfractionated heparin

Low-molecular-weight heparin



Direct Oral Anticoagulants (DOAC)

- The most commonly prescribed: dabigatran and argatroban (Direct Thrombin Inhibitors - DTI's), rivaroxaban and apixaban (Factor Xa inhibitors)
- Cause prolongation in all thrombin-based clotting assays
- All have relatively short half-lives, monoclonal antibodies are being developed as antidotes for reversal
- To measure direct thrombin inhibitors, a “dilute thrombin time” assay is used to roughly measure the drug concentrations, a 4:1 ratio of PNP is mixed with patient plasma, normal range is 13-19 seconds
- For Factor Xa inhibitors, standard curves are used along with the same reagents as the Anti-Xa assay for Heparin to give quantitative measurements for each medication

Retail Cost of Medications goodrx.com

- Coumadin = \$4 for 30 tablets
- Generic Heparin Sodium = \$84 for 60 - 1mL vials
- Lovenox (LMWH) = \$28 for 4 syringe doses
- Dabigatran (Praxada) = \$381 for 60 capsules
- Rivaroxaban (Xarelto) = \$376 for 30 tablets
- Apixaban (Eliquis) = \$405 for 60 tablets
- Fondaparinux (Arixtra) = \$4874 for 10 syringe doses

Non-immune Heparin-Associated Thrombocytopenia (HAT)

- Also referred to as Type I HIT
- A non-immune, transient thrombocytopenia associated with the administration of Heparin caused by the activation and aggregation of platelets due to the interaction of Heparin and PF4
- Can be a result of perioperative hemodilution (contamination) of the blood sample
- Can also be due to a comorbid disorder such as a systemic inflammatory response syndrome
- Can not be predicted, but usually resolves on its own

Immune Mediated Type II HIT Incidence and Predictability

“1% to 5% of patients on Heparin will develop HIT antibodies with thrombocytopenia”

Franchini, M. Heparin-induced thrombocytopenia: an update. Thrombosis Journal 2005 3:14

“Up to 3% of patients treated with UFH will develop HIT antibodies”

Girolami, B. The incidence of heparin-induced thrombocytopenia in hospitalized medical patients treated with subcutaneous unfractionated heparin: a prospective cohort study. Blood 2003 101:2955-2959

Immune Mediated Type II HIT Incidence and Predictability

In a group of 387 patients receiving heparin therapies, 7.8% receiving UFH developed HIT antibodies, and 2.2% receiving LMWH developed HIT antibodies

Warkentin, T. et al. Heparin-induced thrombocytopenia inpatients treated with low molecular weight heparin or unfractionated heparin. N Engl J Med 1995 May 18;332(20):1330-5

****There is no overriding predictive condition associated with HIT antibody development****

HIT can happen to anyone!!

Symptomatic Complications of HIT Antibody Development

- 50% of HIT patients form Deep Vein Thrombosis (DVT's)
- 25% of HIT patients form Pulmonary Embolism (PE's)
- 5% - 15% of HIT patients experience Aortoiliac Thrombosis (Acute Limb Ischemia)
- 5% - 10% of HIT patients experience Acute Thrombotic Cerebrovascular Accident (Stroke)
- 3% - 5% of HIT patients experience Myocardial Infarction
- <3% of HIT patients form Cerebral Sinus Vein Thrombosis
- <3% of HIT patients form Arterial Thrombosis of Upper Limb, Renal, Mesenteric, Spinal or other large arteries

Symptomatic Complications of HIT Antibody Development

- 25% of sensitized HIT patients form Heparin-induced skin lesions upon receiving subcutaneous Heparin injections
- 25% of sensitized HIT patients display acute systemic reactions upon receiving a Heparin bolus injection
- 10% of sensitized HIT patients treated with Coumadin (Warfarin) due to formation of a DVT develop Warfarin-induced venous limb gangrene due to Protein C inhibition

Mechanisms of HIT Antibody Formation

- Microparticles generated from activating platelets were induced by HIT sera and photographed by Mary Hughes, et al., McMaster University Medical Center, Hamilton ON
- In the presence of HIT sera, the platelets lost their discoid shape and numerous sites of swelling were observed on both the platelet body and the newly-formed pseudopodia during the process of activation
- Small, discreet membrane-bound vesicles arose from the pseudopodia and swellings which then separated into distinct microparticles
- The microparticles promote vascular occlusion and have antigenic properties which give rise to PF4 antibodies

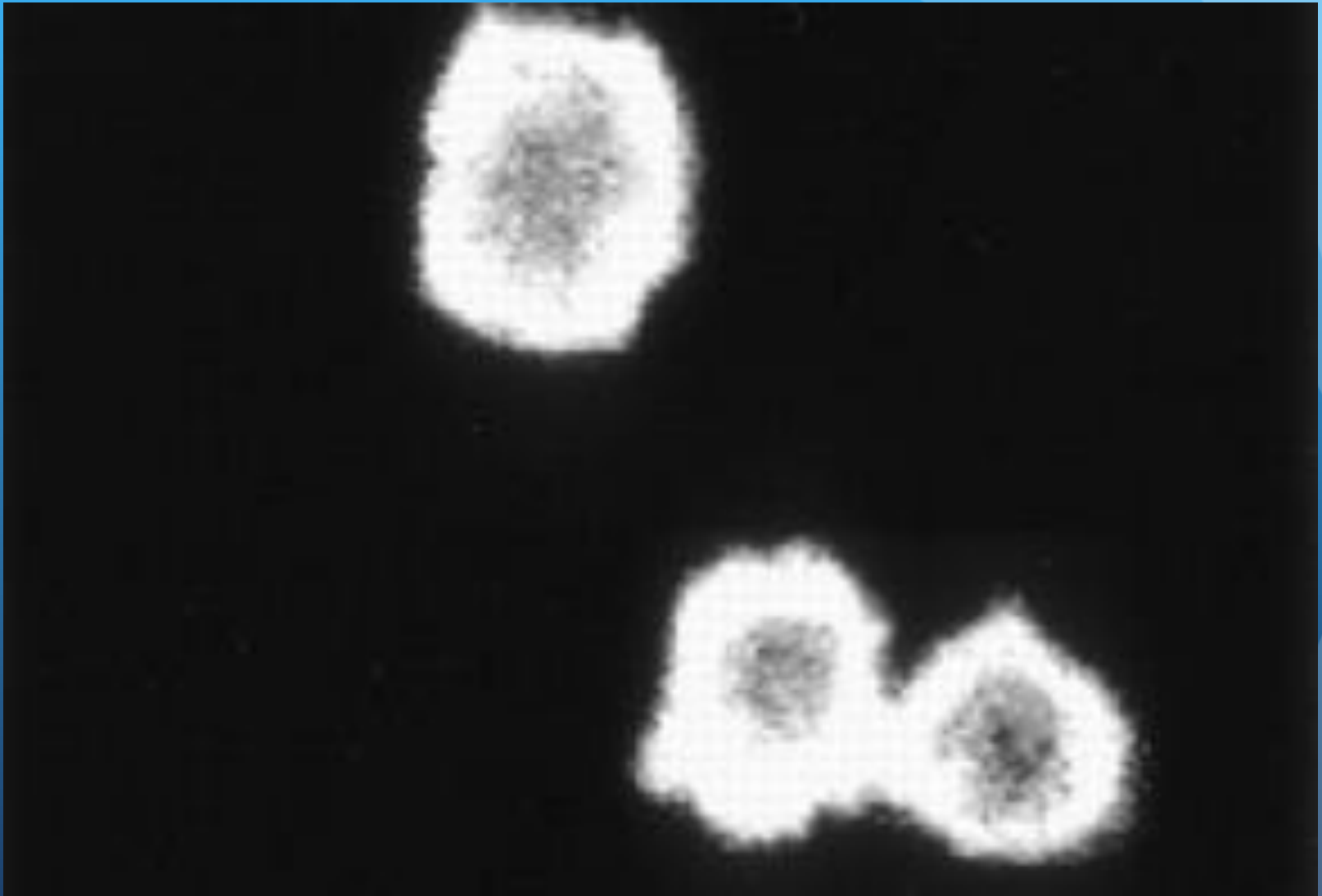
Mechanisms of HIT Antibody Formation

- SEM and TEM were captured and showed the formation of discrete microparticles separating from the pseudopodia of platelets activated by serum containing HIT antibodies with 0.1 U/mL UF sodium Heparin added
- Flow cytometry was performed using mAb anti-GPIIb_α and fluorescent confocal microscopy was performed using mAb's anti-GPIIb_α and anti-GPIIb/IIIa; both methods enabled microparticles to be distinguished from activated platelets based on size measurements
- Microparticles are less than 0.1 to 1.0 μm in diameter, activated platelets are 2.0 to 3.0 μm in diameter
- Reference and Images: Hughes, M. et al., Morphological Analysis of Microparticle Generation in Heparin-Induced Thrombocytopenia. Blood 2000 96:188-194

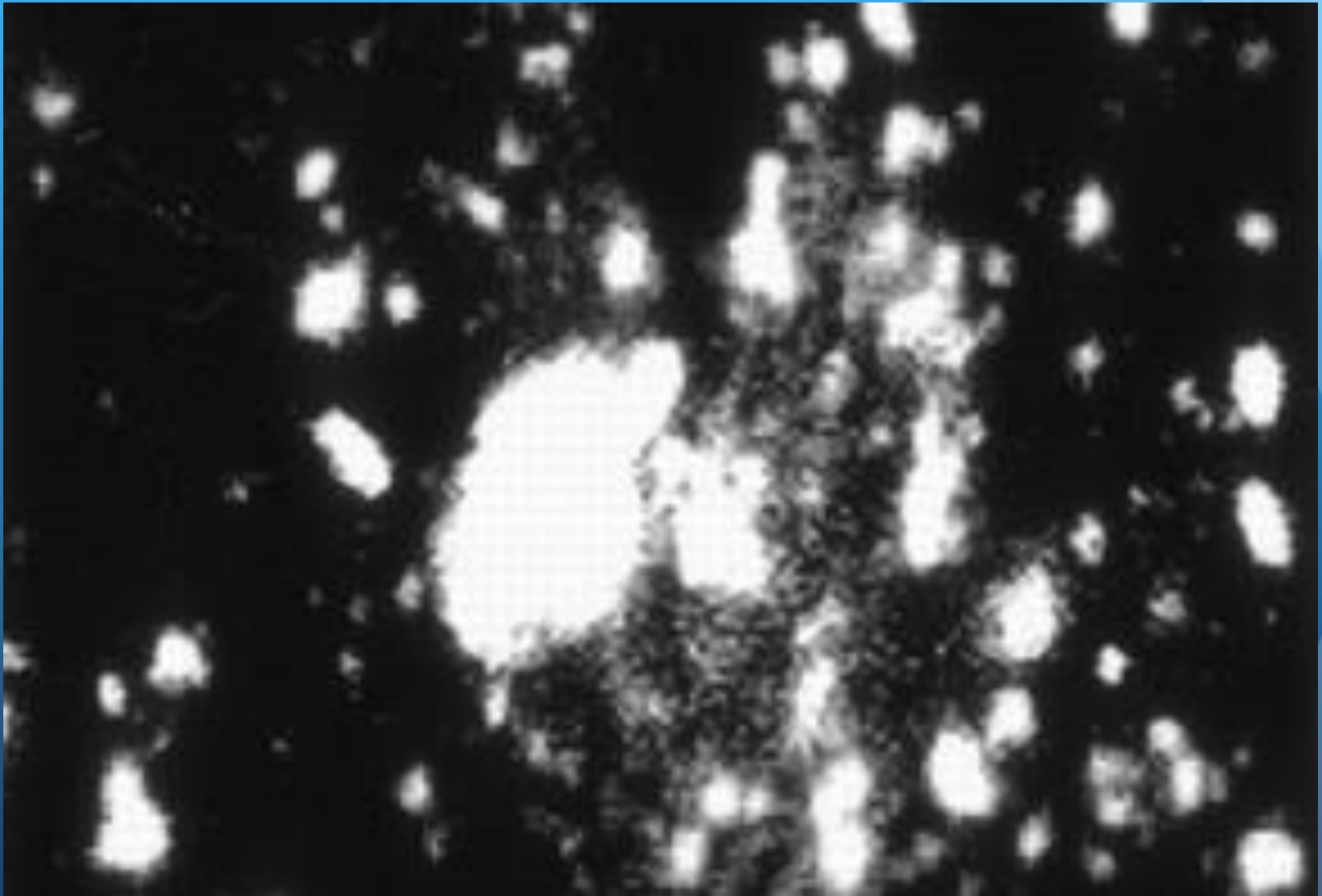
SEM Normal Platelets at Rest and Becoming Activated, Left → Right



Resting Platelets Fluorescent Image

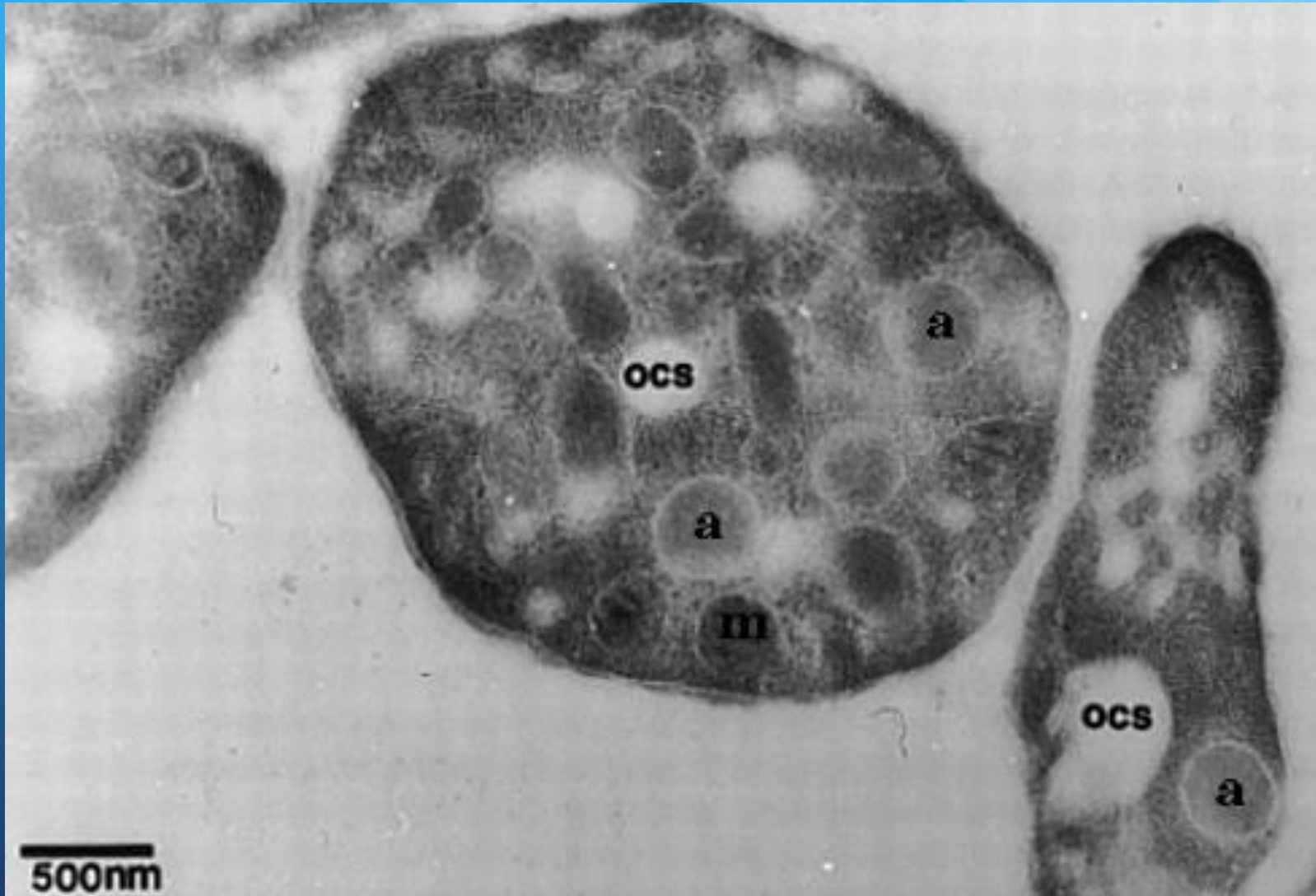


Activated Platelet Fluorescent Image



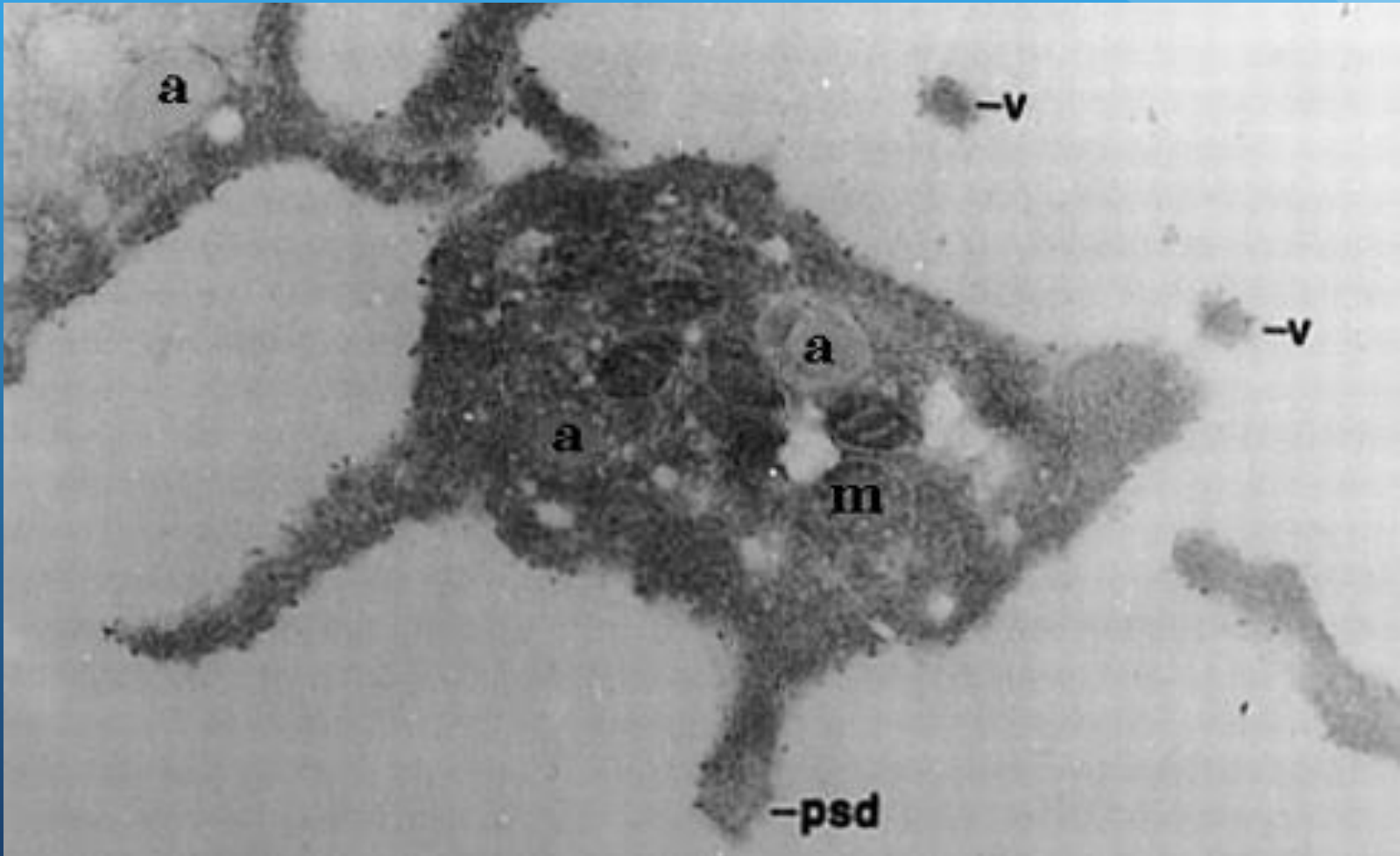
Resting Platelet TEM

a=alpha granules m=mitochondria ocs=open-cannalicular system



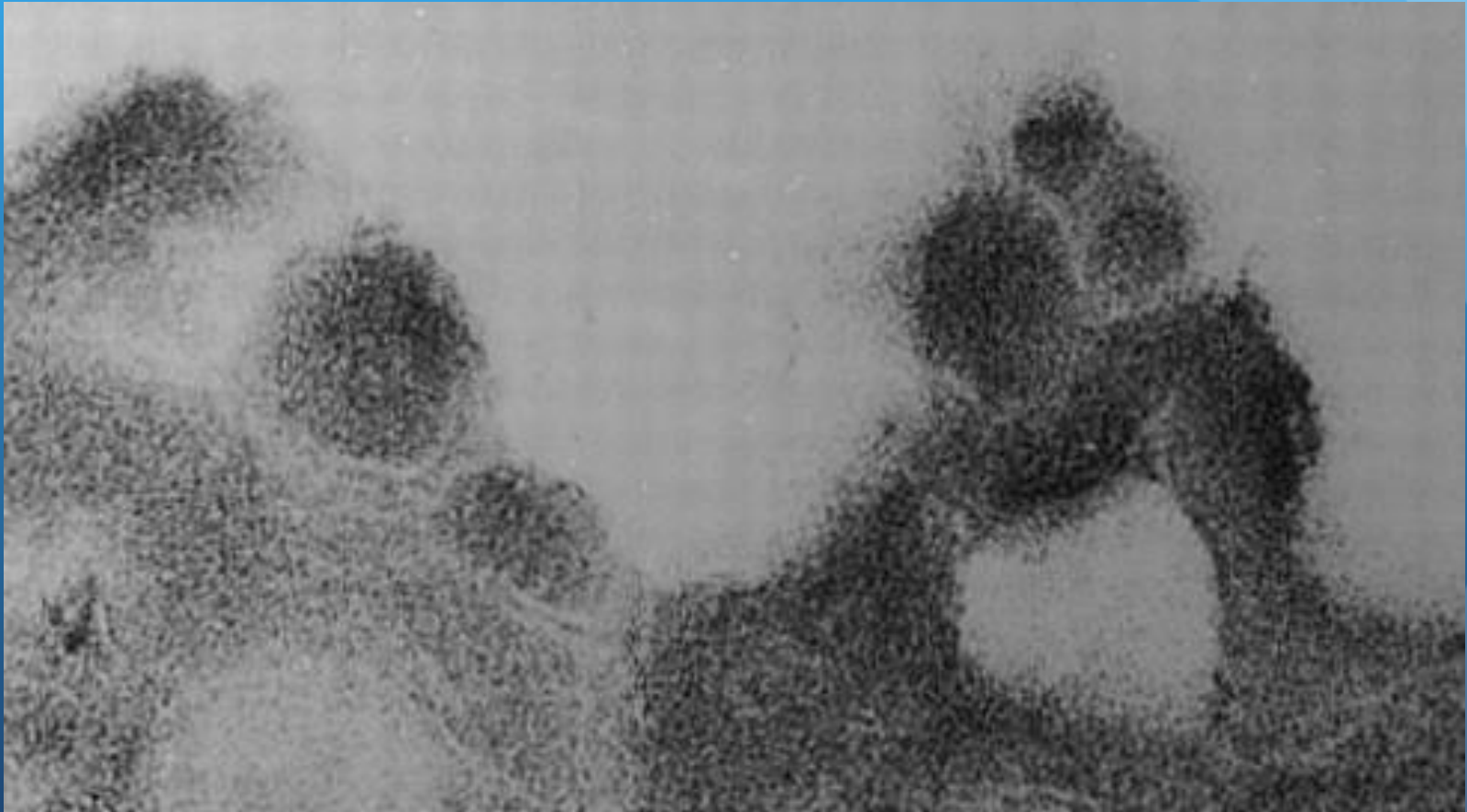
Activated Platelet TEM

a=alpha granules m=mitochondria psd=pseudopod
v=membrane-bound vesicles (microparticles)



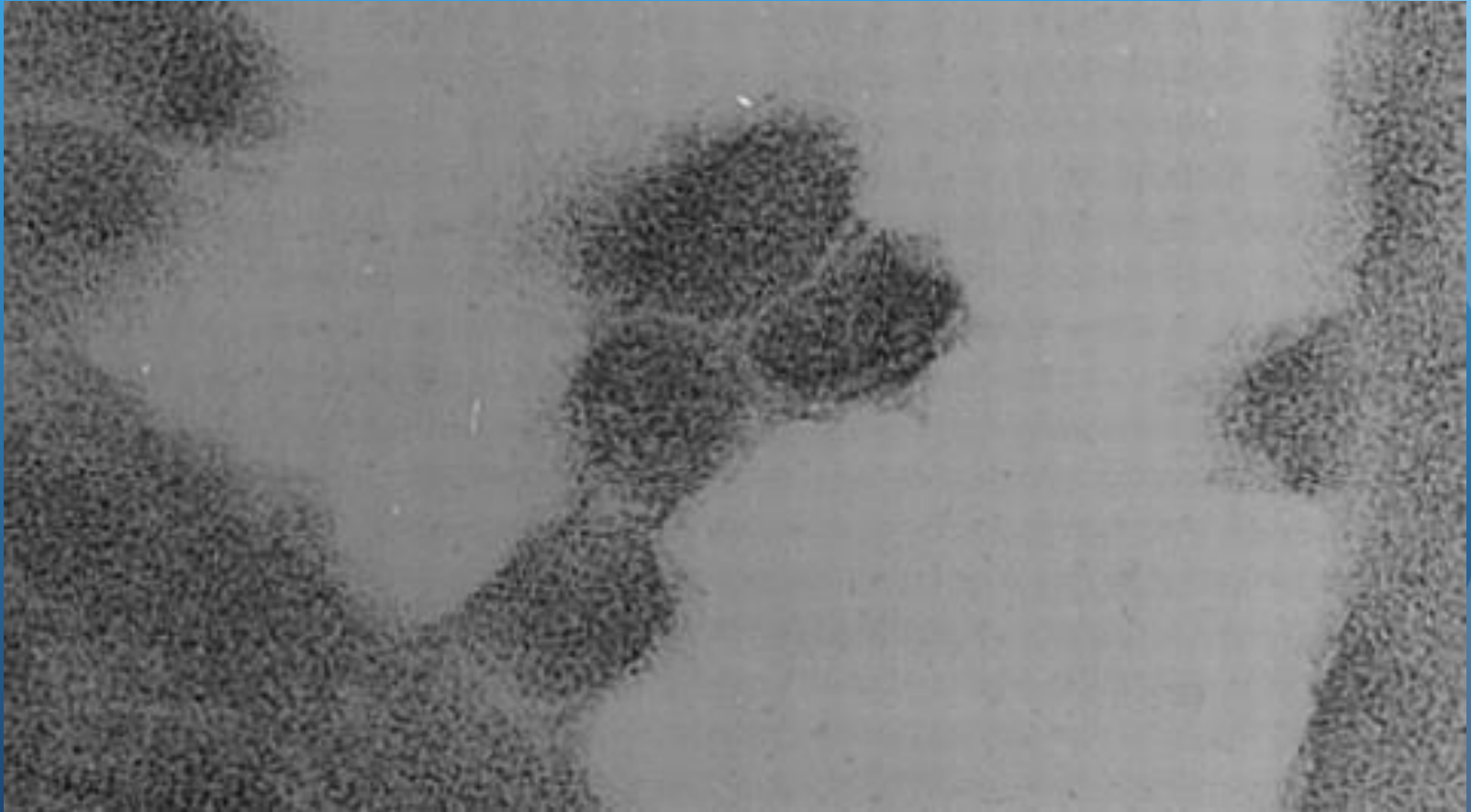
Activated Platelet TEM

displaying membrane-bound vesicle (microparticle) formation along the body of the platelet



Activated Platelet TEM

displaying membrane-bound vesicle (microparticle) formation at the terminal end of a pseudopod



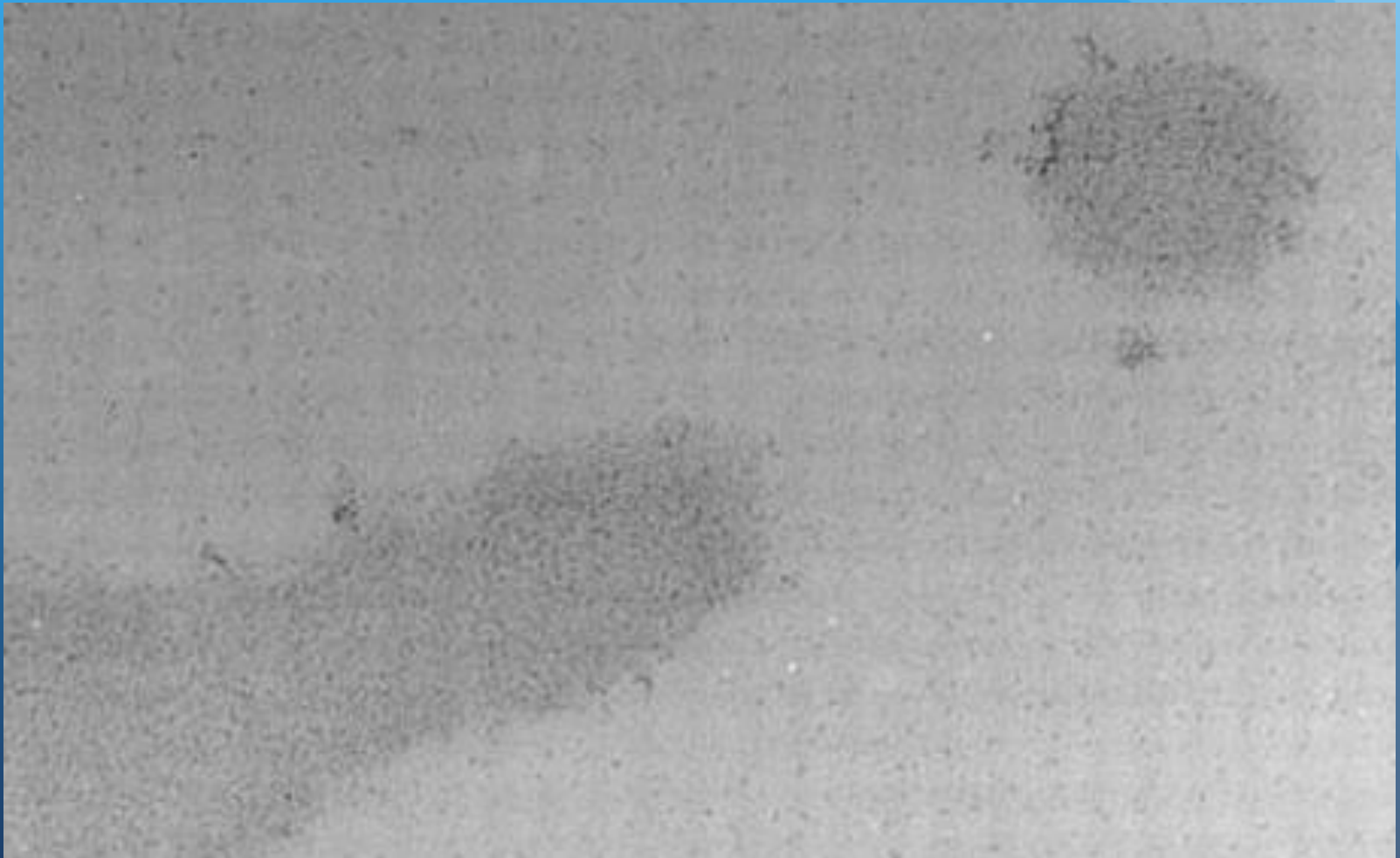
Activated Platelet TEM

displaying membrane-bound vesicle (microparticle)
formation at the terminal end of a pseudopod



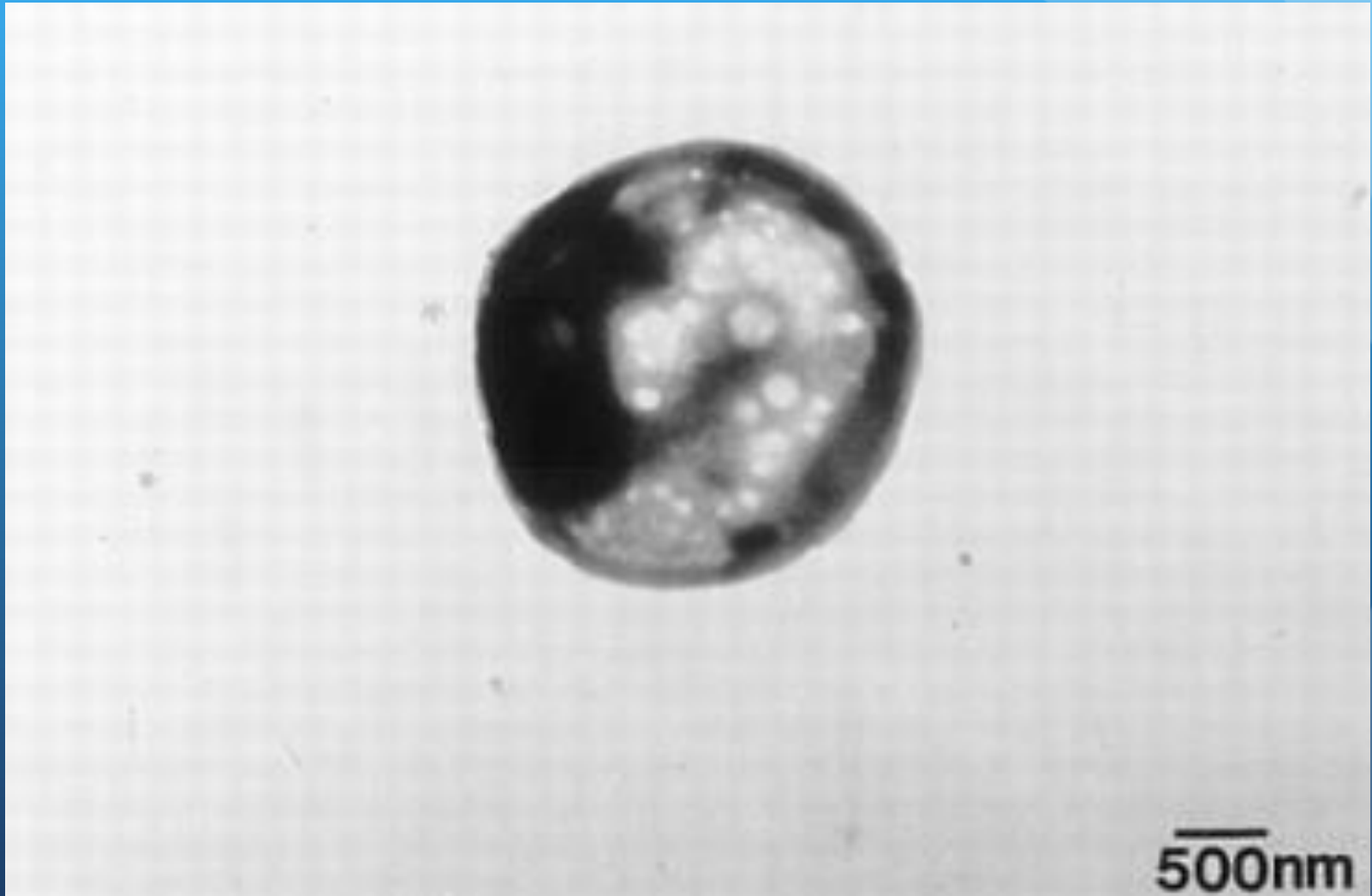
Activated Platelet TEM

displaying membrane-bound vesicle (microparticle)
separation at the terminal end of a pseudopod



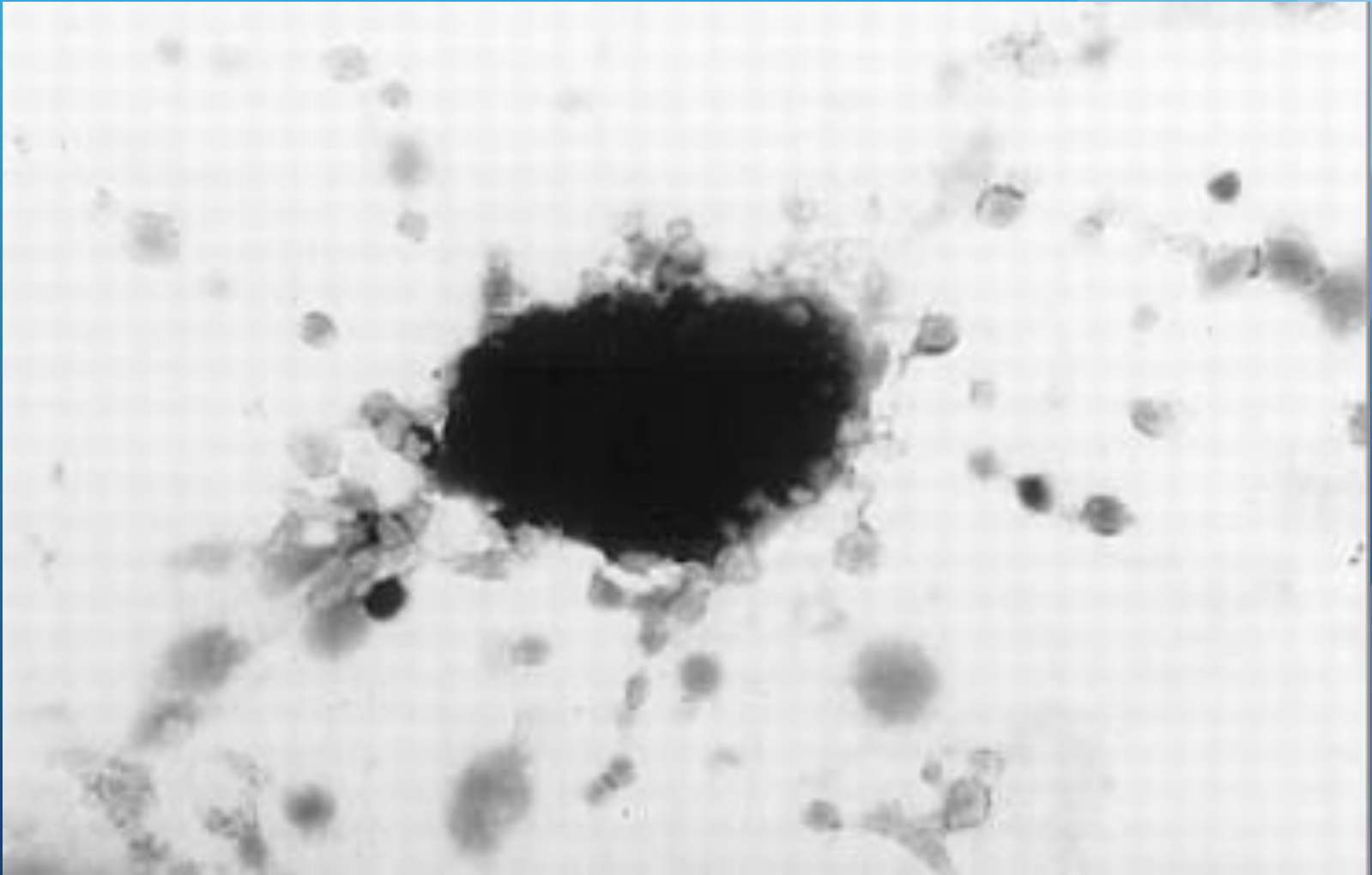
Resting Platelet TEM

negatively stained with 2% phosphotungstic acid

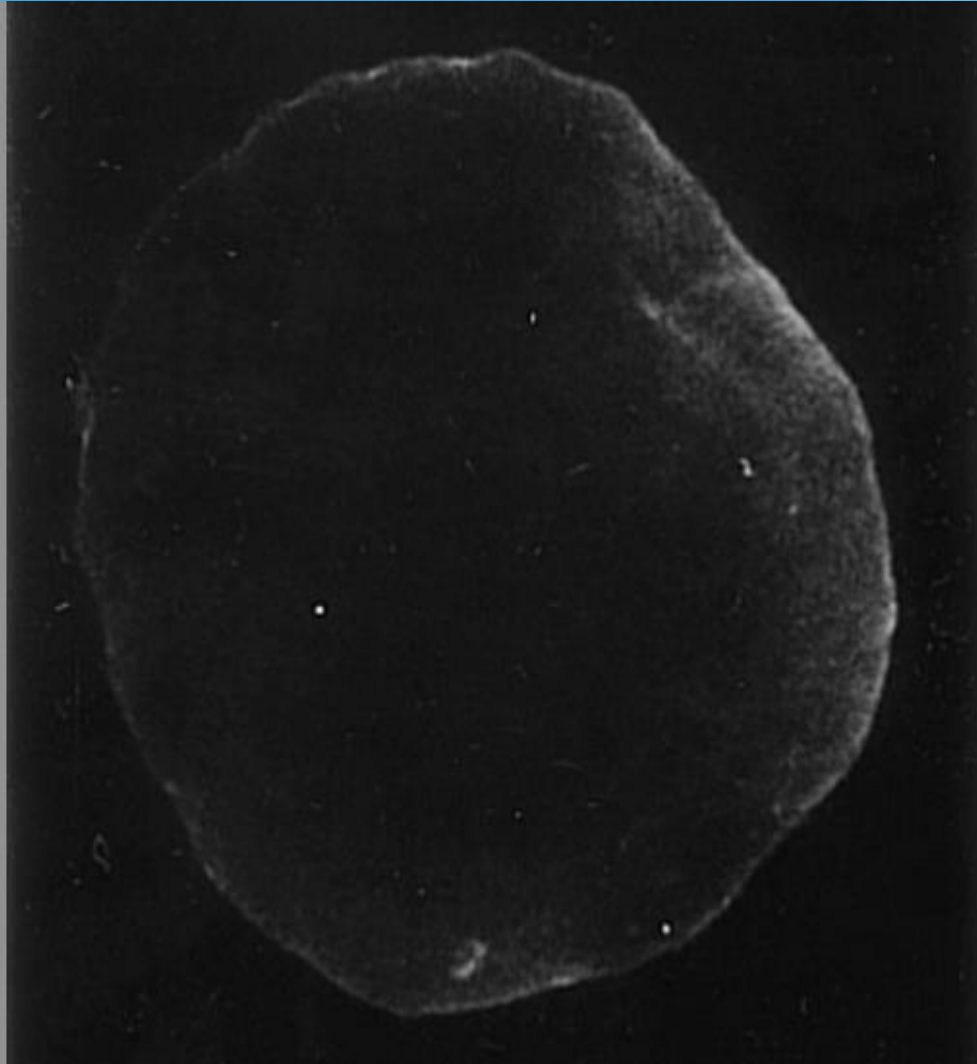


Activated Platelet TEM

negatively stained displaying numerous membrane-bound vesicles (microparticles) and pseudopodia



Resting Platelet SEM



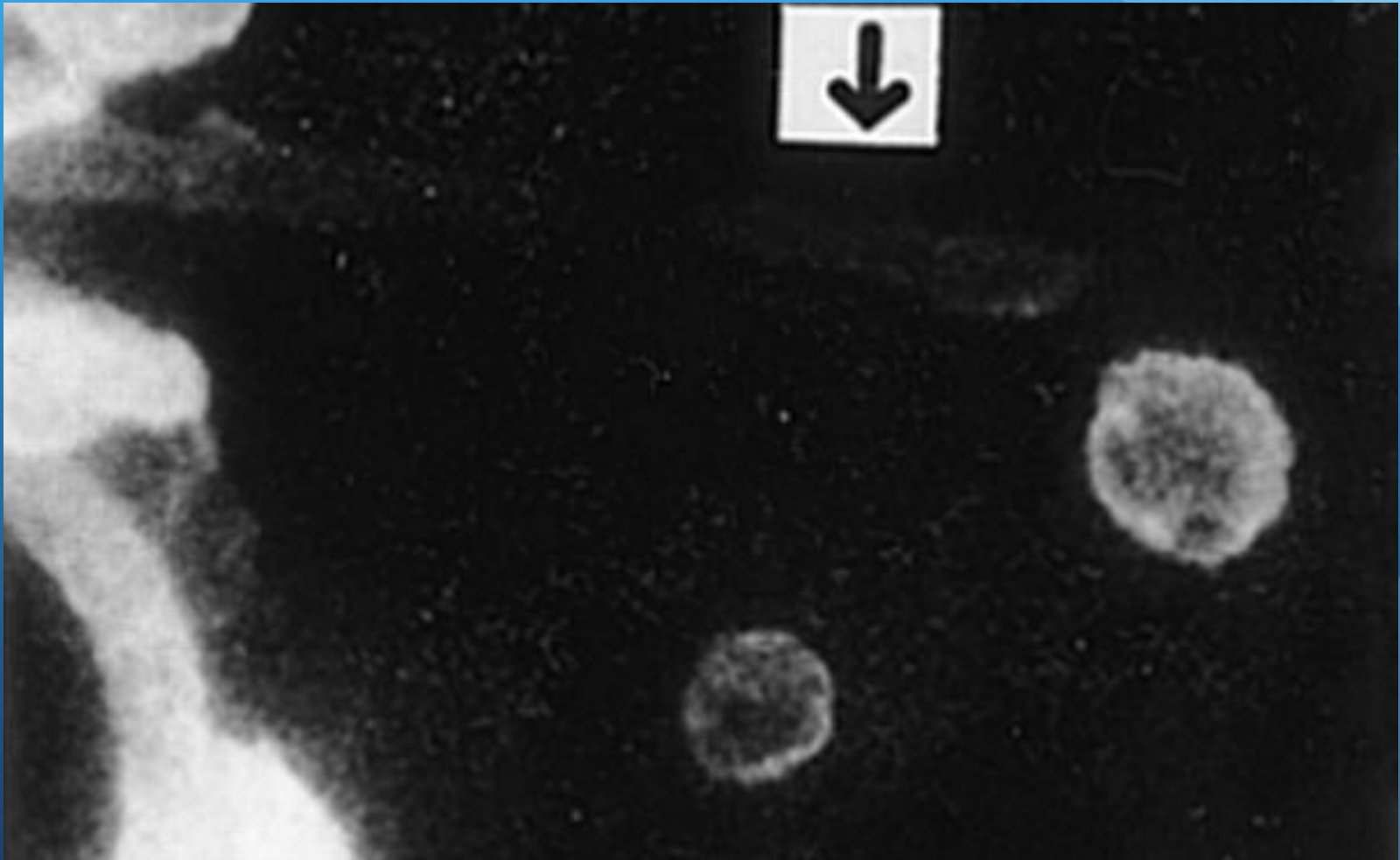
Activated Platelet SEM

displaying membrane-bound vesicle (microparticle) formation on the terminal ends of pseudopodia (↓↑)



Activated Platelet SEM

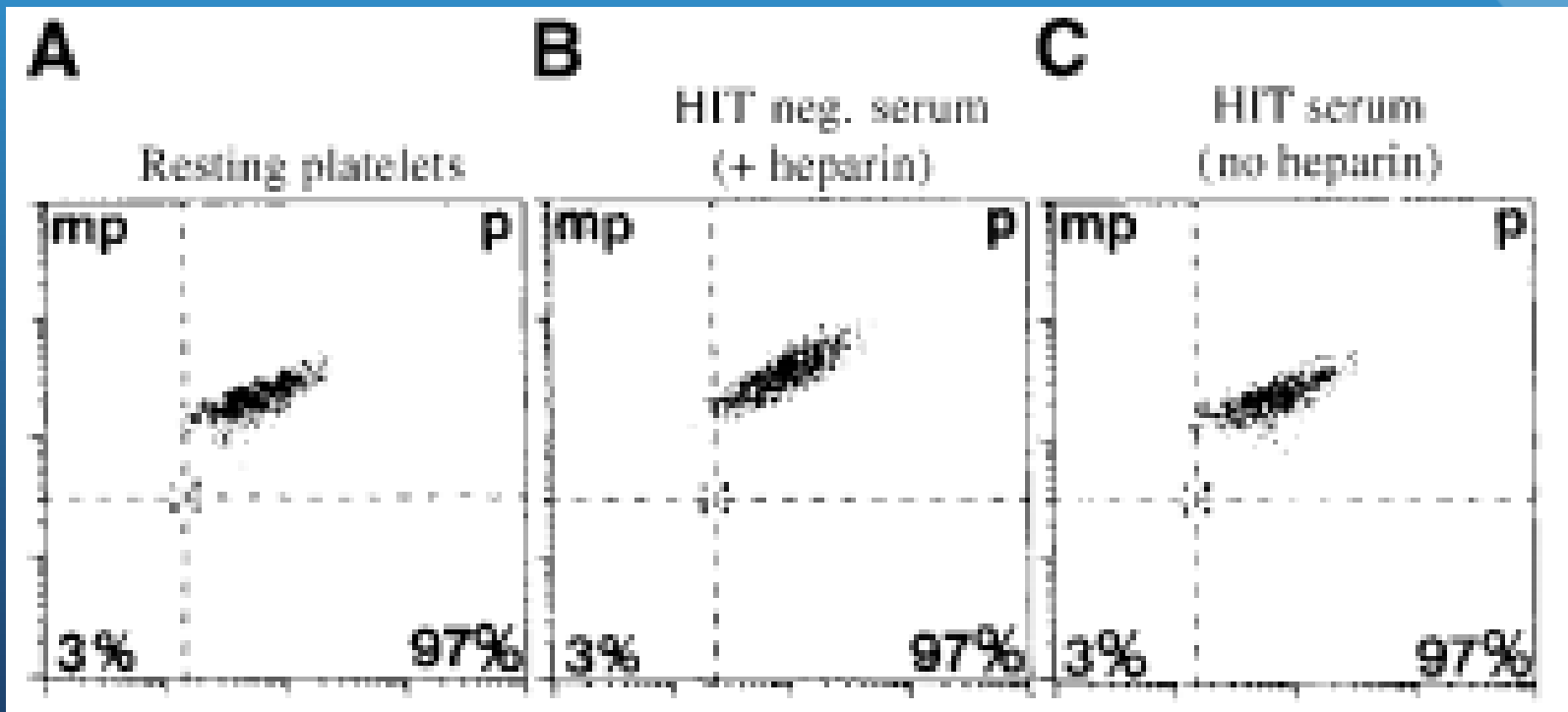
displaying membrane-bound vesicles (microparticles)
discreet and separating from pseudopodia (↓)



Flow Cytometry Scatterplots of Negative Platelet Reactions

Forward Scatter vs. Fluorescence

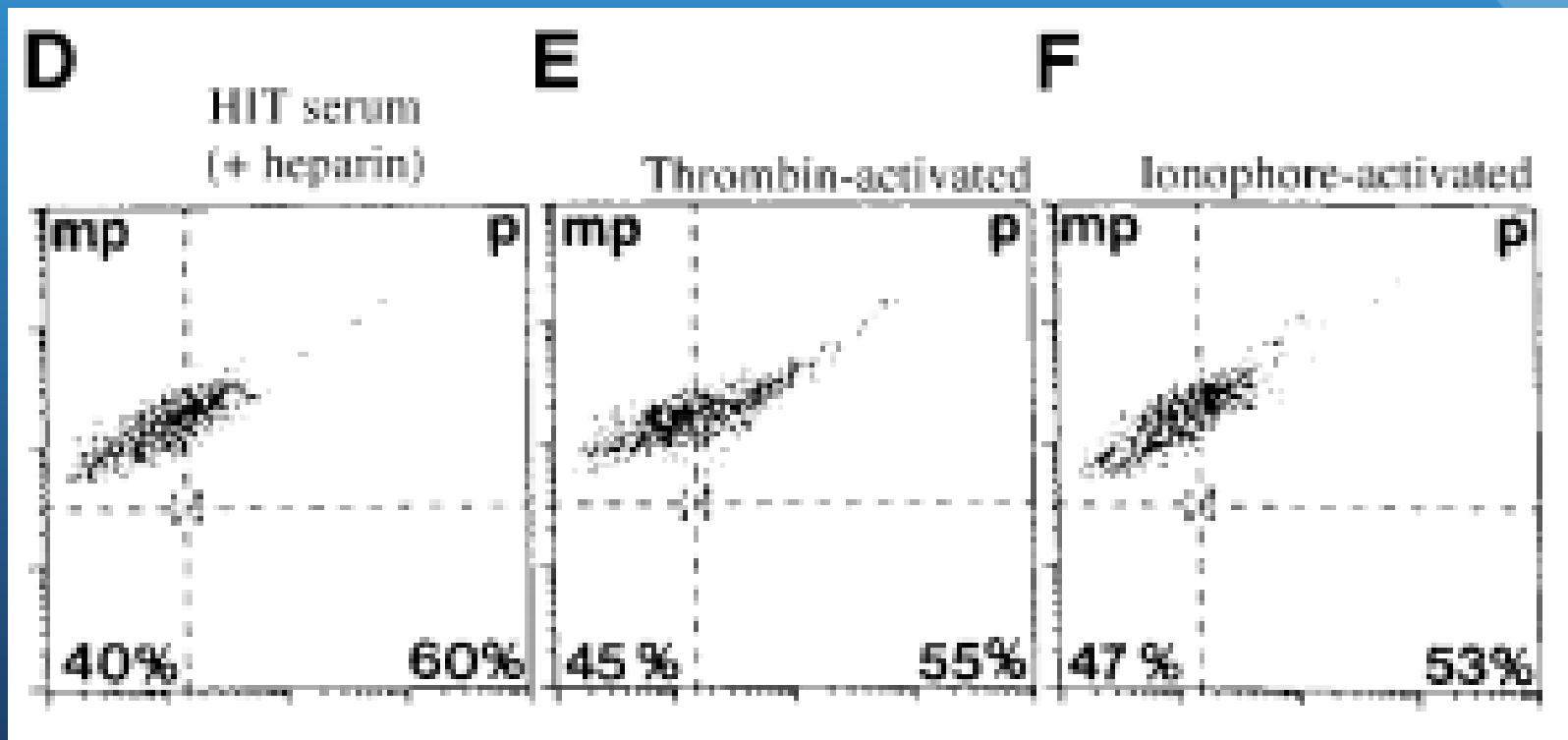
mp = microparticles, p = platelets



Flow Cytometry Scatterplots of Activating Platelet Reactions

Forward Scatter vs. Fluorescence

mp = microparticles, p = platelets



Greinacher, A. Me or Not Me? The Danger of Spontaneity. Blood 2014, 123:3536-3538

- Spontaneous or Autoimmune HIT developed in a 31 year old female with a respiratory infection was given LMWH for thrombosis prophylaxis due to thrombocytopenia, low fibrinogen level, and highly elevated D-Dimer level; the next day, her thrombocytopenia worsened and she developed DVTs, SVT, and intracerebral bleeding
- Both pre-and post-LMWH samples showed high HIT titers
- PF4 binds to polyanions on bacteria due to infection or major surgery; the PF4-coated bacteria can trigger HIT antibody formation
- PF4 also binds to negatively-charged nucleic acids which are released during major surgery; the PF4-nucleic acid complexes can also trigger HIT antibody formation

Clinical Picture of Type II Immune-Associated HIT Syndrome

- 50% to 75% of patients with HIT antibodies will develop thrombosis due to IgG heparin-PF4 immune complexes which activate platelets
- Generally characterized by a platelet count fall of >50% and/or the formation of new thromboses within 5-14 days after initial heparin administration
- In 30% of HIT patients, thrombosis occurs on the same day as a platelet count decrease of >50%
- Delayed onset HIT can occur up to 3 weeks after heparin exposure resulting in antibodies reacting independently of heparin as an autoimmune disease

Clinical Picture of Type II Immune-Associated HIT Syndrome

- The anti-PF4 antibodies can be formed in patients without giving rise to clinical HIT Syndrome
- A negative HIT assay result has good negative predictive value for clinical HIT syndrome
- A positive HIT assay result has poor positive predictive value for clinical HIT syndrome
- The entire clinical picture using the “4T” scoring system **MUST** be taken into account along with the HIT assay results for diagnosis of suspected HIT cases
- Chart on next slide is taken from practical-haemostasis.com

4T Scoring system for Suspected HIT

T	SCORE		
	2	1	0
Thrombocytopenia	>50% fall in platelet count or a platelet nadir of 20-100 x 10 ⁹ /L	30-50% in platelet count or a platelet nadir of 10-19 x 10 ⁹ /L	<30% fall in platelet count or a platelet nadir of <10 x 10 ⁹ /L
Timing	Onset with 5-10 days of exposure to heparin or <1 day if previous exposure to heparin within 100 days	Unclear - Platelet count falls after 10 days	Platelet count falls too early and without recent exposure to heparin
Thrombosis	New thrombosis Skin necrosis	Progressive or recurrent thrombosis Some skin lesions e.g. erythema	None
Other causes of Thrombocytopenia are not evident	No other cause identified	Possible other cause	Other cause clearly identifiable

A score of ≤3 indicates a low pretest probability for Type II HIT [<5% chance of HIT]

A score of 4–5 indicates an intermediate risk.

A score ≥6 is associated with a very high risk of HIT.

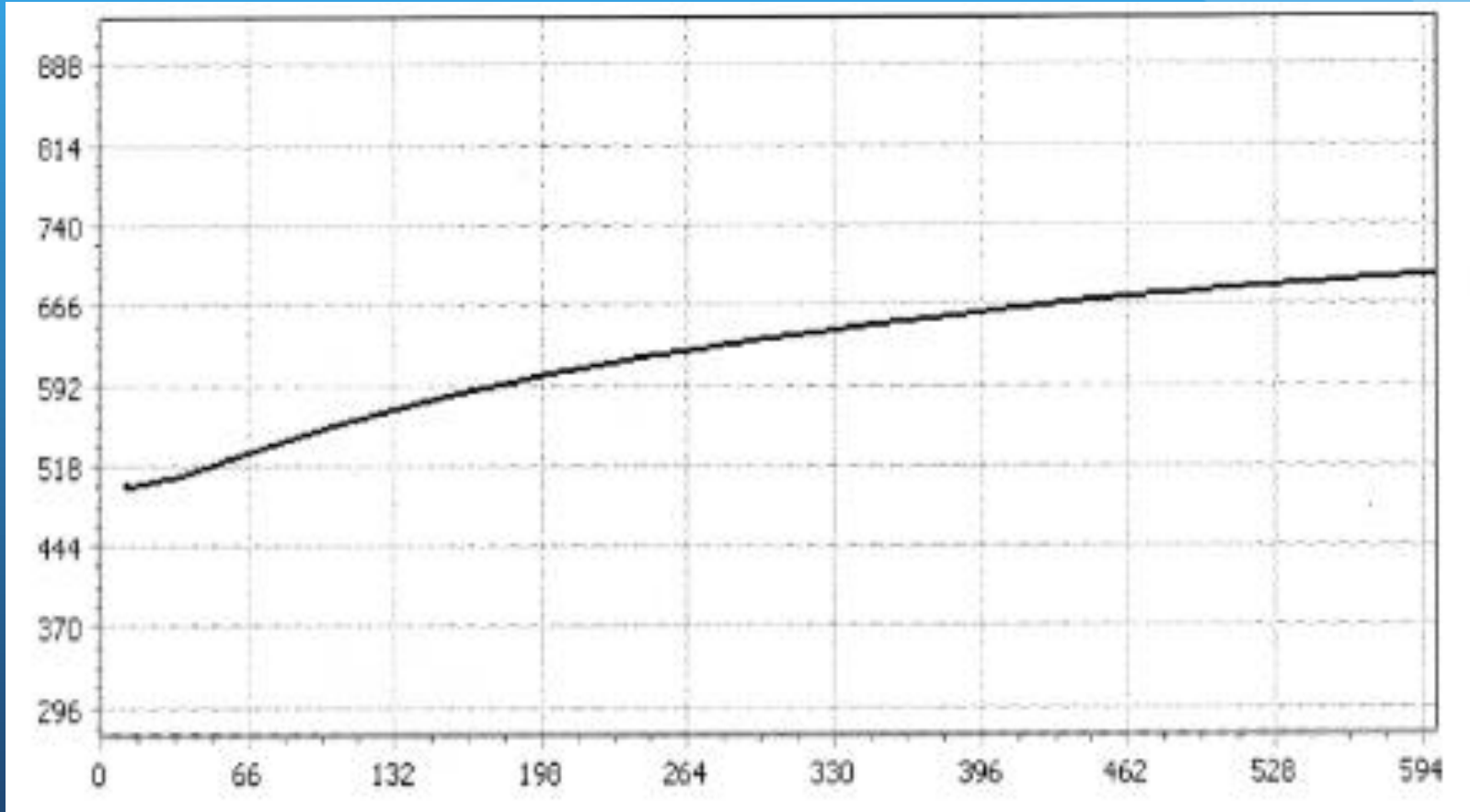
“4T” Scoring System

1. Thrombocytopenia - uses the percentage of the drop in platelet count and a platelet nadir (low point after Heparin administration) threshold to assign a severity score
2. Timing - uses the timing of the platelet count relative to the administration of Heparin with past history of Heparin administration taken into account
3. Thrombosis - uses the presence or formation of thrombosis, skin necrosis and/or skin lesions
4. If Other Causes of Thrombocytopenia Are Not Evident - identifies additional causes of thrombosis formation separate from Heparin administration

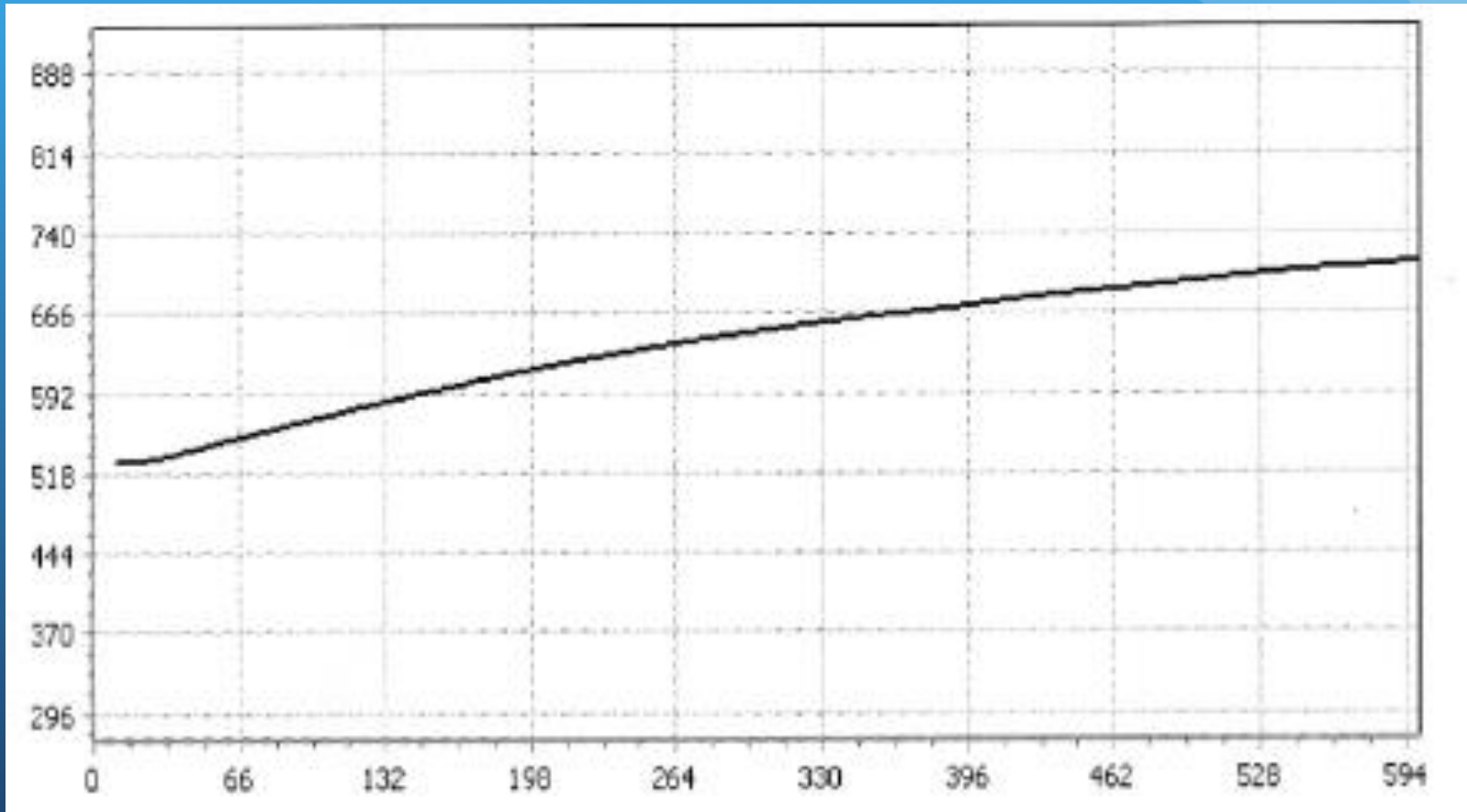
ACL TOP 500 Heparin Induced Thrombocytopenia (HIT) Assay

- Reagents are monoclonal antibodies that mimic HIT coated onto micro-latex particles, and PF4 bound to polyvinyl sulfate
- The patient sample is added and a competitive agglutination reaction occurs, binding the latex particles to each other
- The degree of agglutination is inversely proportional to the amount of HIT present, which is measured as an increase in optical density inside the reaction well
- A graph is generated with OD and Concentration axes, results ≥ 1.0 U/mL are considered positive values

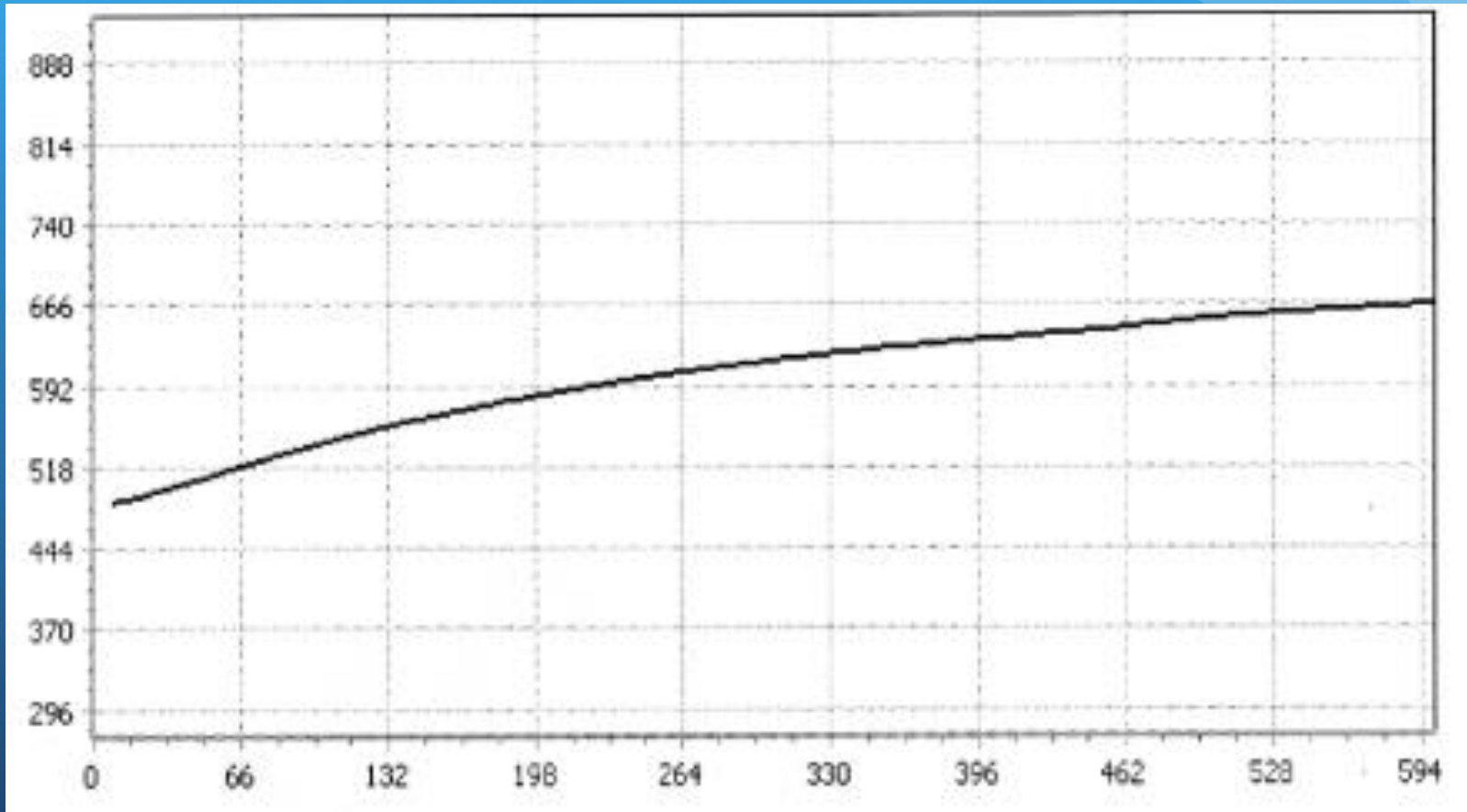
Negative HIT Reaction Curve, Result = 0.0 U/mL, $\Delta mAbs = 193$



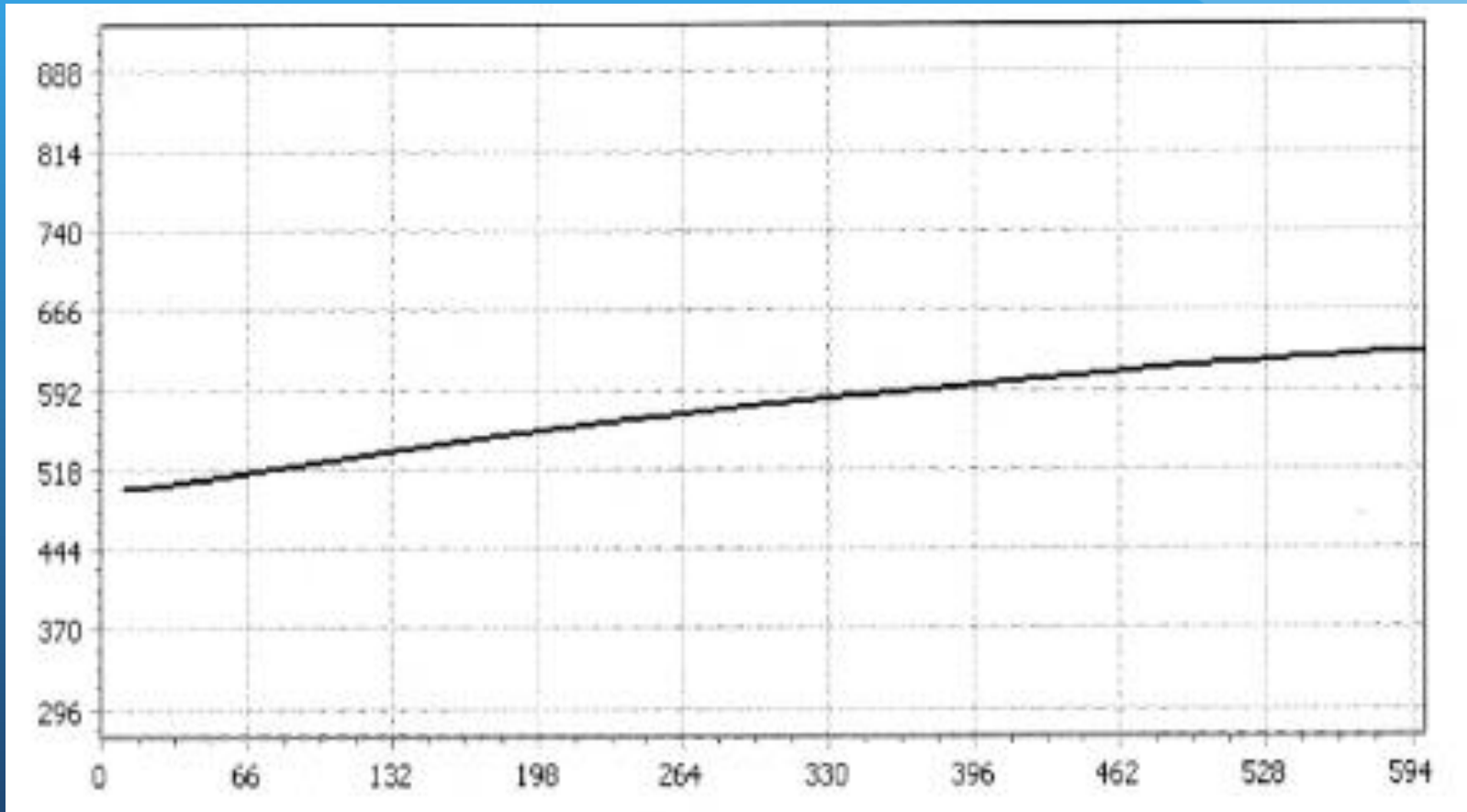
Negative HIT Reaction Curve, Result = 0.1 U/mL, $\Delta mAbs = 186$



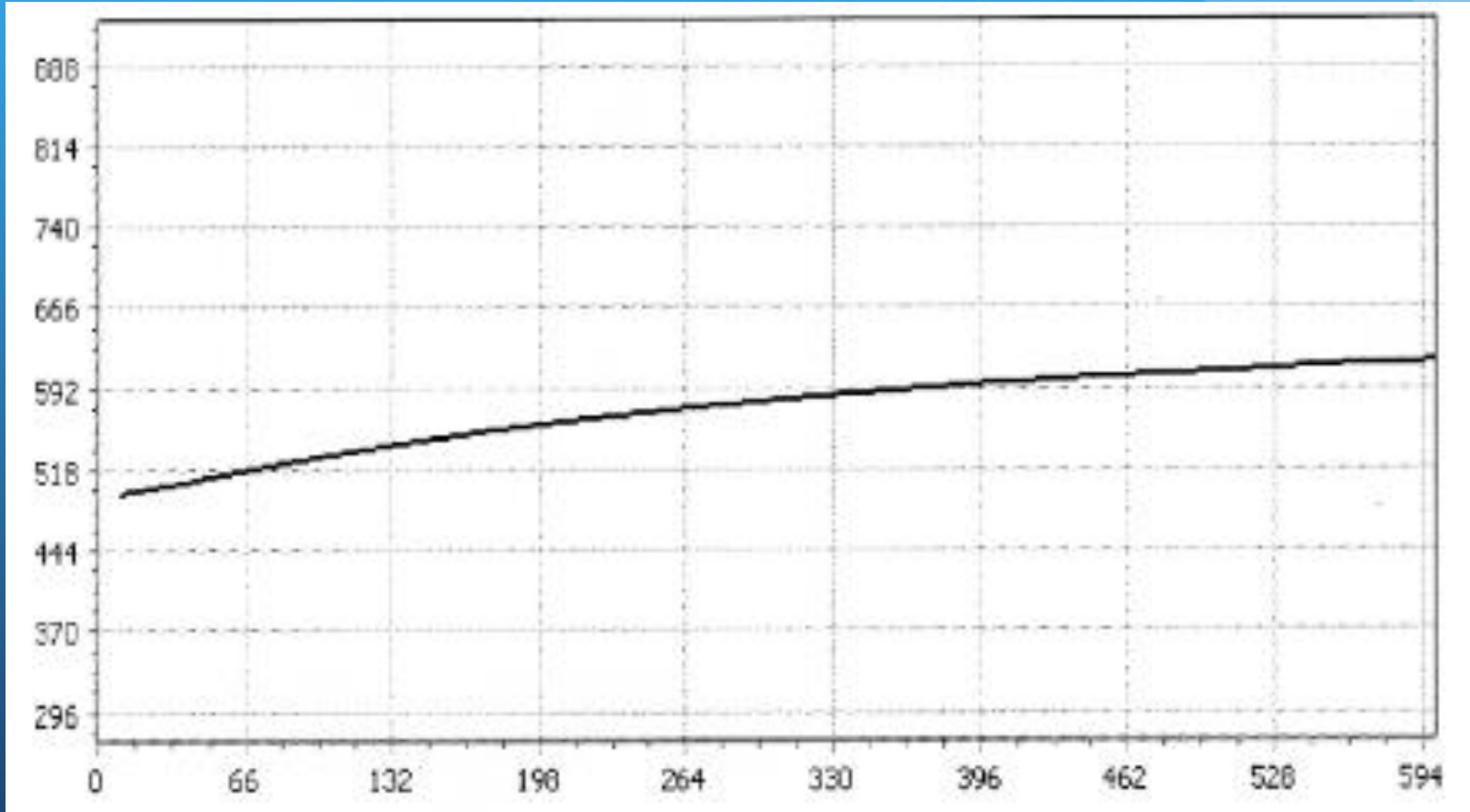
Negative HIT Reaction Curve, Result = 0.5 U/mL, $\Delta mAbs = 178$



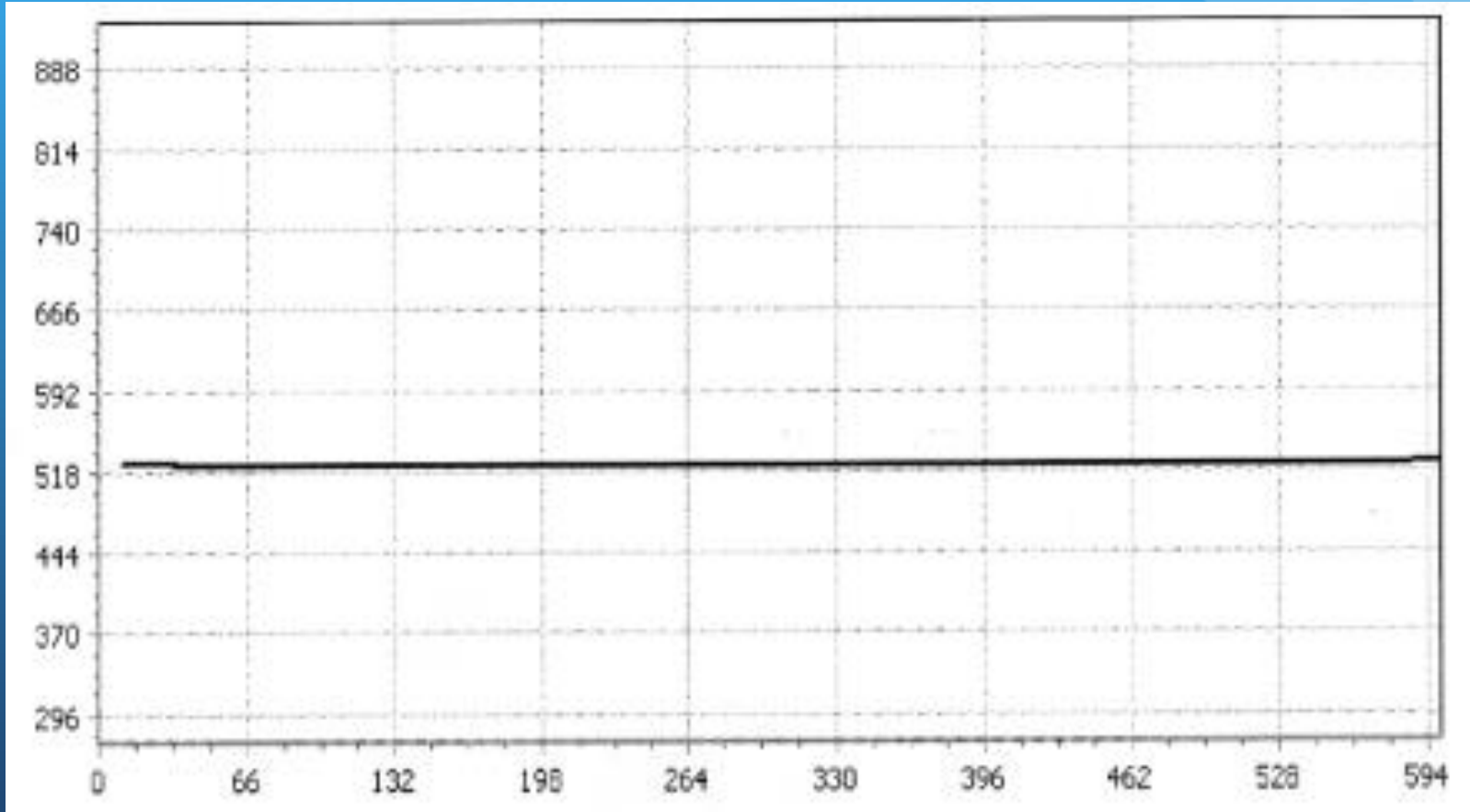
Positive HIT Reaction Curve, Result = 2.0 U/mL, $\Delta mAbs = 128$



Positive HIT Reaction Curve, Result = 2.4 U/mL, $\Delta mAbs = 119$



Positive HIT Reaction Curve,
Result = 27.4 U/mL, $\Delta mAbs = 1$



Confirmatory Immunoassays for HIT Antibodies

- Platelet Serotonin Release Assay = Gold Standard
Performed at McMaster University, Hamilton, ON
 - Normal donor platelet-rich plasma is preincubated with ^{14}C -serotonin which enters the platelet dense granules
 - The platelets are washed and incubated with patient serum or plasma along with varying amounts of Heparin
 - The reaction mixture is centrifuged
 - The radioactivity of the supernate is measured by scintillation counting on a β -counter and is directly proportional to the degree of platelet activation
 - Positive Result = Platelet serotonin release of >20% at therapeutic heparin levels with inhibition at high heparin levels due to disruption of the PF4-Heparin complexes which prevents activation of the platelets

Confirmatory Immunoassays for HIT Antibodies

- Anti-PF4/Heparin ELISA
 - Reaction wells are coated with PF4/Heparin complex
 - Duplicate 1:50 dilutions of HIT serum are incubated
 - Chromogen-conjugated rabbit secondary antibodies allow for separate detection and measurement of IgG, IgA, and IgM HIT antibody fractions
- Flow cytometry methods
 - CD61 (GPIIIa) and CD41 (GPIIb/IIIa) identify resting platelets; CD62P (P-selectin) and Annexin V identify activated platelets in the presence of HIT serum
 - GPIb_α, and Annexin V are used to identify activated platelets and platelet microparticles employing forward-scatter plotting for size-based differentiation

Confirmatory Immunoassays for HIT Antibodies

- Assessment of ADP release by activated platelets using luminography (chemiluminescence)
- Particle Gel Immunoassay (column agglutination)
- Particle Immunofiltration Assay
- Platelet Activation Endpoint by Platelet Aggregation
- Washed Platelet Assay
- Heparin-Induced Platelet Aggregation Test
- Other Aggregation Assays using platelets and Heparin

** In all Aggregation Assays, the platelet preparation is more important than the assay methodology used**

Additional References

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2. Gulati, G. et al., Case Studies in Hematology and Coagulation. ASCP Press, 2012 ISBN 978-0-89189-585-5
3. McKenzie, S. et al., Textbook of Hematology, 2nd Edition. Williams and Wilkins, 1996 ISBN 0-683-18016-9
4. Monagle, P. et al., Haemostasis Methods and Protocols. Humana Press, 2013 ISBN 978-1-62703-338-1
5. Rodak, B. et al., Hematology: Clinical Principles and Applications, 3rd Edition. Saunders, 2007 ISBN 978-1-4160-3006-5
6. Rodgers, G and Alday, A., Hemostasis Casebook, Lab Diagnosis and Management. ASCP Press, 2013 ISBN 978-089189-6173

Any Questions?

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KGH Core Lab

Thank You!