

Diagnostic Platelet Electron Microscopy

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VA Health Systems Employee Education series

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Topic overview

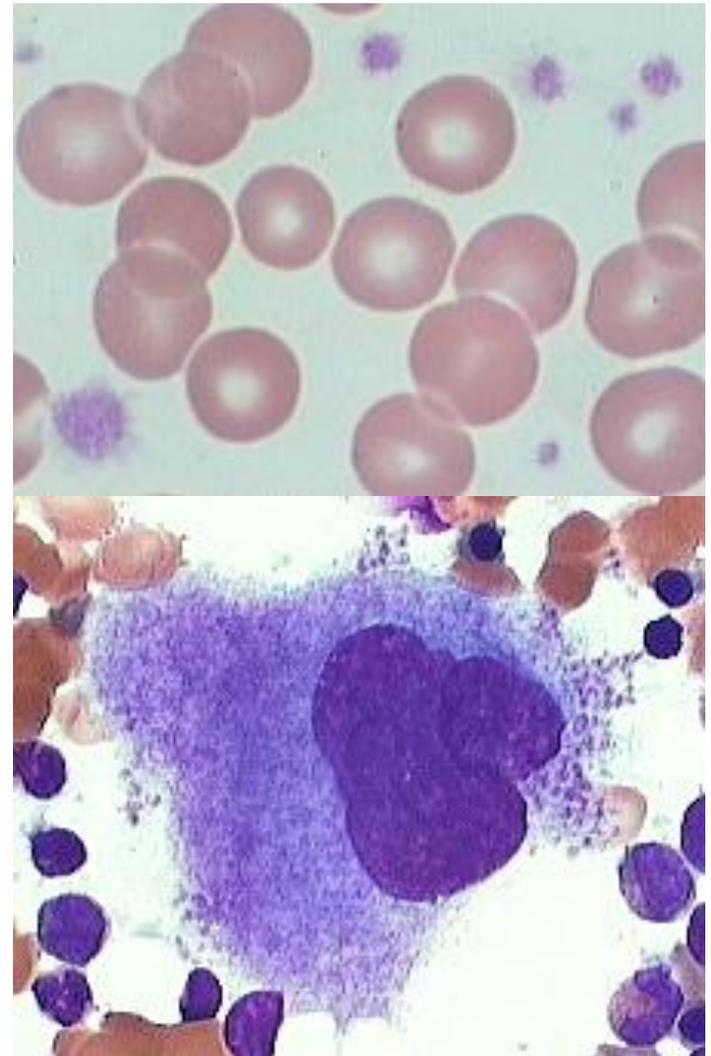
- Review the role of platelets in blood coagulation.
- Overview of acquired and congenital platelet disorders.
- Laboratory assessment of platelet disorders.
- Platelet sample collection and processing for electron microscopy.
- Illustrate diagnostic findings in platelet electron microscopy.

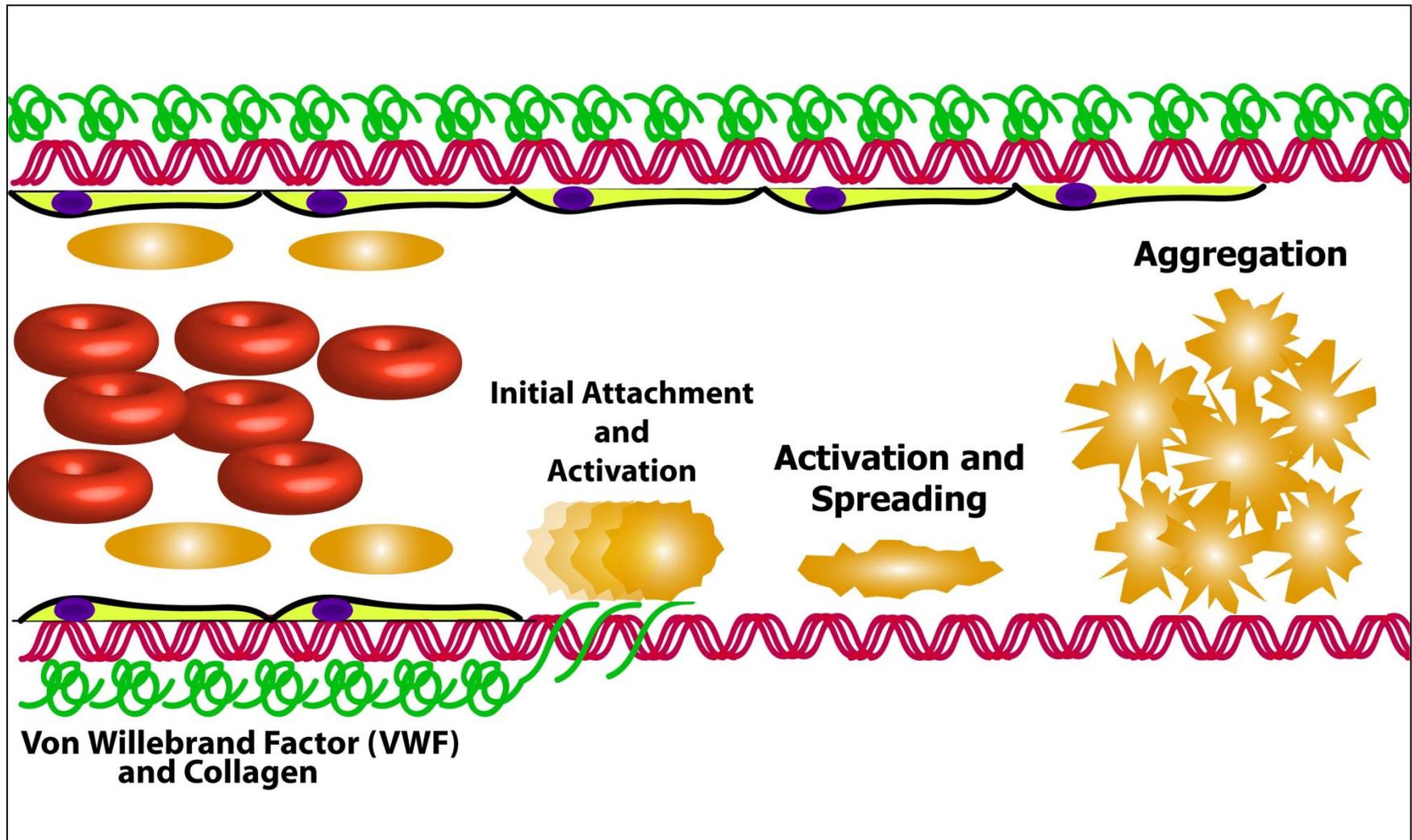
Four Stages of Hemostasis

1. Vasoconstriction
2. Primary Hemostasis: platelet adhesion and aggregation
3. Secondary Hemostasis: formation of a fibrin clot
4. Fibrinolysis: dissolution of the fibrin clot

Platelets: the key player in primary hemostasis

- Made in the bone marrow, produced by megakaryocytes
- 2-4 μm in diameter
- Live in circulation for 7-10 days
- Normal platelet count: 150,000-400,000/mL
- $2/3^{\text{rd}}$ in circulation, $1/3^{\text{rd}}$ in spleen





Acquired Platelet Disorders

Intrinsic disorders

- Chronic myeloproliferative disorders
- Leukemias and myelodysplastic syndromes
- Paroxysmal nocturnal hemoglobinuria

Extrinsic disorders

Drugs (aspirin, other NSAIDs, clopidogrel, etc.)

Foods and food additives:

- ω 3-fatty acids, vitamin E, ethanol, Chinese black tree fungus, garlic extract

Chronic renal failure

Liver disease / cirrhosis

Cardiopulmonary bypass

Antiplatelet antibodies

Myeloma / Dysproteinemias

Disseminated intravascular coagulation

Hypothermia



Congenital Platelet Disorders

Disorders of Adhesion:

- Bernard-Soulier

Disorder of Aggregation:

- Glanzmann thrombasthenia

Disorders of Granules:

- **Grey Platelet Syndrome**
- **Storage Pool deficiency**
- **Hermansky-Pudlak syndrome**
- Chediak-Higashi syndrome

Disorders of Cytoskeleton:

- Wiskott-Aldrich syndrome

Disorders of Primary Secretion:

- Receptor defects (TXA₂, collagen, ADP, epinephrine)

Disorders of Production:

- Congenital amegakaryocytic thrombocytopenia
- **MYH9 related disorders**
- Thrombocytopenia with absent radii (TAR)
- **Paris-Trousseau/Jacobsen's Syndrome**



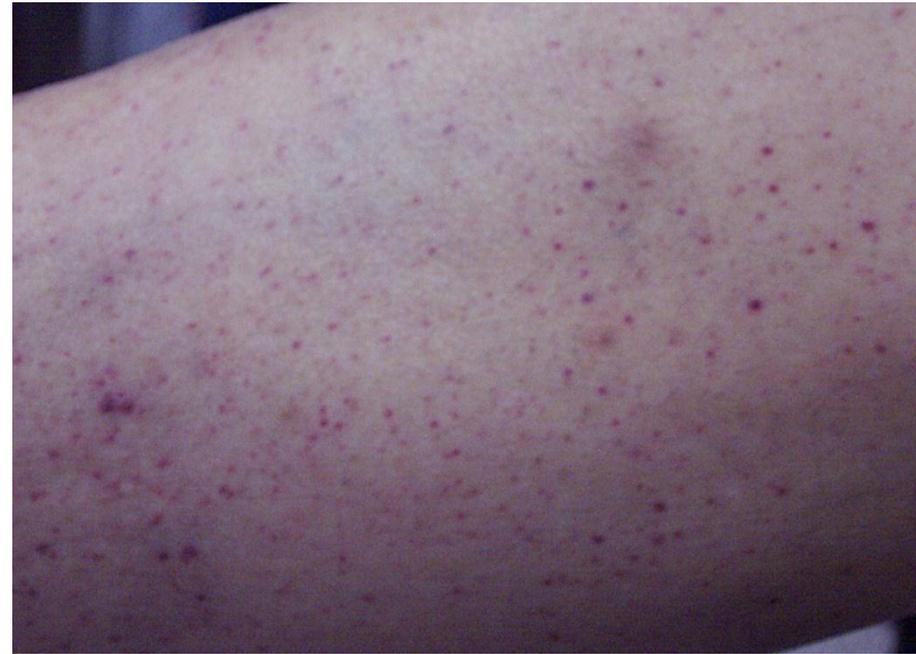
Balduini, et al., *Haematologica*

88(05): 582-592 May 2003

Clinical assessment of primary hemostasis

Bleeding history

- Mucocutaneous hemorrhage
 - Epistaxis
 - Menorrhagia or obstetric hemorrhage
 - Easy bruising, petechiae
 - Excessive bleeding during shaving, flossing
 - G.I. bleeds, hematuria
 - Family history
- Surgical bleeding
- Bleeding during dental procedures
- History of transfusion



ASH Image Bank 2008;2008:8-00089

Petechiae, Peter Maslak

An approach to laboratory diagnosis of platelet disorders

Initial testing

- Complete Blood Count and blood smear review
- Mean Platelet Volume (normal range 6-10 fL)
- Rule out von Willebrand disease
- PFA-100 +/- bleeding time

Secondary Testing

- Platelet aggregometry

Tertiary testing

- Platelet flow cytometry
- ADP and serotonin release assays
- Electron microscopy

Laboratory assessment of platelet function

- 1. Bleeding time**
Time for cessation of bleeding from a standardized wound

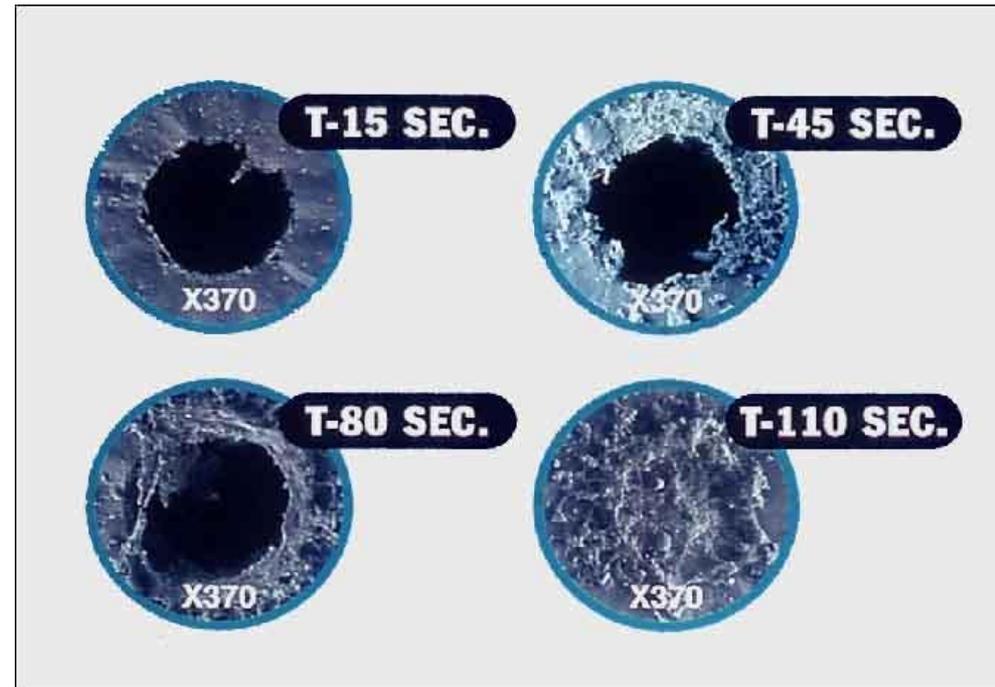


Laboratory assessment of platelet function

1. Bleeding time

2. PFA-100

- Whole blood flowing under high shear stress .
- Agonist-lined cartridges (C-Epi, C-ADP).
- Platelet/fibrin aggregates occlude aperture.
- Prolonged by anemia or thrombocytopenia



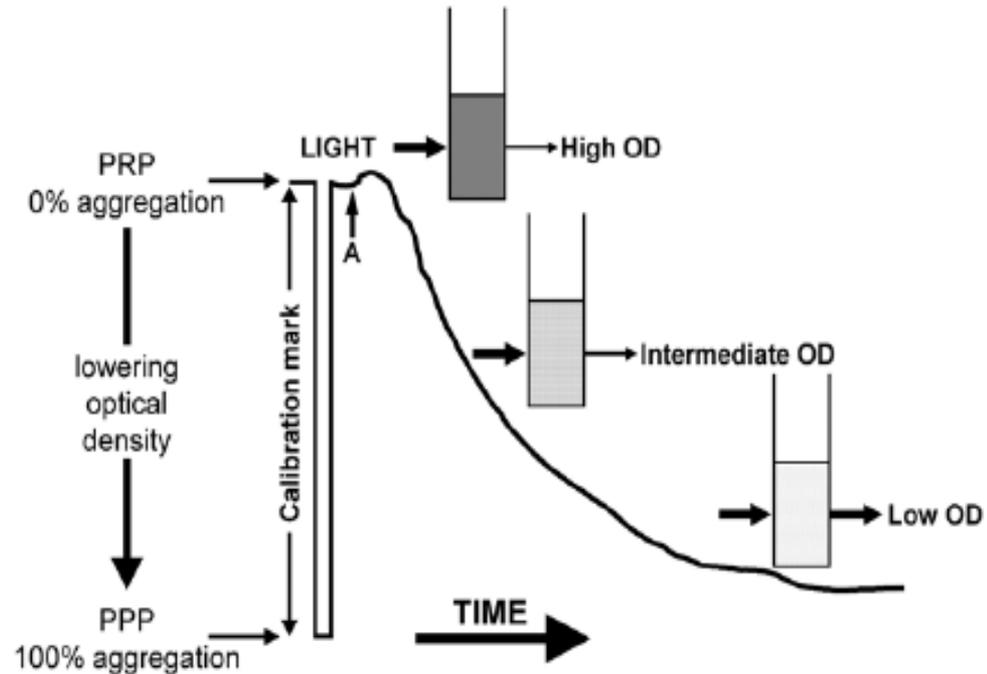
Laboratory assessment of platelet function

1. Bleeding time

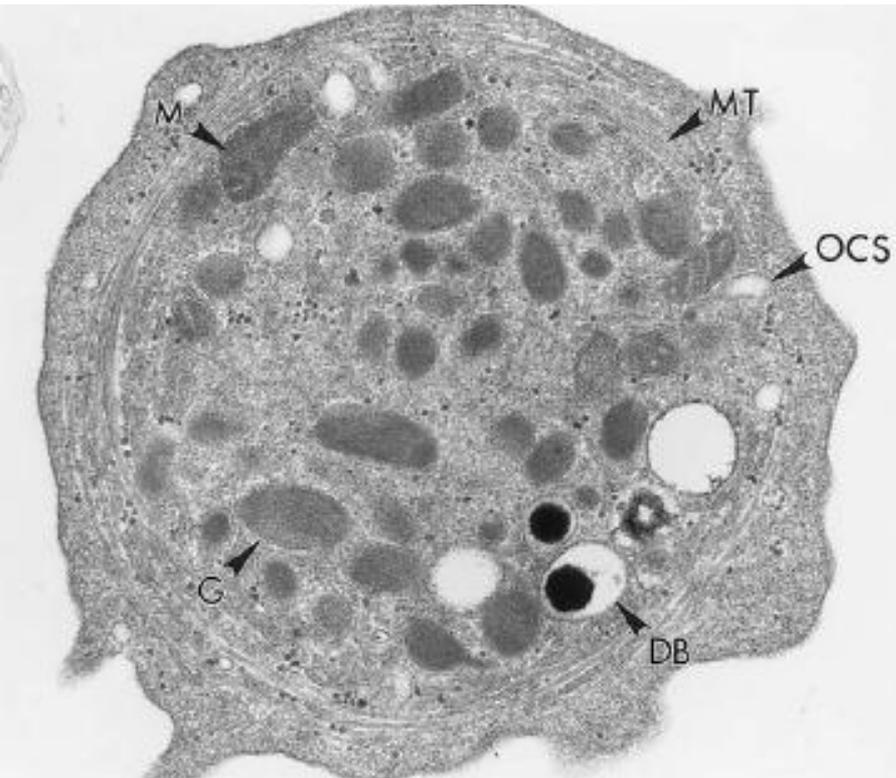
2. PFA-100

3. Platelet aggregation

Agonist addition to platelet-rich plasma to induce formation of platelet aggregates.



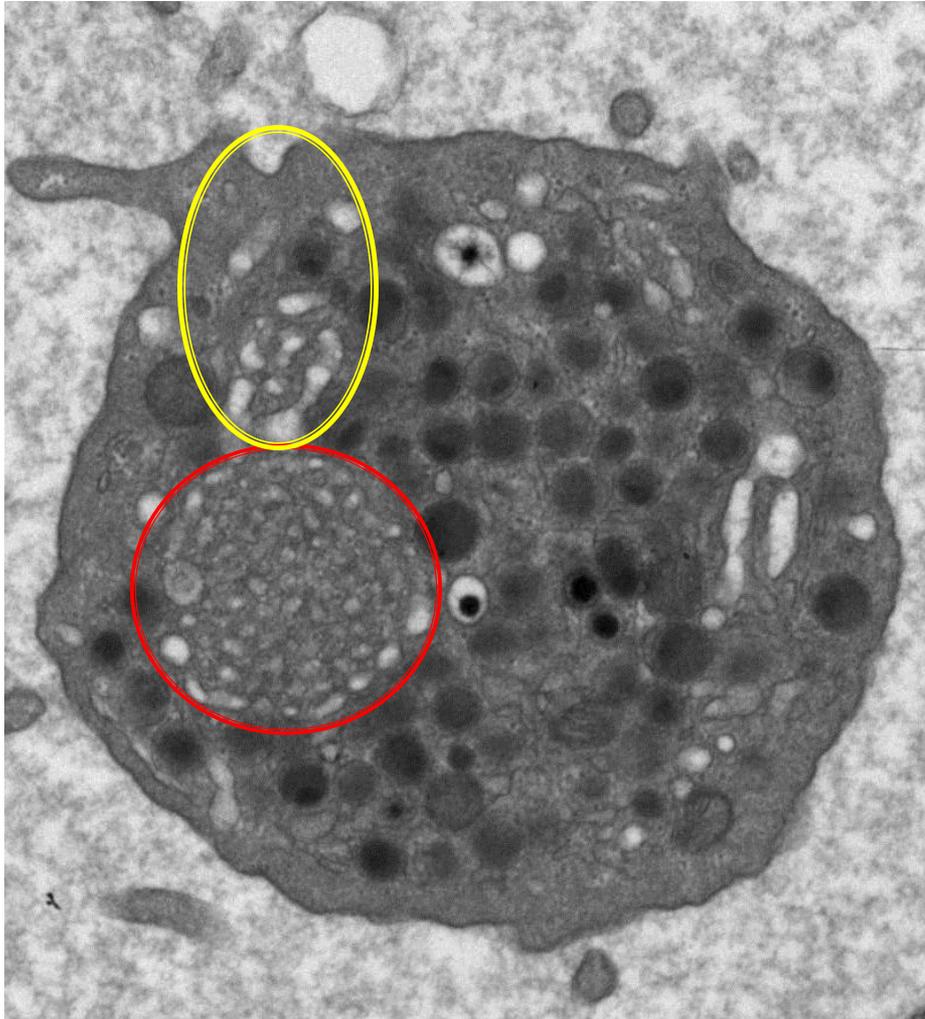
Normal Platelet Ultrastructure



- Lipid bilayer membrane
- Gel-like cytoplasm with fibrous matrix
- Microtubules: 8-12 circumferential profiles
- Mitochondria
- Lysosomes
- Glycogen

Photo by Dr. James White, in "Methods in Molecular Biology: Platelets and Megakaryocytes, Vol. 1", Gibbins, J.M., and Mahaut-Smith, M.P., [eds.], 2004, pg. 48.

Normal Platelet Ultrastructure



- Open canalicular system
 - Invaginations of the surface membrane
 - Allows for four-fold increase of the platelet surface area
- Dense tubular system
 - Derived from MK R.E.R. and S.E.R., Ca^{++} storage, prostaglandin synthesis

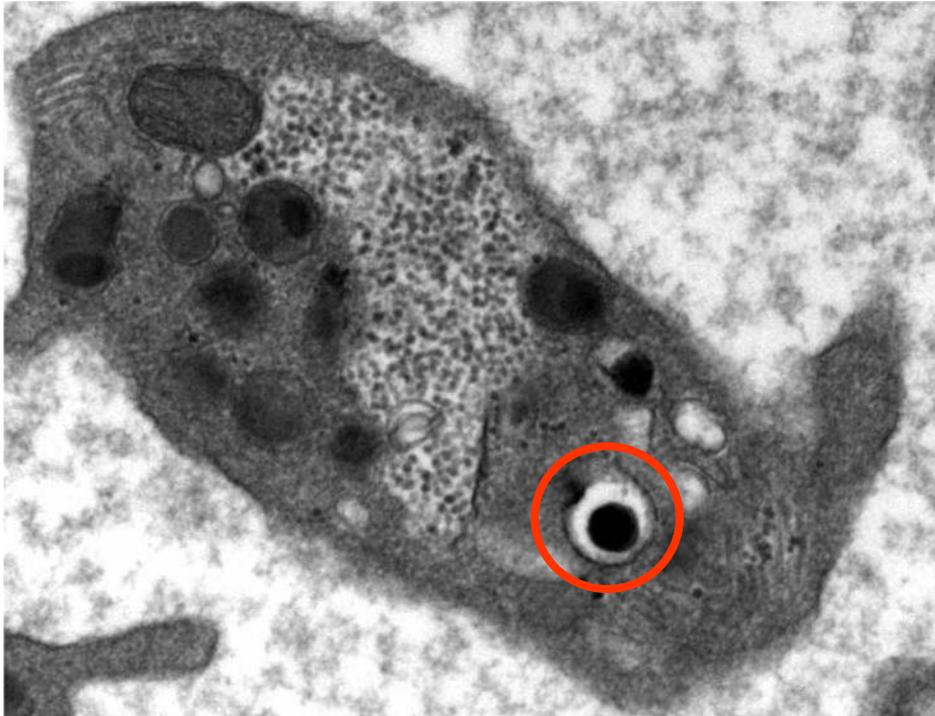
Normal Platelet Ultrastructure



Alpha granules:

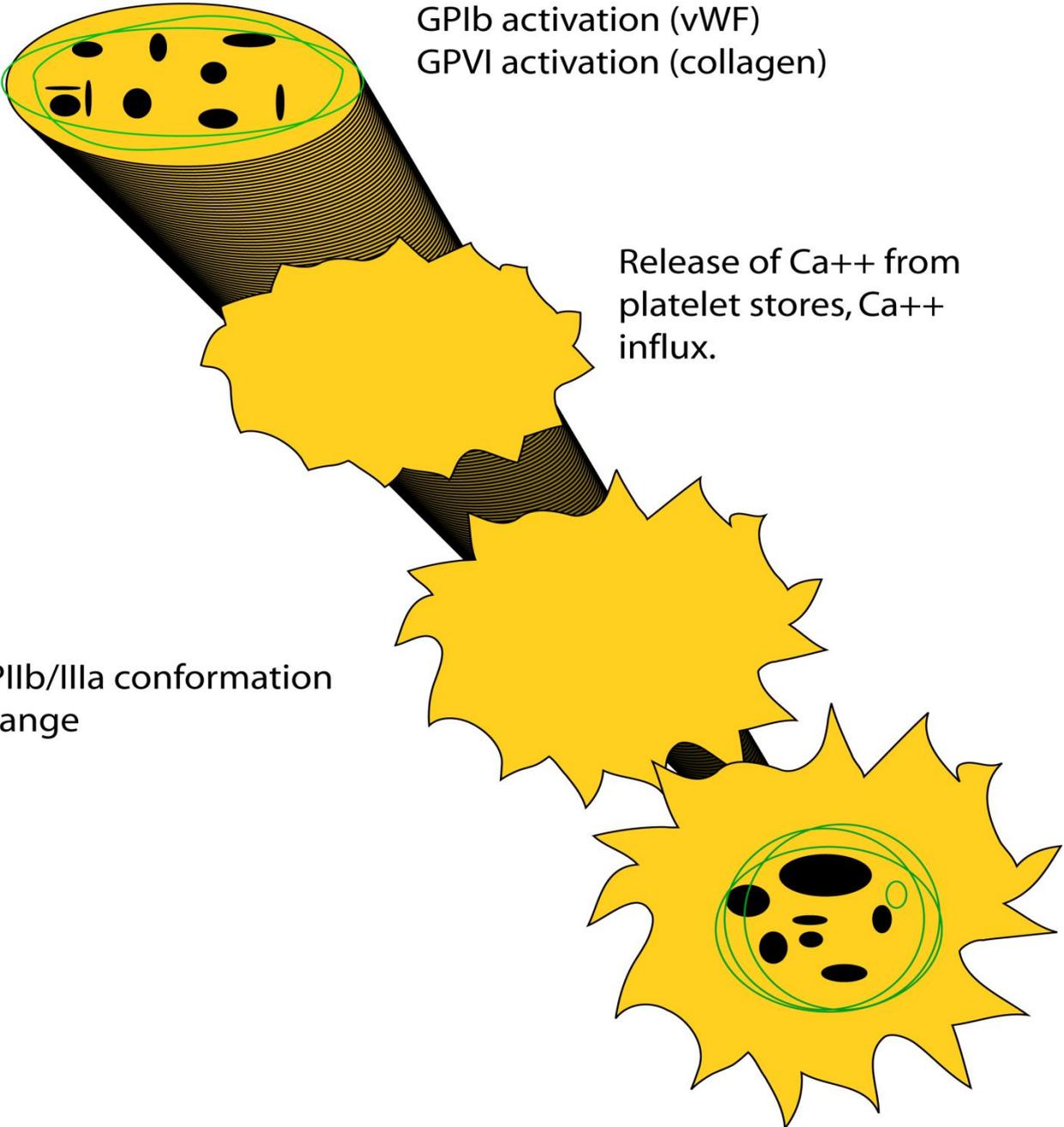
- 40-80 per platelet
- Round-to-oval, 200-500 nm in diameter
- Contains proteins synthesized in MKs and taken up by endocytosis
 - Von Willebrand factor
 - Factors V and XIII
 - Fibrinogen
 - Fibronectin
 - P-selectin
- Variable electron density, often with a nucleoid of greater density
 - Calcium-poor

Normal Platelet Ultrastructure



Dense Granules:

- 4 to 8 D.G. per platelet
- Electron-dense Ca^{++} → 60-70% of platelet calcium stores
- Also contains ADP, serotonin, polyphosphate



GPIIb activation (vWF)
GPVI activation (collagen)

Release of Ca^{++} from
platelet stores, Ca^{++}
influx.

GPIIb/IIIa conformation
change

Sample processing for platelet E.M.



Platelet E.M.:

Sample requirements

- Minimize shear stress and agitation
- No Vacutainers!
- No pneumatic tube transport

- Phlebotomists need to be carefully trained
- Recommend direct supervision of phlebotomists while establishing testing.

Recommend concurrent control sample from normal individual (if possible)

Platelet E.M.:

Sample requirements

- Draw blood slowly into syringe using 19-21 gauge butterfly needle
- Drip into citrate anticoagulant , gently invert 3-5x to mix
 - Acid Citrate Dextrose (yellow-top tube) most easily accessible anticoagulant
- Keep sample warm (between 20-37 C) prior to processing
 - Process within 4 hours of collection
- Use room temperature centrifuge

Platelet processing for TEM

PLATELET-RICH PLASMA (PRP) method*:

1. Sample: citrated whole blood
2. Separate PRP from leukocytes/RBC after low speed centrifugation.
3. Combine PRP in 1:1 ratio with 0.1% glutaraldehyde in Ca⁺⁺-rich buffer for 15 min.
4. Centrifugation to form pellet; 3% glutaraldehyde fixation for 1 hour.

*White JW, in "Methods in Molecular Biology: Platelets and Megakaryocytes, Vol. 1", Gibbins, J.M., and Mahaut-Smith, M.P., [eds.], 2004, pg. 48

Platelet processing for TEM

PLATELET-RICH PLASMA (PRP) method*:

Advantages

- Excellent preservation of ultrastructural features.
- Ca^{++} rich buffers (i.e. White's saline) helps preserve dense granule morphology in thin section.

Disadvantages

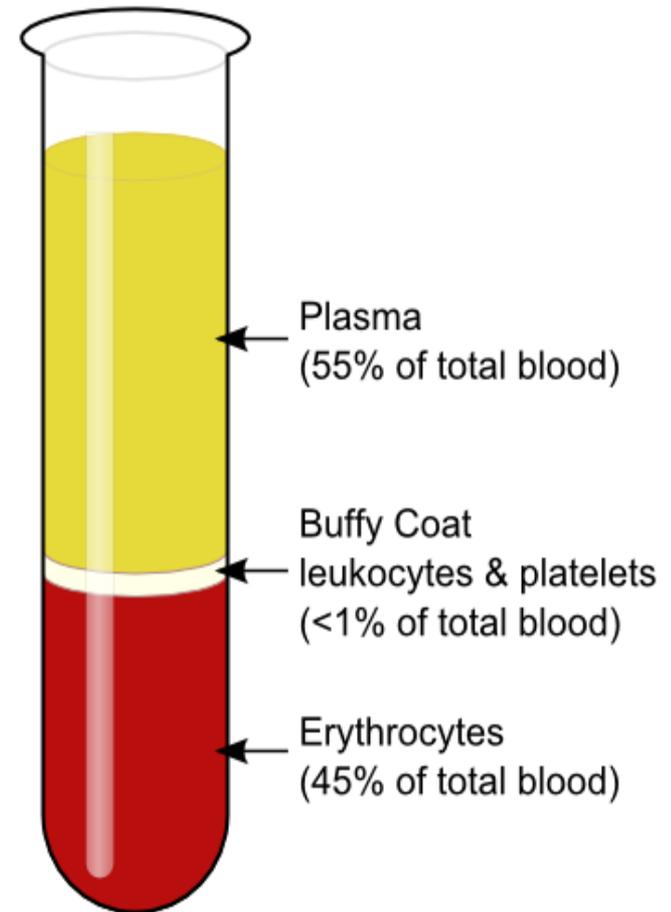
- At least 3 mL blood necessary.
- No leukocyte ultrastructure.
- Difficult in thrombocytopenia.

*White JW, in "Methods in Molecular Biology: Platelets and Megakaryocytes, Vol. 1", Gibbins, J.M., and Mahaut-Smith, M.P., [eds.], 2004, pg. 48

Platelet processing for TEM

BUFFY COAT:

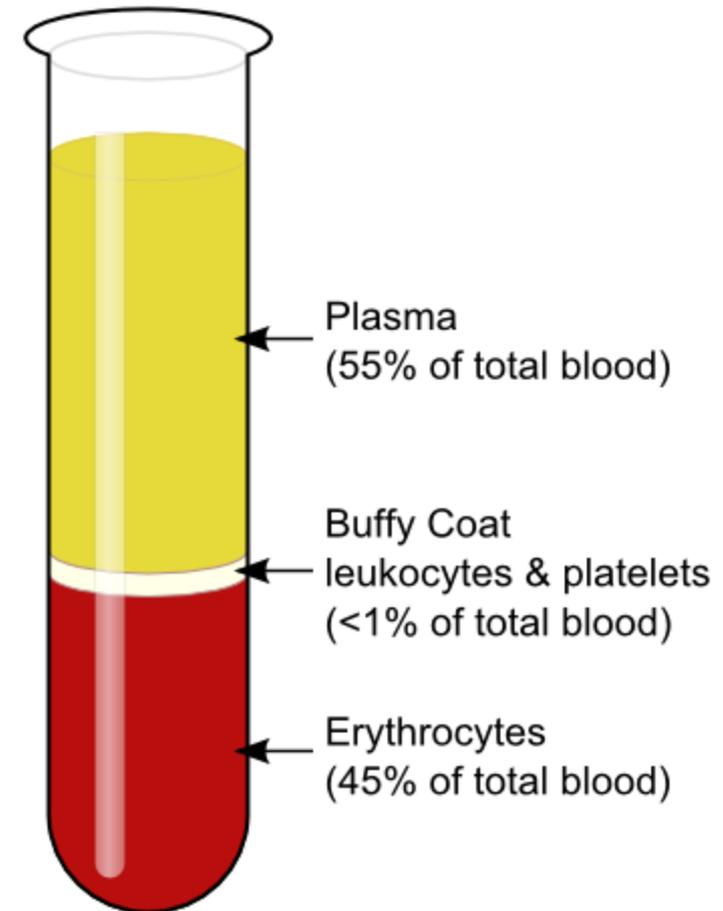
1. Citrated whole blood
2. Centrifugation at low-to moderate speed (300-500 g x 20 min) to form buffy coat
3. Removal of upper plasma layer
4. Layering of 3% glutaraldehyde buffer on top of buffy coat with fixation *in situ*



Platelet processing for TEM

BUFFY COAT:

- Useful in thrombocytopenic patients--should be performed with known macrothrombocytopenia
- Minimal blood needed; useful in neonatal patients



Platelet processing for EM

WHOLE MOUNT¹:

1. Place drop of PRP on Formvar-coated grid for 10-15 seconds.
2. Rinse 3-4x with drops of distilled water.
3. Dry edge of grid with filter paper, let grid air dry.
4. Ready for E.M.—no fixation, no staining.
5. Count dense granules in 50-100 platelets.

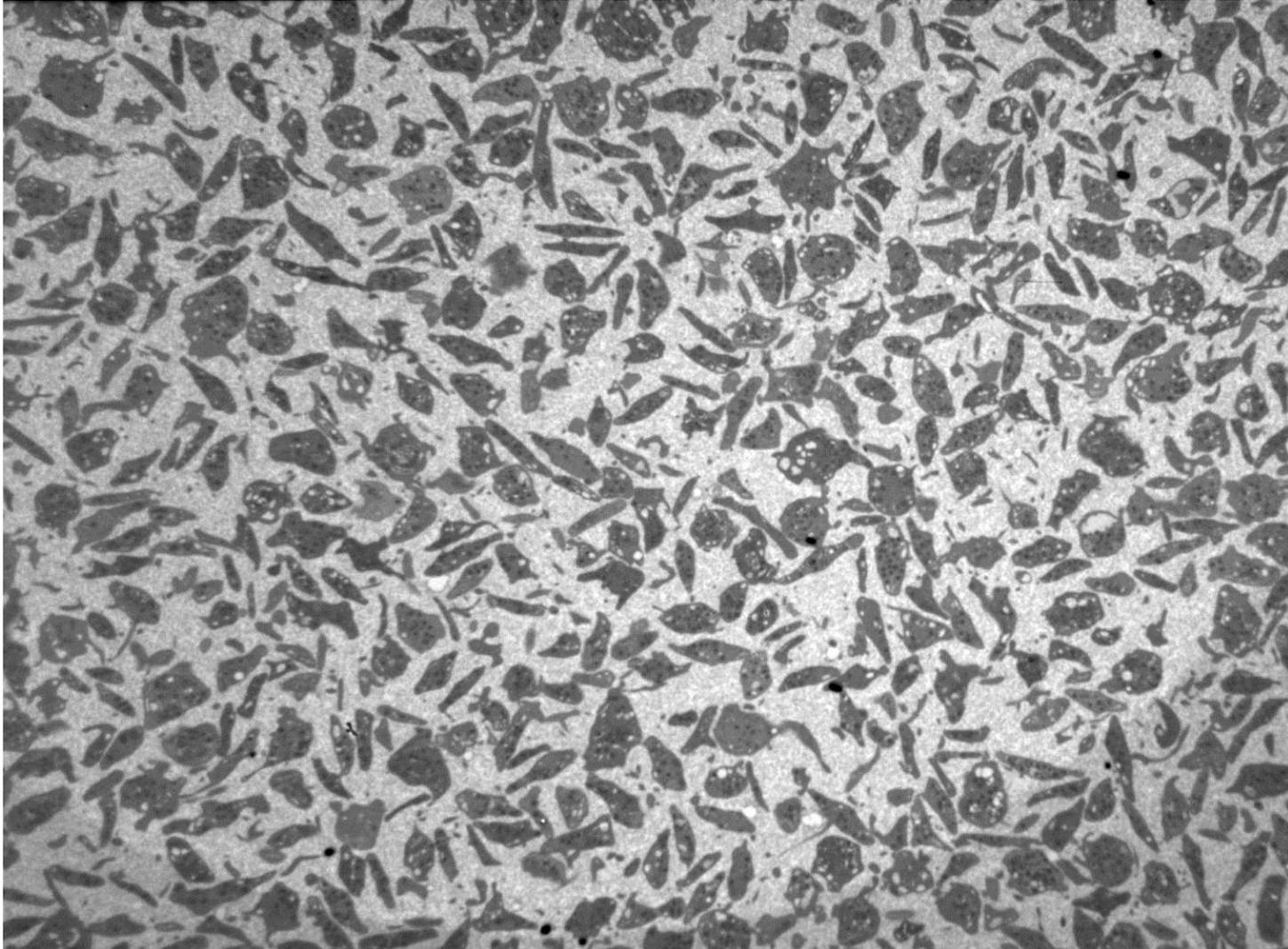
1. White JW, in "Methods in Molecular Biology: Platelets and Megakaryocytes, Vol. 1", Gibbins, J.M., and Mahaut-Smith, M.P., [eds.], 2004, pg. 48.

Platelet processing for EM

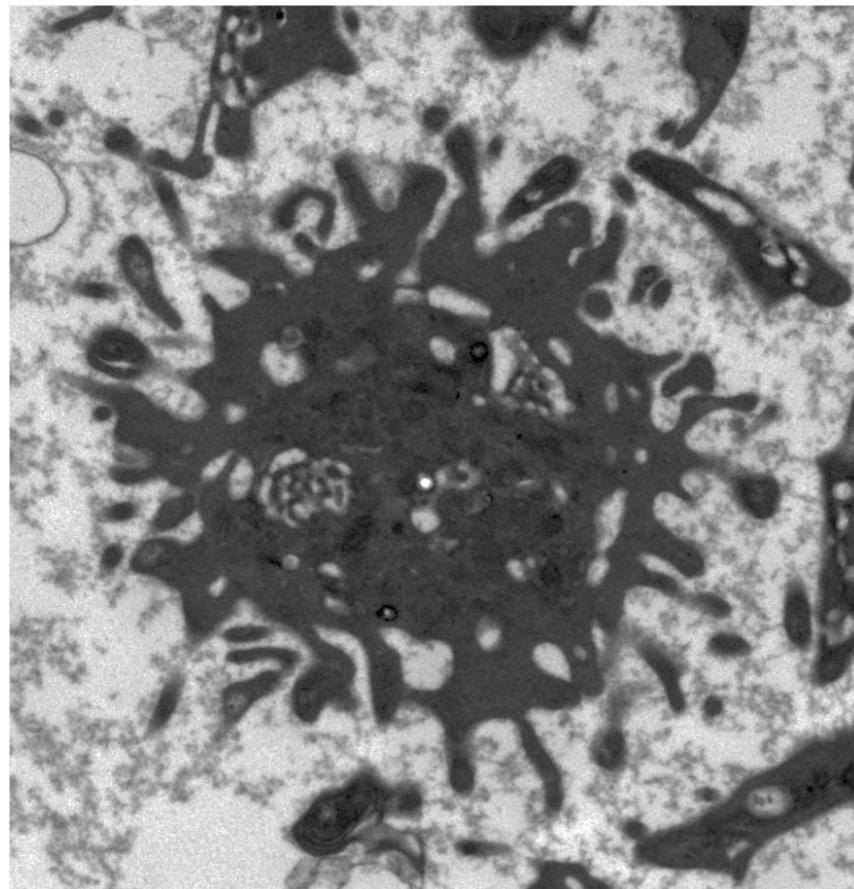
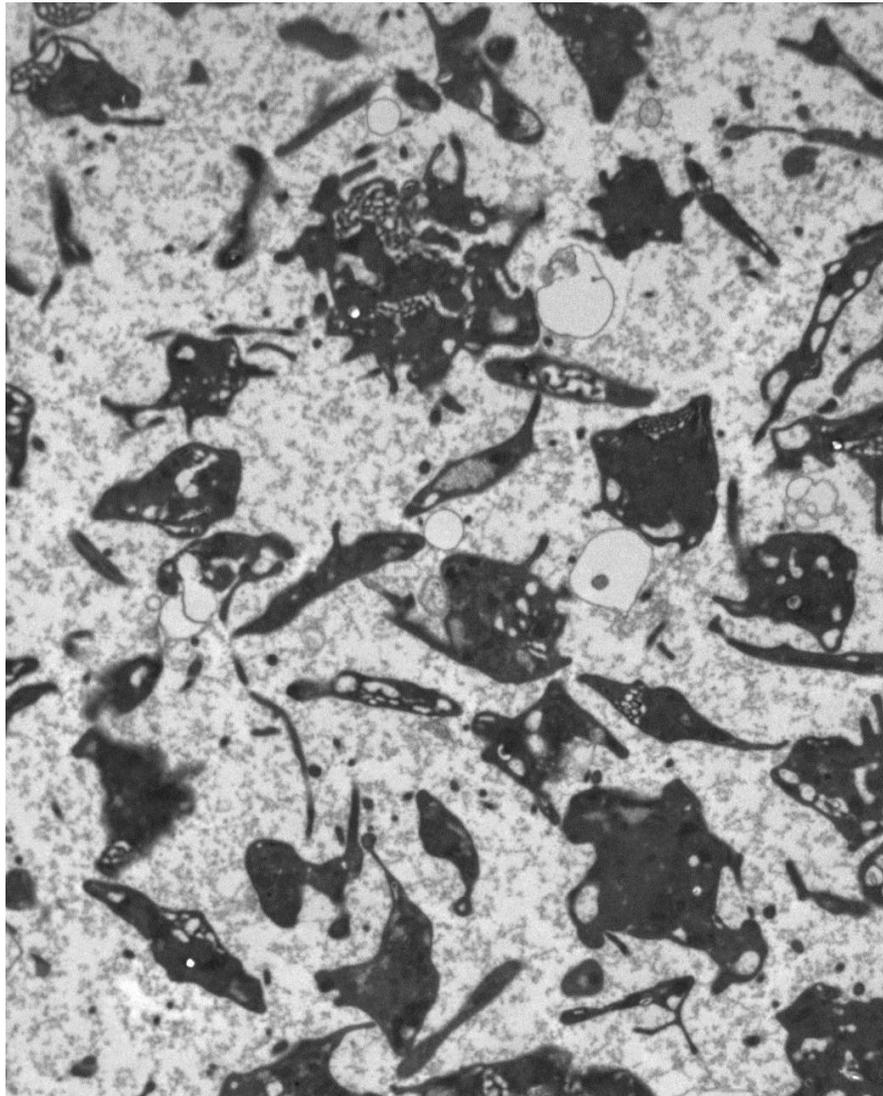
WHOLE MOUNT:

- Can be performed in thrombocytopenic patients.
- Minimal blood needed (as little as 20 μ L PRP).
- Rapid analysis (~2 hours from sampling to data).
- Care must be taken in evaluating dense granule morphology.
- Platelet should be examined at magnifications $>3,000\times$.¹

Unactivated platelets: discoid morphology

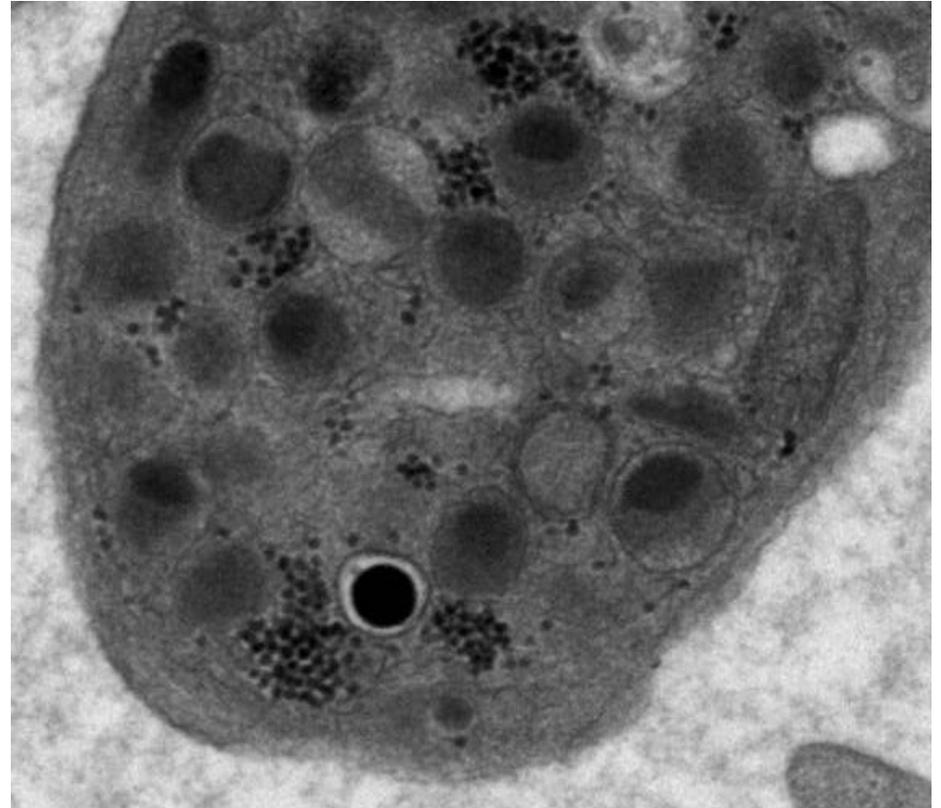


Mistreated platelets



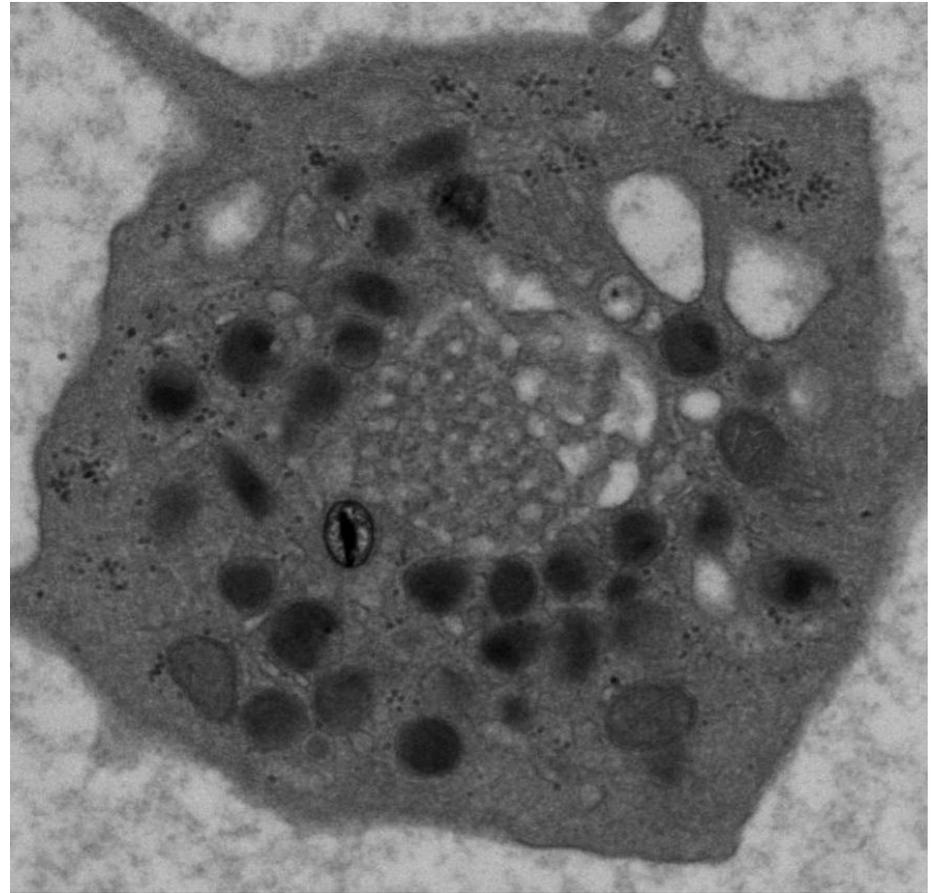
Dense granules in TEM

- Classic morphology: “Bulls Eye”: electron-dense core retracting from the granule membrane



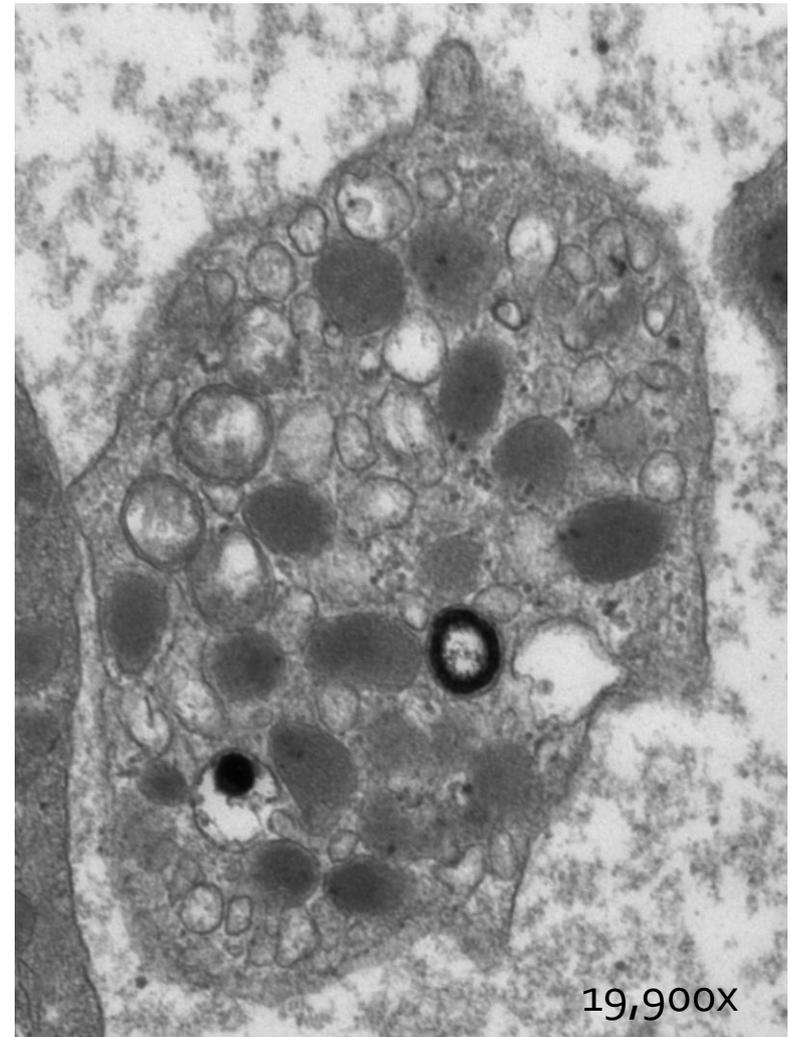
Dense granules in TEM

- Classic morphology: "Bulls Eye"
- Sharp edged cores



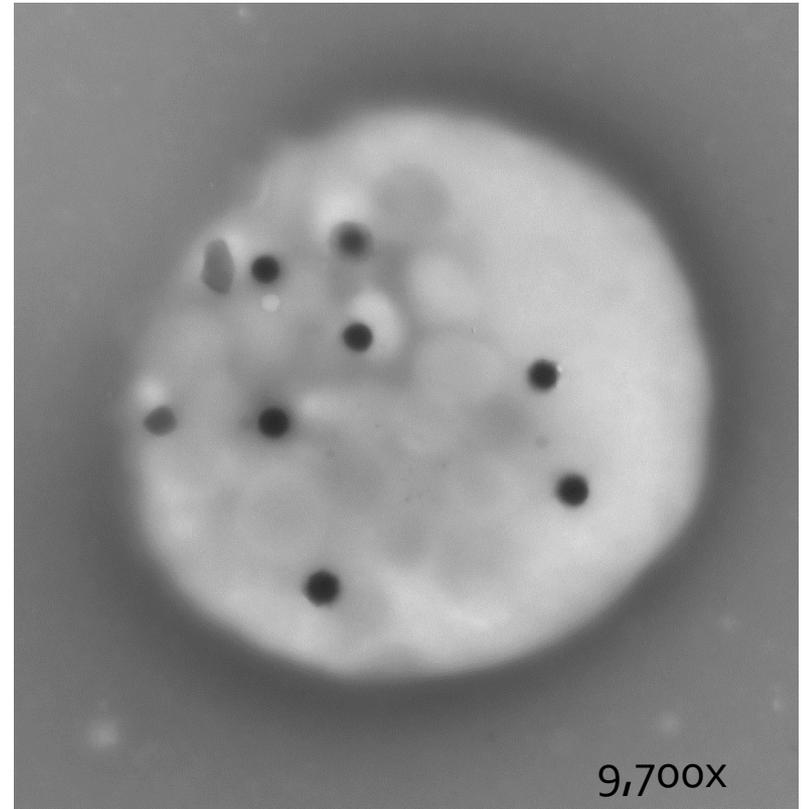
Dense granules in TEM

- Classic morphology:
- Sharp Edges
- Ringed



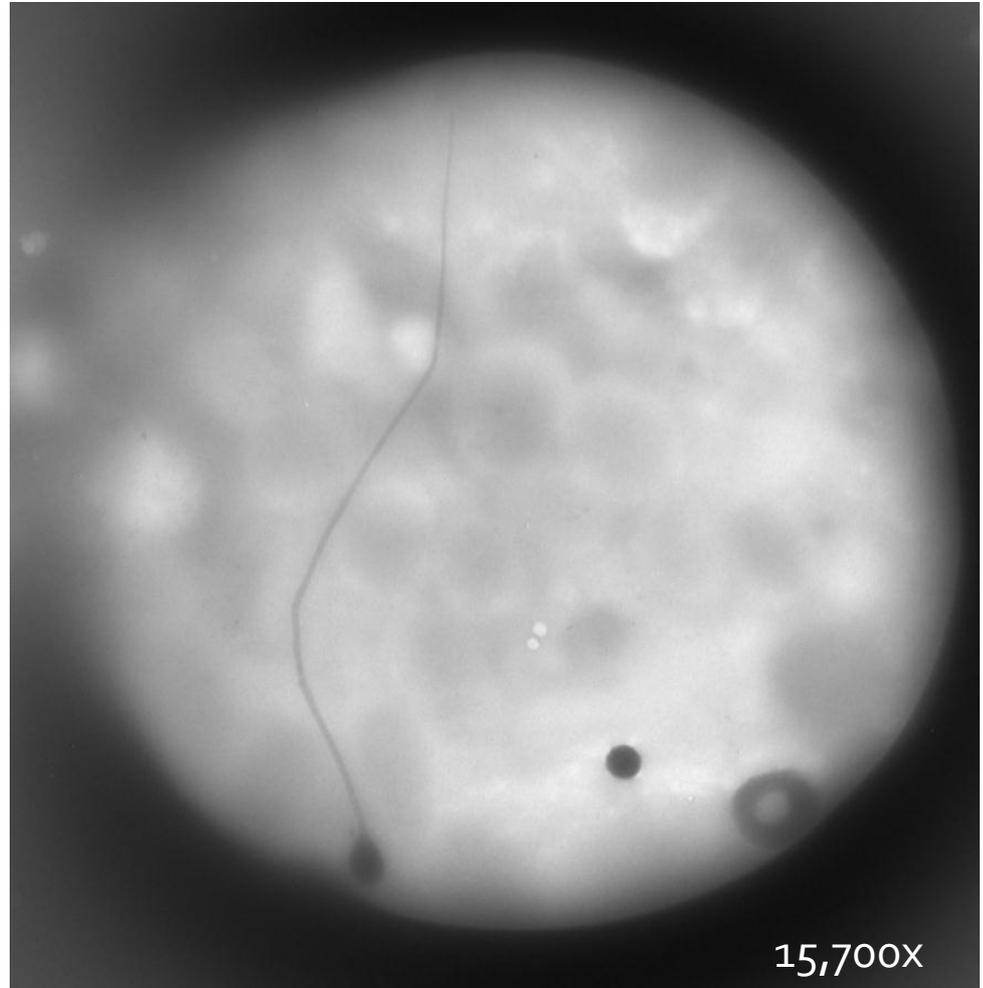
Whole mount dense granule morphology

- Classic: sharply spherical, smooth



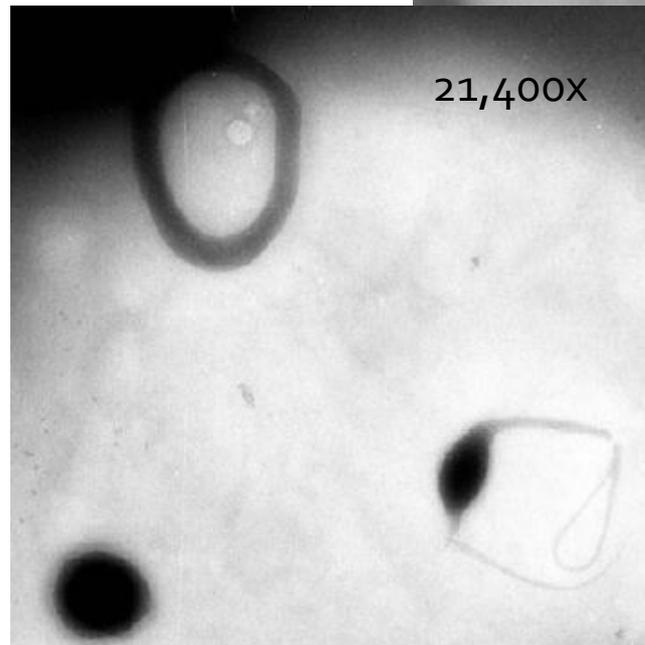
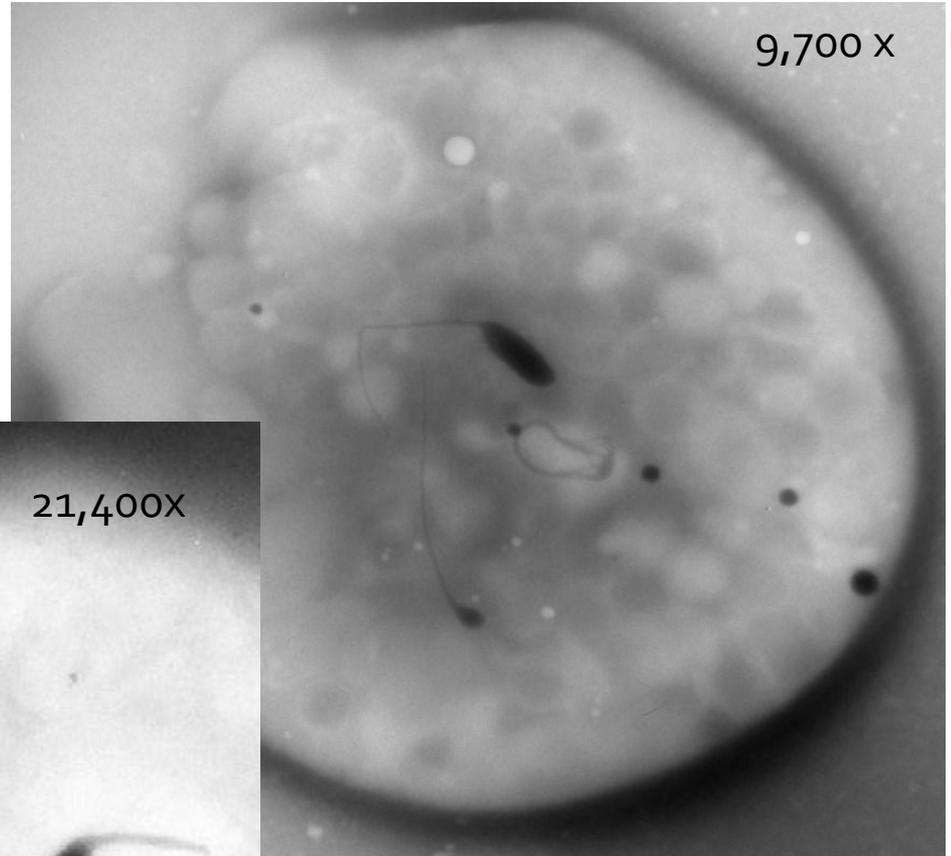
Whole mount dense granule morphology

- Classic
- “Whip-like” tail



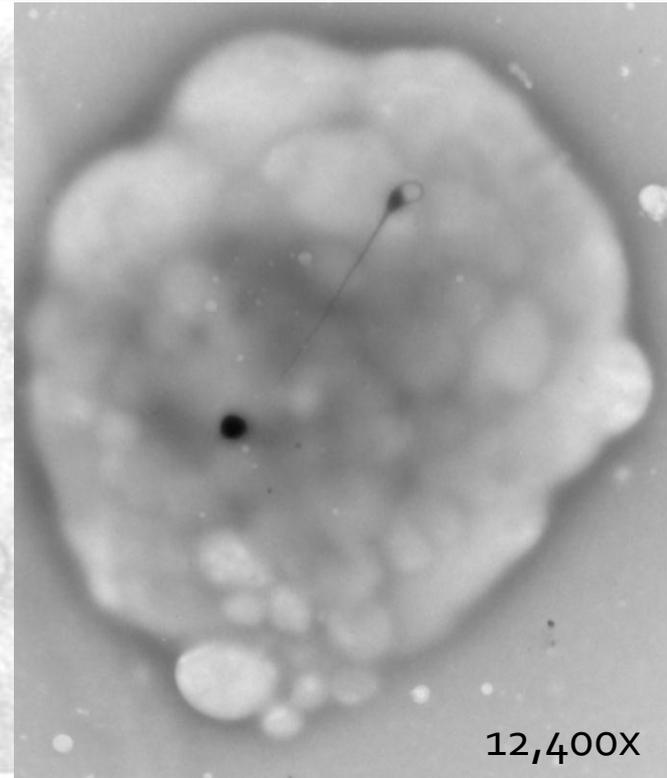
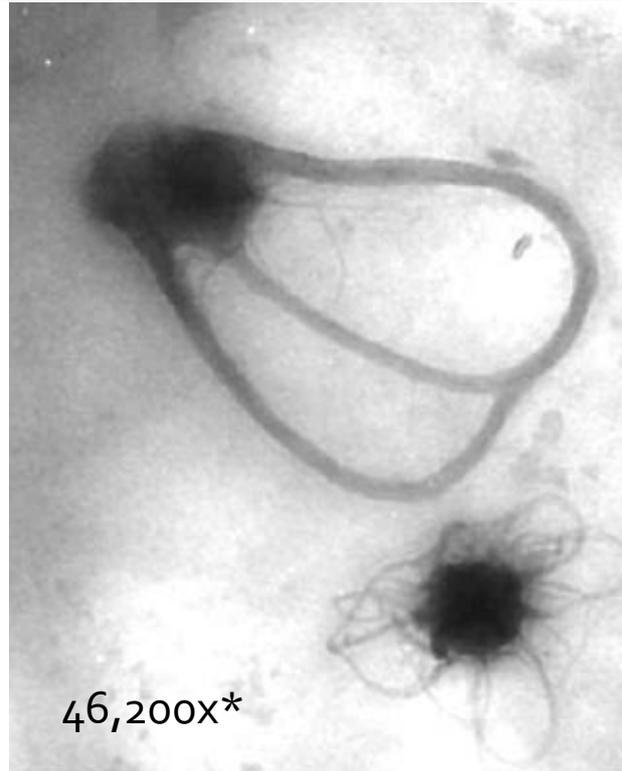
Whole mount dense granule morphology

- Classic
- “Whip-like”
- Ringed



Whole mount dense granule morphology

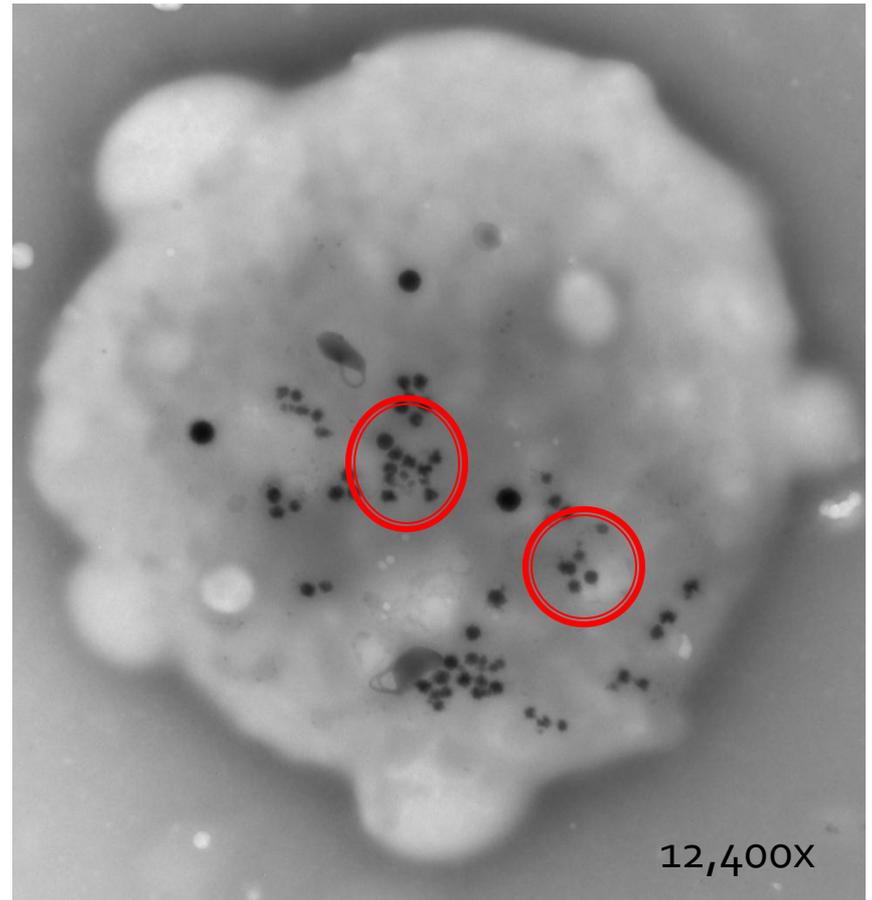
- Classic
- “Whip-like”
- Ringed
- Complex forms



*White, J.W., *Platelets* 2008, 19:455-466

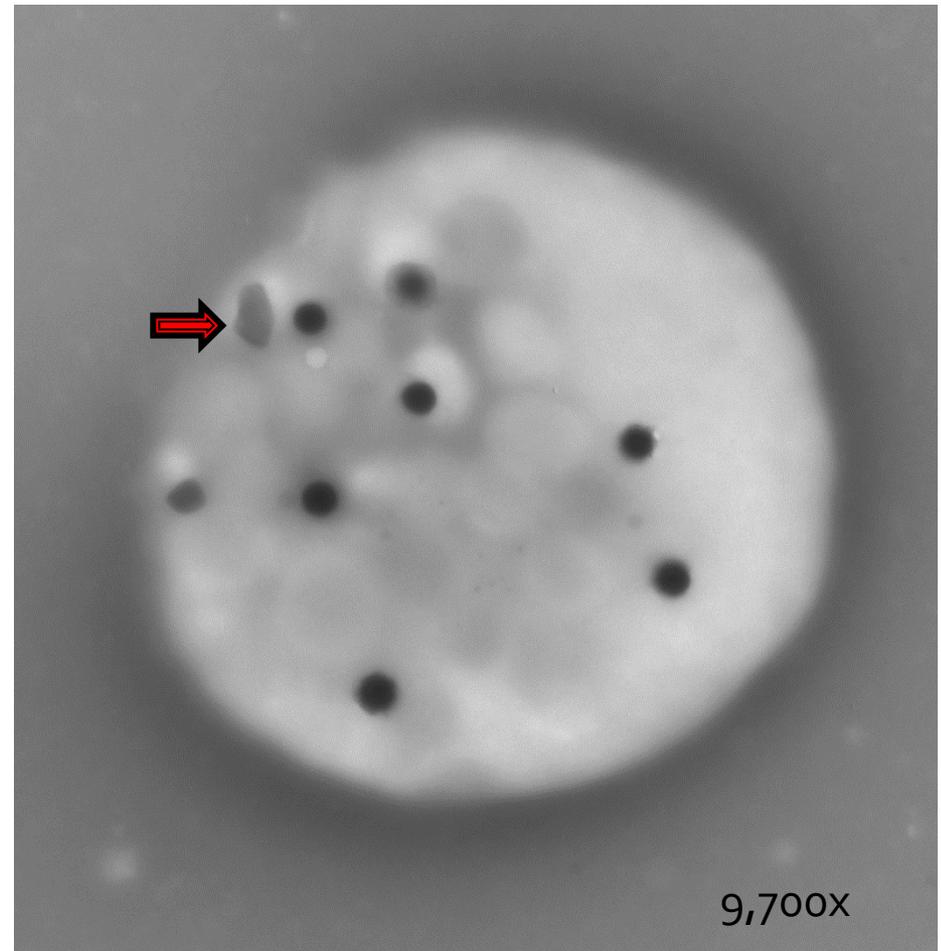
Dense granule red herrings

- **Electron-dense chains and clusters:** may represent Ca^{++} in the dense tubular system



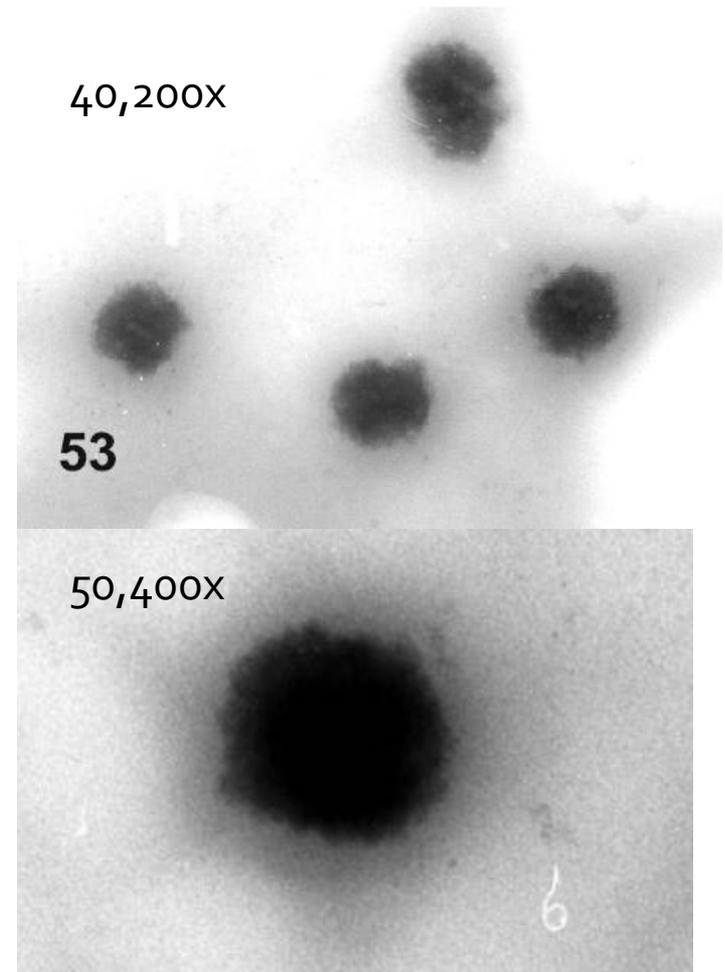
Dense granule red herrings

- Electron-dense chains and clusters
- **Alpha granules:** less electron-dense than D.G.s, irregular contours

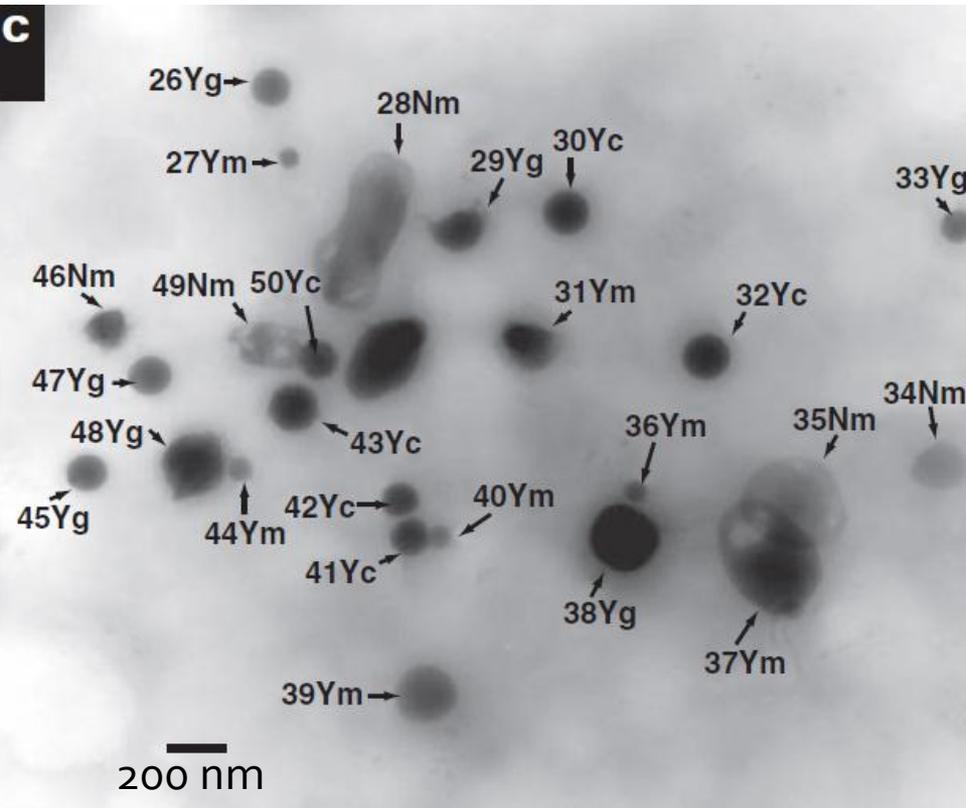


Dense granule red herrings

- Electron-dense chains and clusters
- Alpha granules
- “Fuzzy Balls”:
 - Irregular contours



NASCOLA Whole Mount E.M. Proficiency Testing Survey



- NASCOLA: North American Specialized Coagulation Laboratory Association
- Survey of 8 centers (6 with prior whole mount experience)
- 55 structures examined in standardized images
 - Overall consensus: 84%
 - 20 complete
 - 16 good (86%)
 - 19 moderate (57-71%)

Hayward, et al., *Am J Clin Path* 2009; 131:671-675

Delta Storage Pool Deficiencies

- Heterogeneous group of disorders due to deficiency in dense granules or granule content
- Often autosomal dominant
- Variable degree of bleeding symptoms
- Under-recognized due to insensitivity of platelet function testing
 - 51 patients with confirmed congenital SPD showed normal platelet aggregations (Nieuwenhuis et al., Blood 1987; 70 (3): 620)

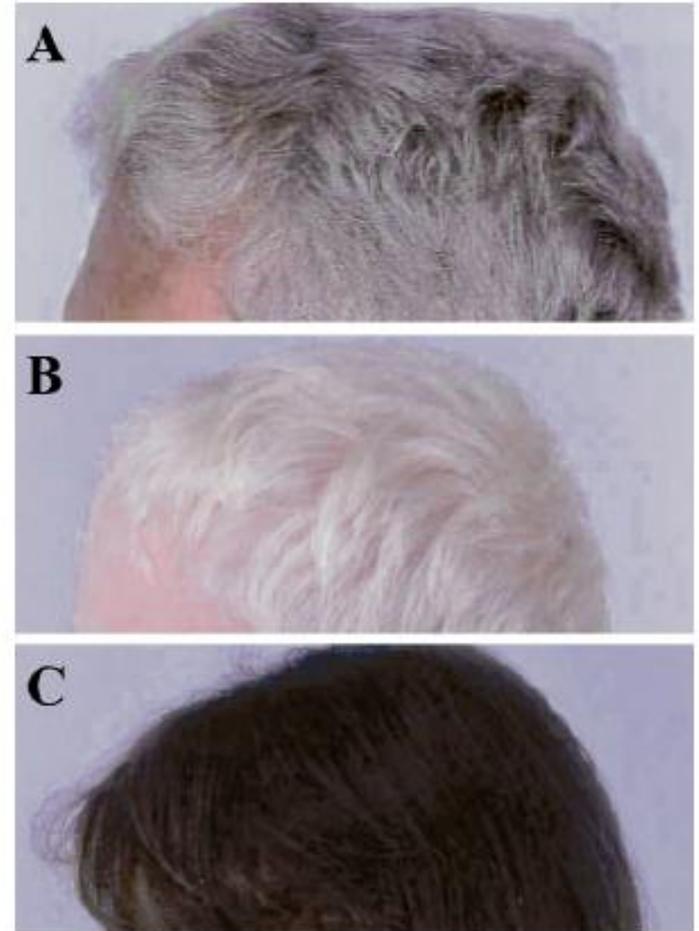
Hermansky-Pudlak Syndrome

Clinical Features

- Oculocutaneous albinism
- Congenital nystagmus
- Bleeding diathesis
- Ceroid-lipofuscin accumulation
 - Pulmonary fibrosis
 - Granulomatous colitis

Epidemiology

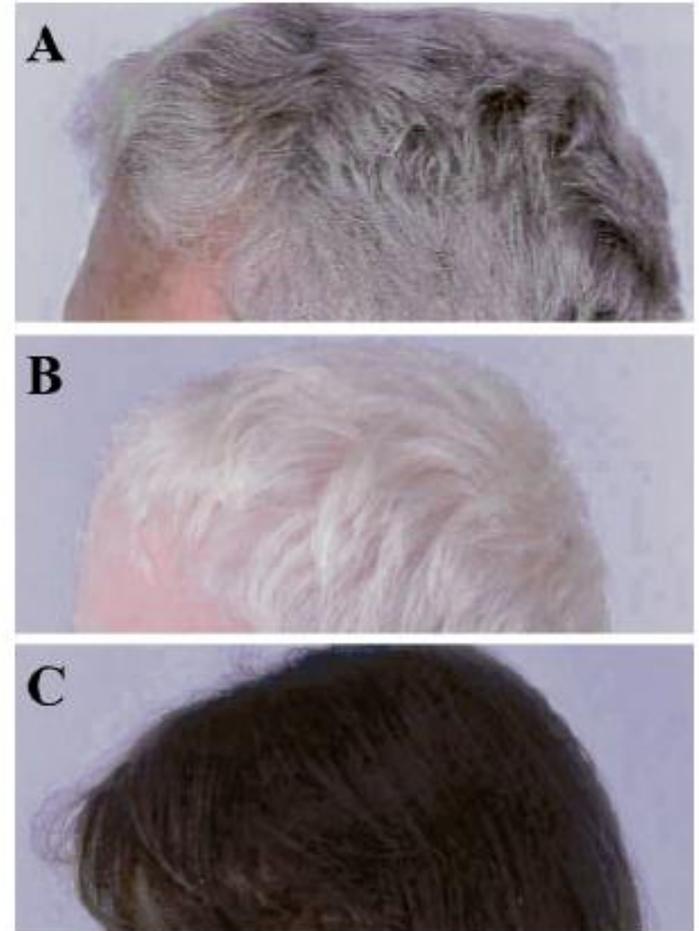
- 1 : 1800 Puerto Ricans



Hermansky-Pudlak Syndrome

Pathophysiology

- Autosomal recessive disorder of lysosomal-related organelle biogenesis:
 - Melanosomes
 - Lytic granules
 - Platelet dense granules
 - Basophil/neutrophil granules
- HPS₁ and HPS₃: most common mutations
- **Absence of DG by whole mount is gold standard for diagnosis**



Storage pool disease case study

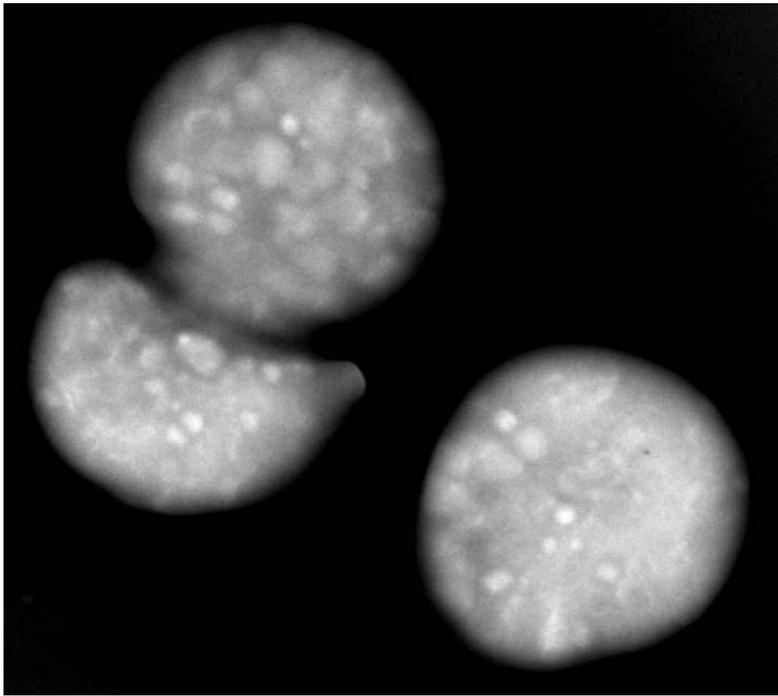
Patient: 3 y.o. boy with bleeding diathesis, chronic upper respiratory infections

Family history: mucocutaneous bleeding in father and older brother

Laboratory findings:

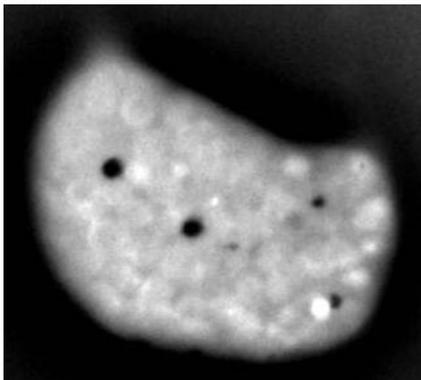
- PFA-100: Prolonged C-Epi, normal C-ADP
- Mepacrine flow cytometry showed minimal dense granule deficiency (5% of platelets with no dense granules).
- Platelet aggregation: mild abnormalities

Case study platelet E.M.



Whole mount analysis: 0.21 D.G. per platelet

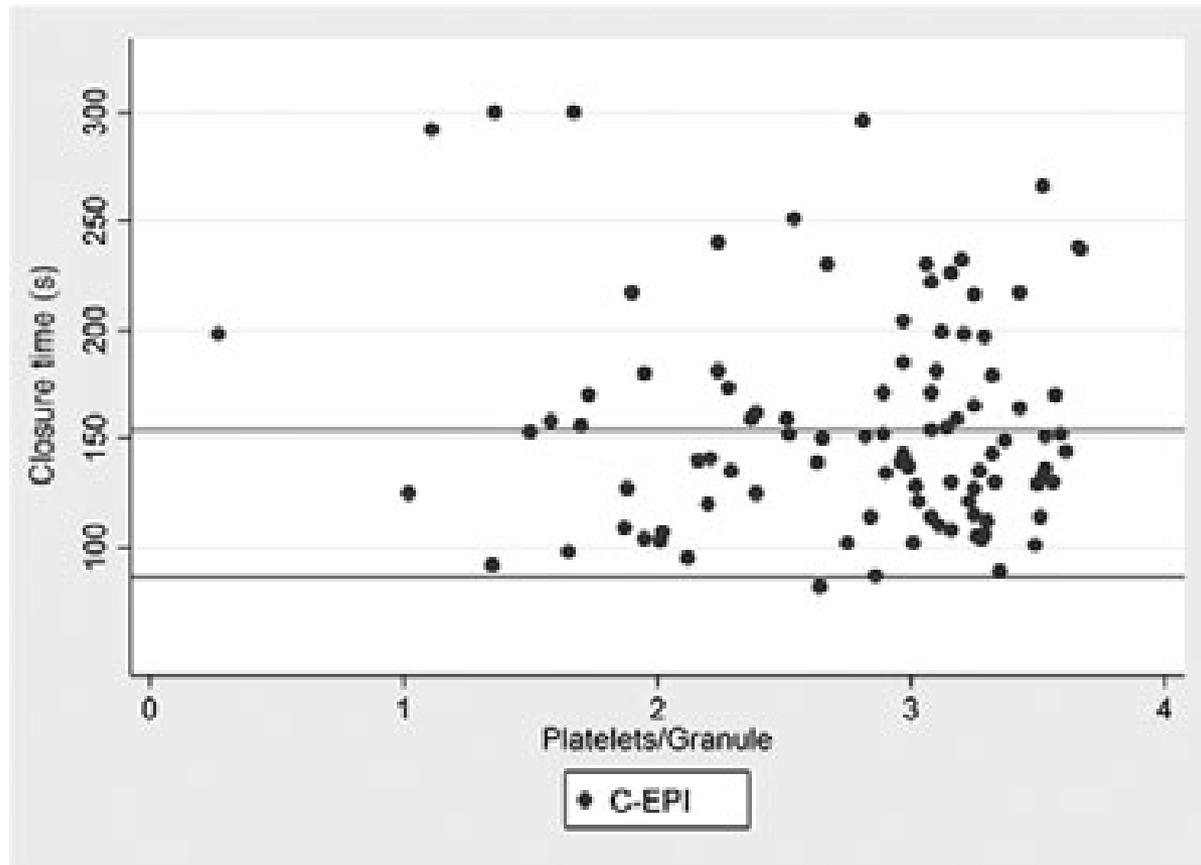
T.E.M.: Rare dense granules present.



“Empty Sack” syndrome

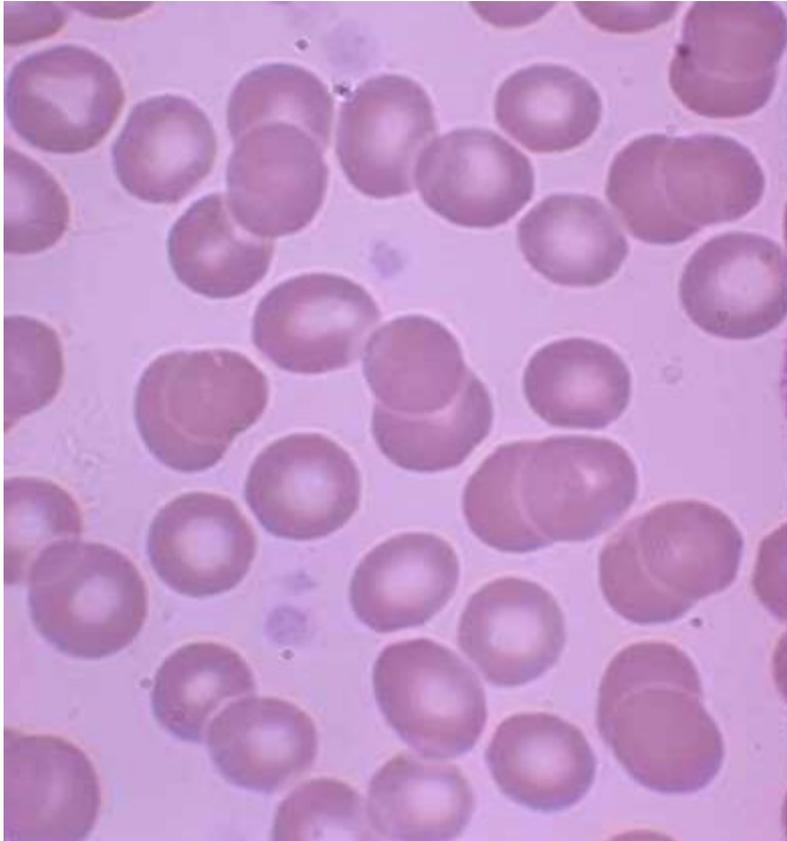
- McNichol, et al., Br. J. Haematol 1994; 86:574-82
 - Two sisters with bleeding tendency, abnormal platelet function testing
 - Dense granule deficiency by whole mount and mepacrine I.F.
 - Granulophysin (D.G. transmembrane protein) present in normal amounts
 - Conclusion: D.G. membrane present, but deficient D.G. contents
- Patient case: dense granules appeared deficient in (at least) calcium.

PFA-100 and dense granule deficiency in pediatrics



n=99

Gray Platelet syndrome



[image.bloodline.net/stories/storyReader\\$794](https://image.bloodline.net/stories/storyReader$794)

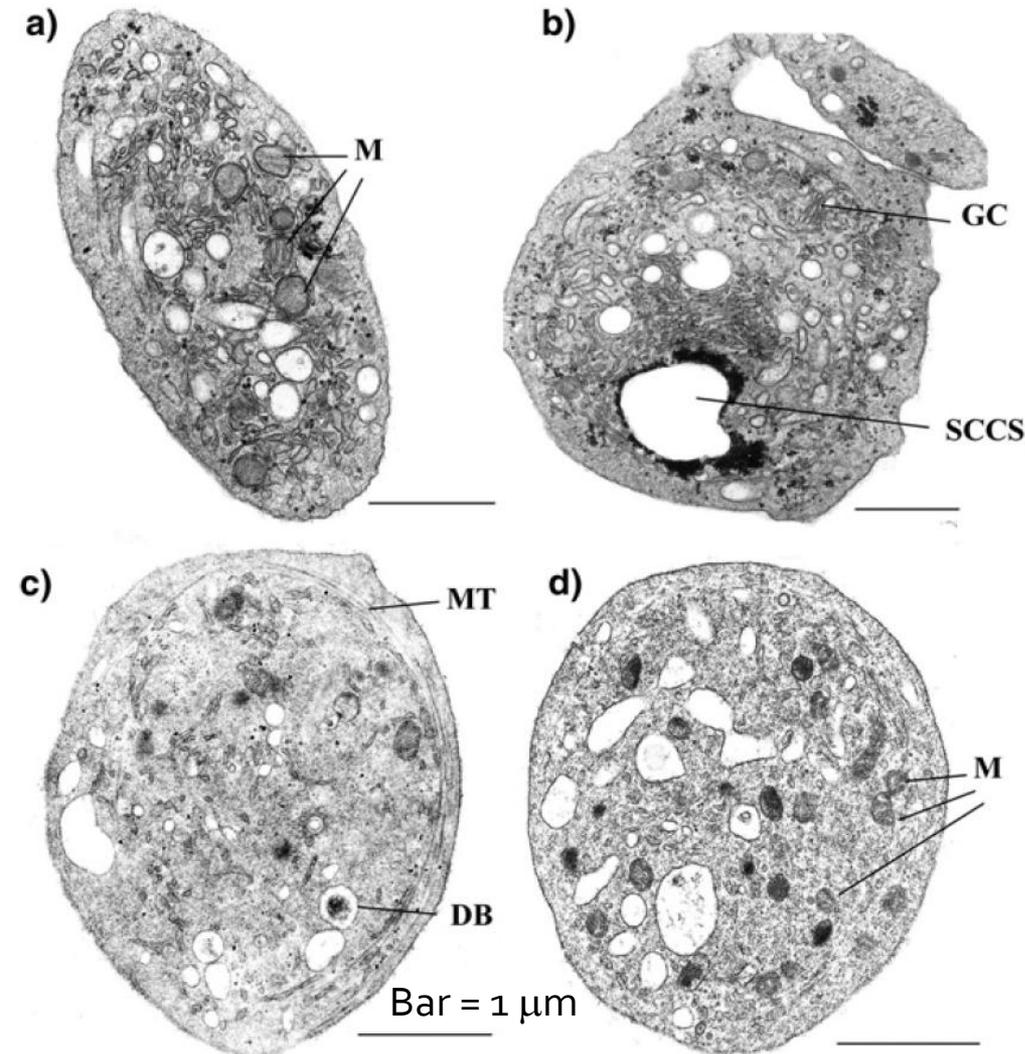
Clinical Features:

- A.k.a. α -storage pool disease
- Mild-moderate thrombocytopenia
- Myelofibrosis +/- splenomegaly
- Large, hypogranular platelets on peripheral blood smear

Pathophysiology:

- Near absence of α -granules
- Release of α -granule content to the MK and platelet cytosol
- Genetically mixed: ~50 cases reported with both AD and AR transmission

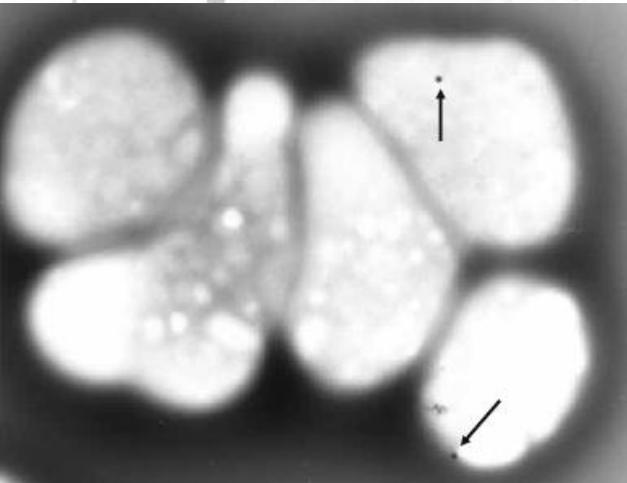
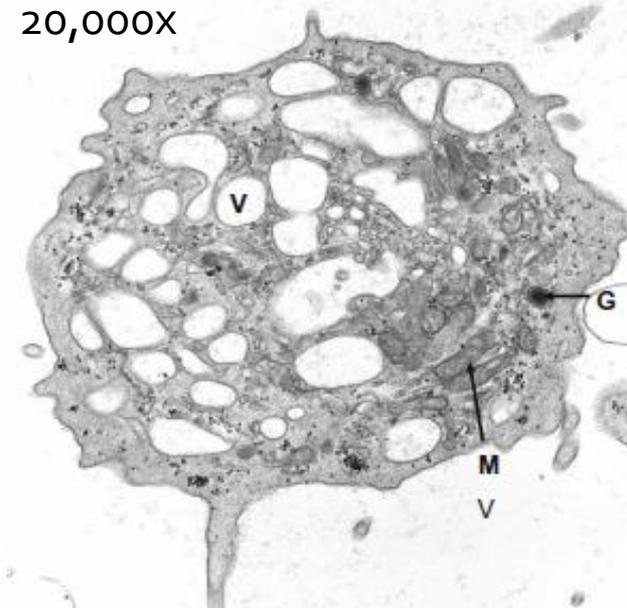
Gray Platelet syndrome



Ultrastructural features:

- Large platelets, but variability in platelet size
- Alpha granules significantly diminished in number (<15% of normal platelets)
- Fewer organelles, but dense granules and mitochondria present
- Dilated vacuoles thought to be empty alpha granules

$\alpha\delta$ -storage pool deficiency



- 3 generations of one family described
- Significant decrease in dense granules (0.01-0.05 D.G./plt in family members)
- Significant decrease in α -granules
 - Normal: 2.37 per μm^2
 - Family: 0.71-0.82 per μm^2
- May represent a different pathophysiology than other hypogranular syndromes.

Paris-Trousseau / Jacobsen Syndrome



Wenger, S., et al., *Am J Med Genetics*, 2006; 140A: 704-708

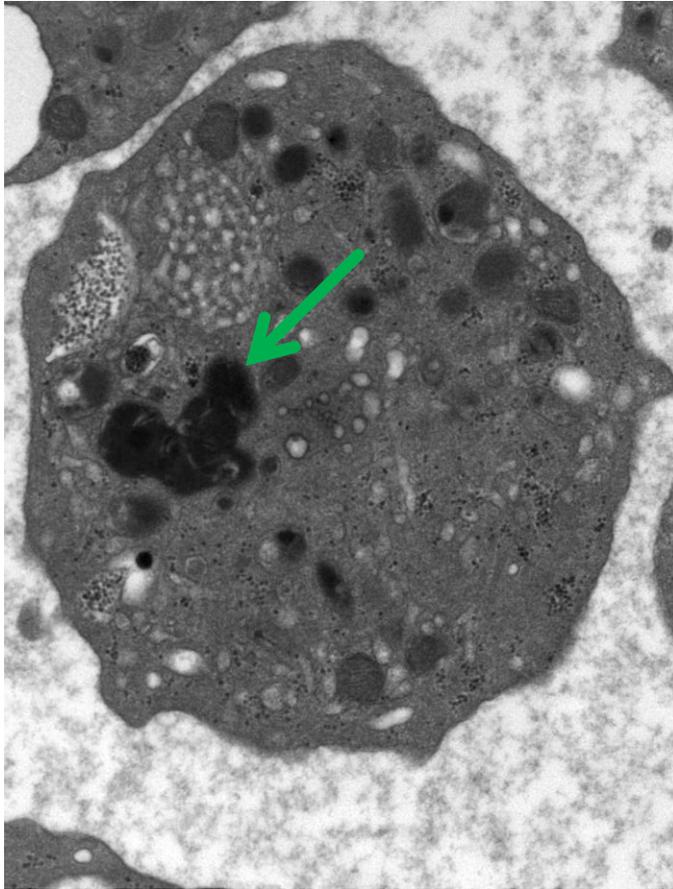
Clinical Features

- 1:100,000 live births
- Developmental delay
- Cardiac defects, dysmorphic
- Moderate macrothrombocytopenia
- Most common cause of death: cardiac and bleeding.

Pathophysiology

- Autosomal dominant 11q23 terminal chromosome deletion
- Lysis of mature megakaryocytes
- Impaired alpha granule maturation

Paris-Trousseau / Jacobsen Syndrome



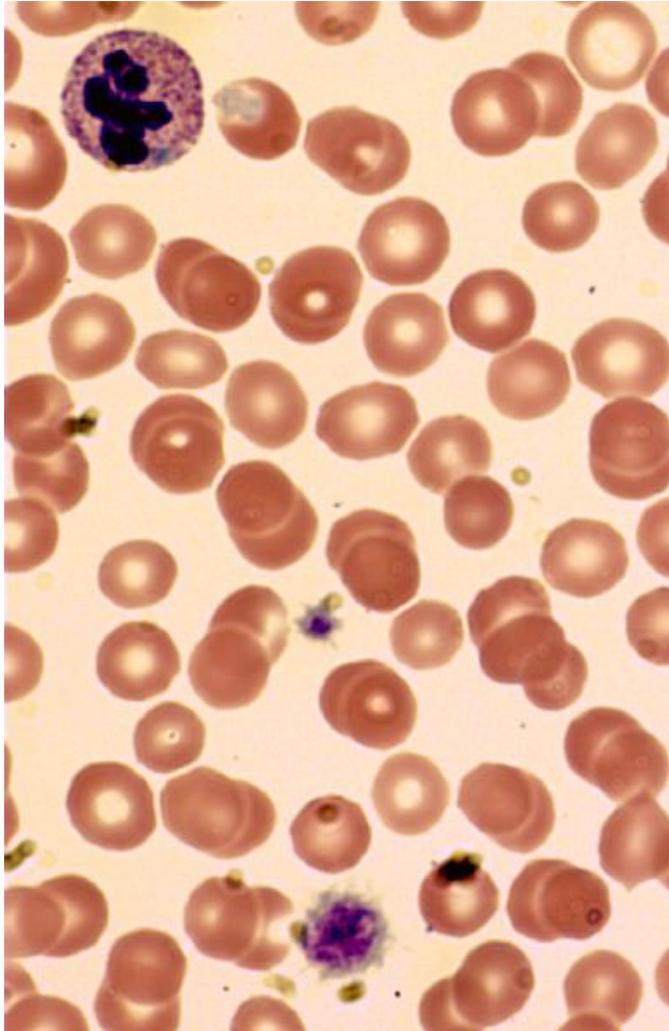
Laboratory Diagnosis:

- Macrothrombocytopenia (may correct with age)
- Increased immature megakaryocytes
- Cytogenetic analysis: 11q chromosomal deletion

Electron Microscopy:

- **Fusion of alpha granules:** giant granules (1-2 micron) in 1-15% of platelets
- Remaining ultrastructure normal

Disorders of Production: MYH9-related disorders (MYH9-RD)

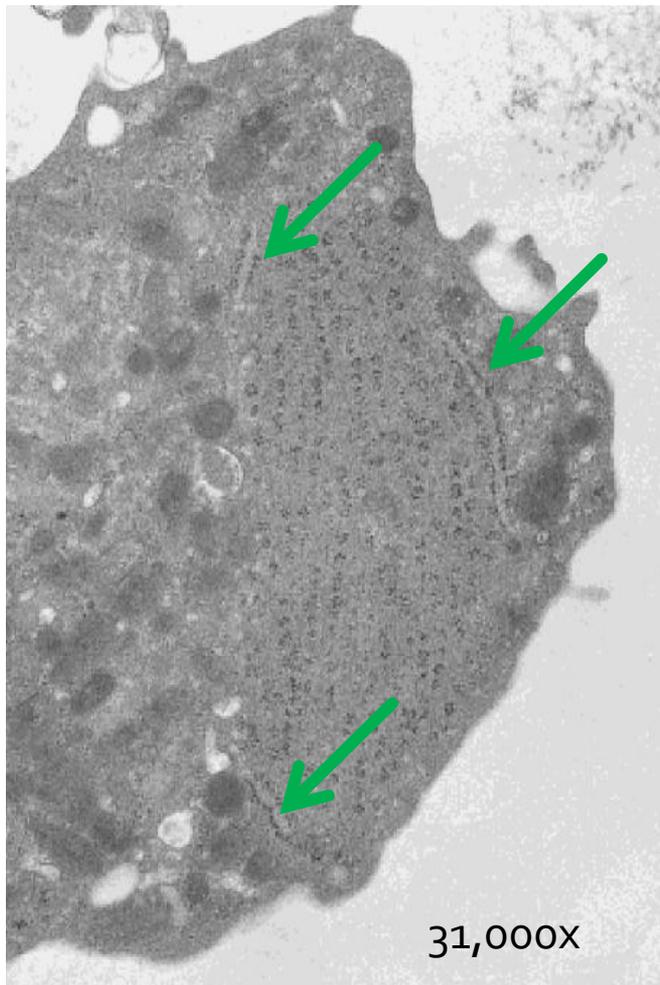


- Autosomal dominant macrothrombocytopenia
 - >20% of platelets >4 μm in diameter.
- Mutation of non-muscle myosin heavy chain type II-A (MYH9)*: expressed in platelets, kidney, leukocytes, cochlea.
- Normal megakaryocyte number, normal platelet survival \rightarrow defective production.

Disorders of Production: MYH9-related disorders (MYH9-RD)

Clinical features	May-Hegglin	Sebastian	Fechner	Epstein
Macrothrombocytopenia	Yes	Yes	Yes	Yes
Leukocyte inclusions	Yes	Yes	Yes	No
Hearing impairment	No	No	Yes	Yes
Cataracts	No	No	Yes	No
Nephritis	No	No	Yes	Yes

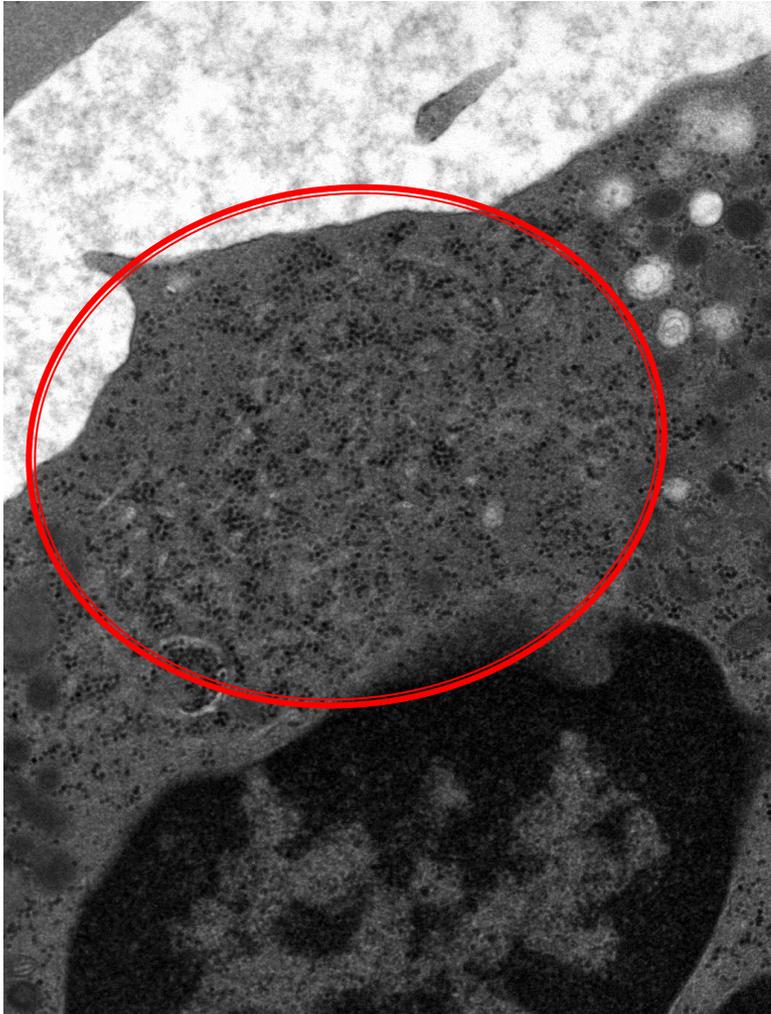
Leukocyte inclusions in MYH9 related disorders



Döhle-like bodies

- **Partially limited by fragments of rough endoplasmic reticulum**
- Fine electron-dense filaments oriented longitudinally

Leukocyte inclusion variations in MYH9 related disorders

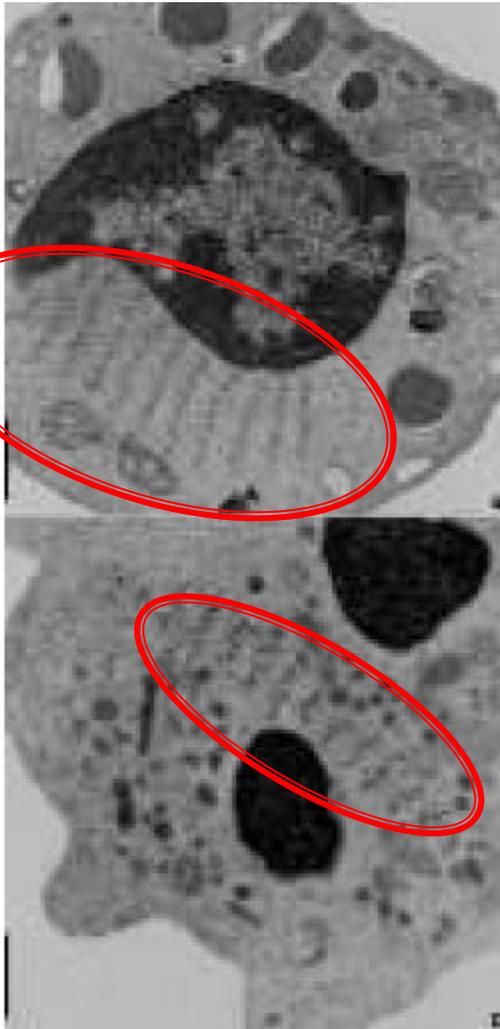


Döhle-like bodies

- Partially limited by fragments of rough endoplasmic reticulum
- Fine electron-dense filaments oriented longitudinally
- **Ribosomal rows and clusters**

Leukocyte inclusion variations in MYH9 related disorders

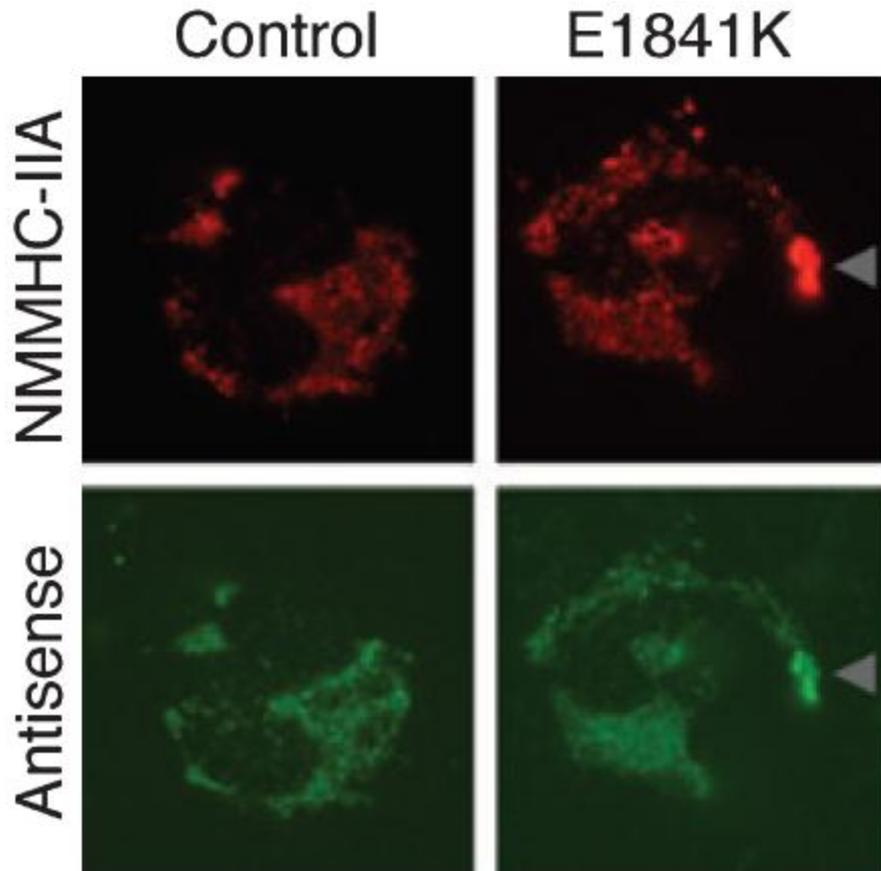
Bar =
1 μm



Döhle-like bodies

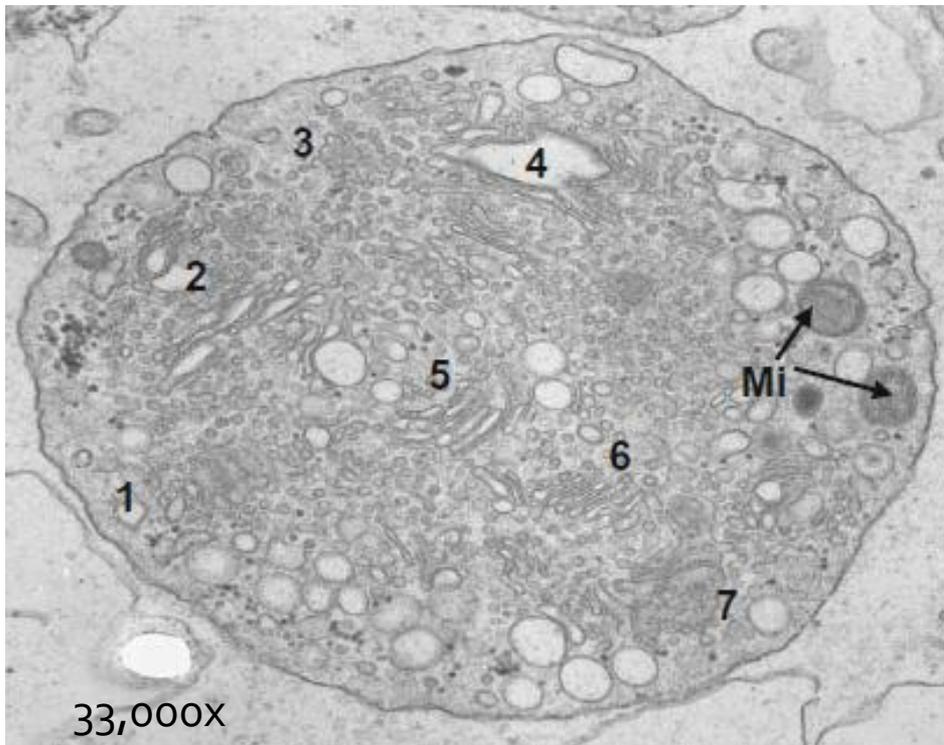
- Partially limited by fragments of rough endoplasmic reticulum
- Fine electron-dense filaments oriented longitudinally
- Ribosomal rows and clusters
- **Occasional prominent cross-striations**

MYH9 immunofluorescence



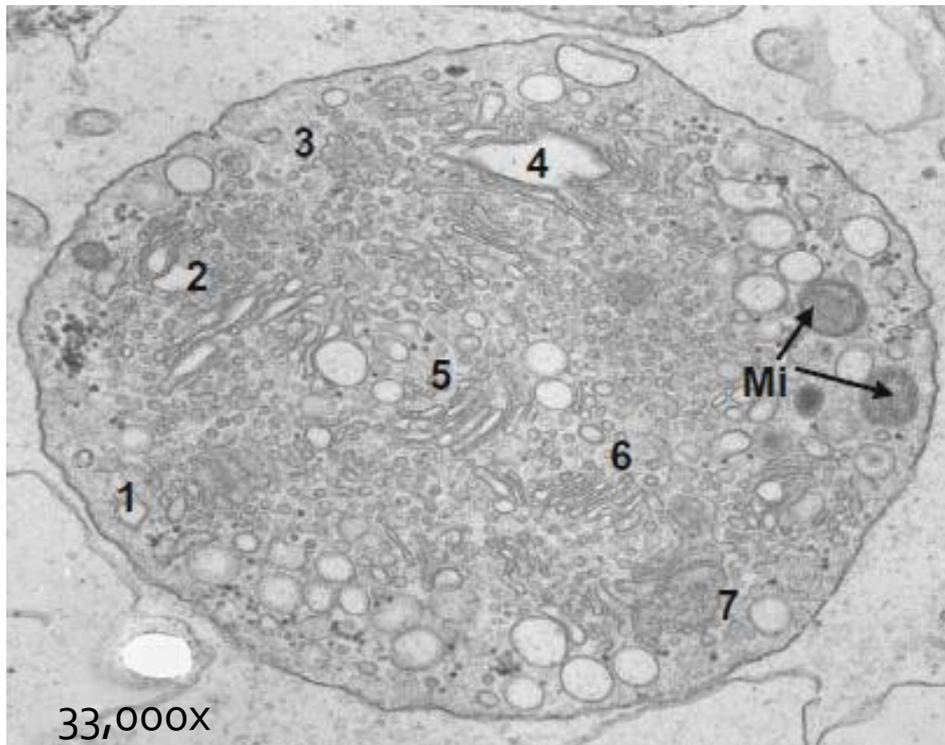
- Normal leukocytes: MYH9 evenly distributed in cytoplasm
- MYH9 aggregates present in varying size and shape
- MYH9 RNA co-localizes to aggregates
 - Sensitivity and specificity of IF is 100% and 95%, respectively (Savoia, et al., *Thromb. Haemostas.* 2010; 103:826-832)

White platelet syndrome



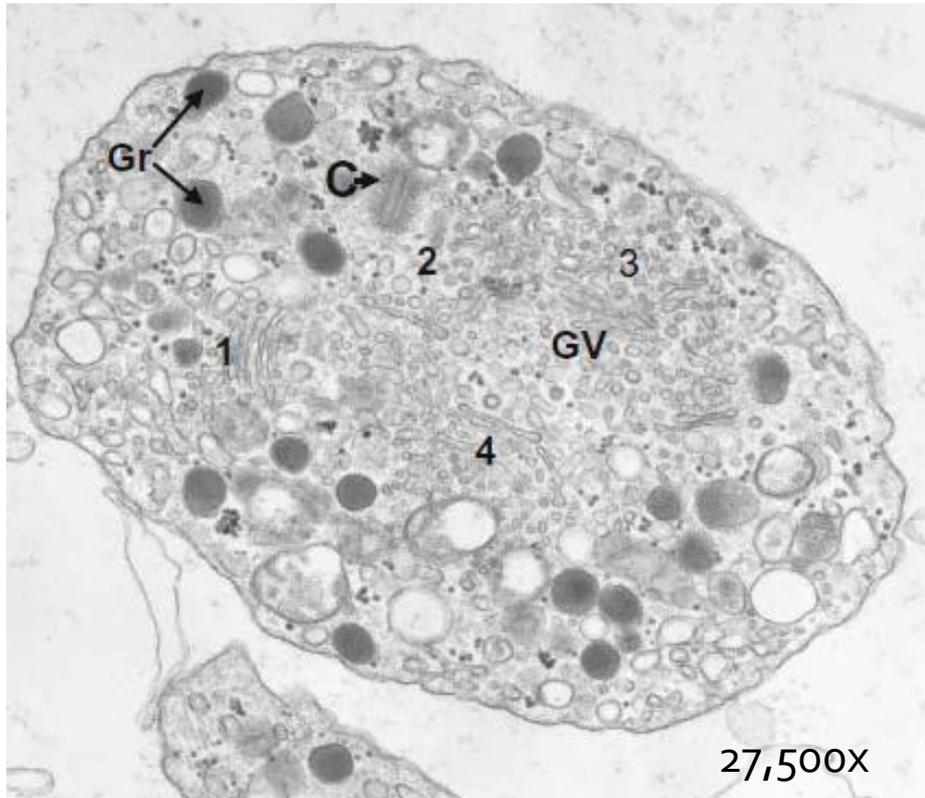
- Autosomal dominant platelet disorder described in four generations of one family (21 affected members)
- Mild macrothrombocytopenia
 - Plt count 120-150,000
 - MPV 9-11 fL

White platelet syndrome



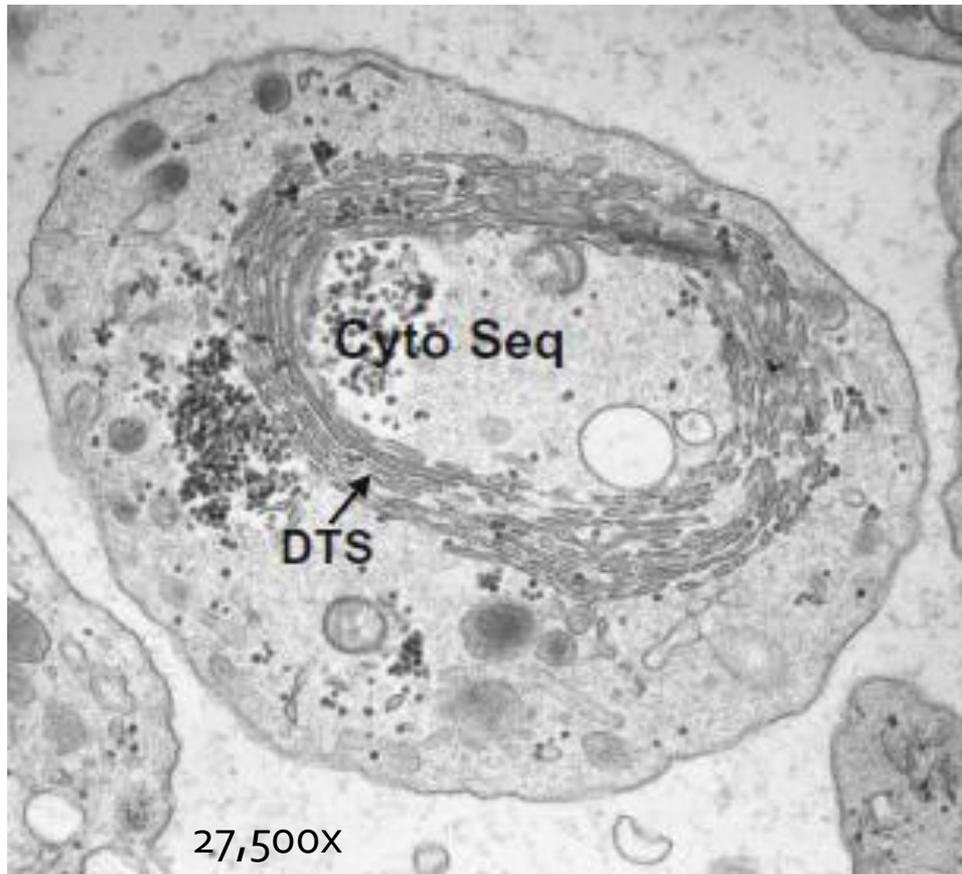
- Prominent Golgi complexes

White platelet syndrome



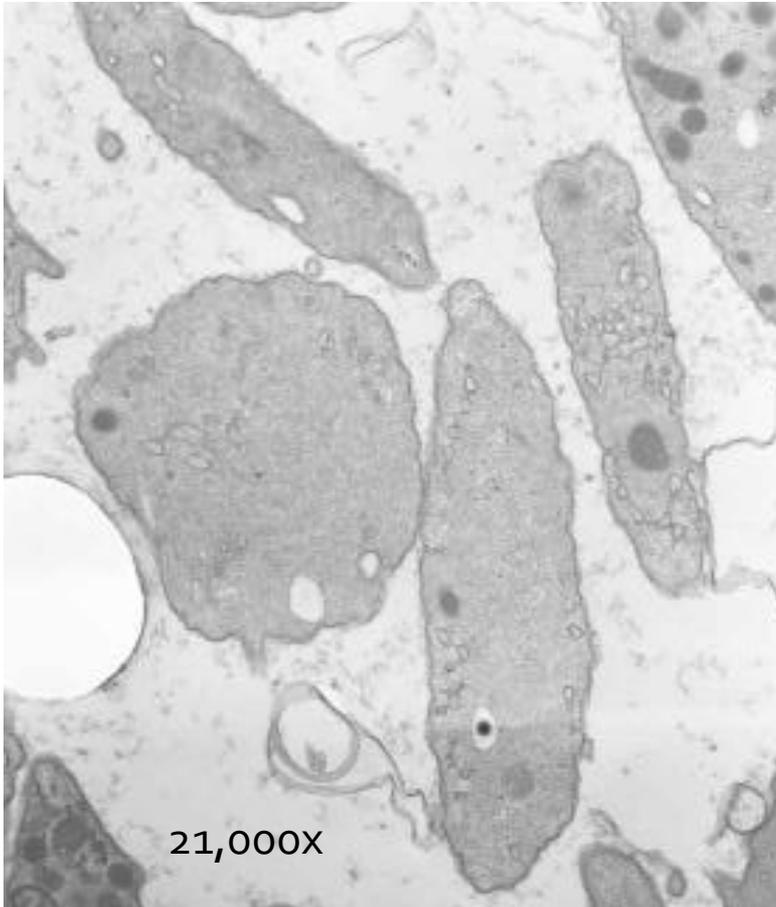
- Prominent Golgi complexes
- Occasional centrioles

White platelet syndrome



- Prominent Golgi complexes
- Occasional centrioles
- Cytosolic sequestration by abnormal dense tubular system

White platelet syndrome

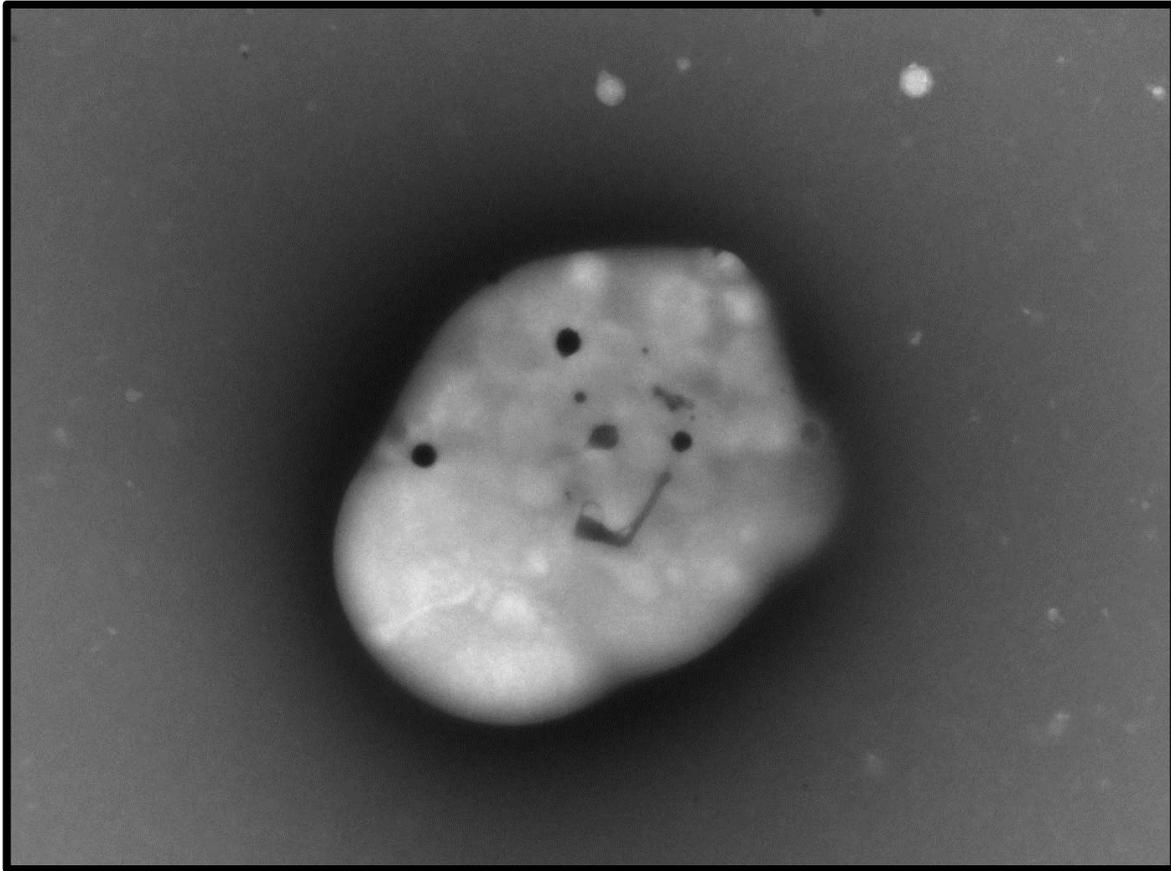


- Prominent Golgi complexes
- Occasional centrioles
- Cytosolic sequestration by abnormal dense tubular system
- Grey platelets (~30%)

White JW, *Platelets* 2004, 15:173-184

Summary

- Platelet E.M.: a valuable tool for the diagnosis of congenital platelet disorders
- Prior to E.M. analysis, review the case with hematologist
- Negative platelet function assays do not always exclude a platelet disorder which can be diagnosed by E.M.
- Careful, supervised sample collection is critical in avoiding pre-analytical errors
- Autosomal dominant platelet disorders (such as storage pool diseases or MYH9-RD) are a more common congenital platelet disorder; E.M. can play a significant role in their diagnosis



Questions?

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