? Medical mystery ?
A case report

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03-10-2017

Case report

- 34 year male
- Medical history (Peru)
  - **2014**: anemia (**Hb 7-8 g/dL**) => endoscopy: negative, R/ iron
  - April 2015: acute cholecystitis (and gallstones) => laparoscopic CCE, **Hb 8,3 g/dL** (PLT 78.000/µL and WBC 2890/mm3)
  - 2015: hemolytic anemia, R/ prednisolone
  - April 2016: splenectomy, 1 unit of PC, stable Hb
  - **May 2016: Hb 6 g/dL**, evaluation by rheumatologist: no arguments for CTD
  - August 2016: arterial hypertension, R/ Micardis
  - **September-december 2016**: Hb values between **8.3 and 9.7 g/dL**
  - February 2017: occipital left headache
  - March 2017: acute gastro-enteritis and coitis (E. Coli), sever anemia, temporary acute (prerenal) insufficiency, R/ AB, hydratation, 2 units PLT
  - **March 2017**: **Hb 10.3 g/dL**

Case report

- Laboratory and other test (Peru):
  - Hemolysis parameters: positive (eg. LHD: increased +++)
  - Protein EF: normal
  - Cryoglobulins and cryo-agglutinins: negative
  - IgM, IgA, IgG: normal
  - ANF, ANCA, SSA (anti-Ro) and SSB (anti-1a), RF: negative
  - DAT: negative
  - Hb-electrophoresis: normal
  - Glucose-6-phosphatase: normal level
  - Osmotic fragility: decrease of osmotic fragility curve
  - HAM-test: negative
  - Flow PNH: negative
  - Gastroscopy: negative, no HP
  - APO spleen: congestion of red pulp

Case report

- 2nd opinion UZL (July 2017): anamnesis
  - Continuously tired (‘used to it because it lasts since 3 years’), fatigue ++
  - Continuous icterus
  - Dark urine
  - No abdominal or thoracal pain
  - No increased frequency of infections (exception early 2017)
  - No thrombosis in past

Case report

- 2nd opinion UZL: laboratory test
  - **Hematological parameters**
    - Hb: 8.1 g/dL ↓
    - Hematocriet: 0.266 (0.400 - 0.540) ↓
    - RBC: 3.06 10^{12}/L (4.50 - 6.00) ↓
      - MCV: 86.9 fL (76.0 - 96.0)
      - MCH: 26.5 pg (27.0 - 32.0) ↓
      - MCHC: 30.5 dL (30.0 - 35.0)
      - RDW: 20.9% (11.7 - 14.5) ↑
    - Reticulocyten telling: 297 10^{9}/L (20 – 100) ↑
      - Immature reticulocyten fractie: 42.9% (5.0 - 21.0) ↑
    - Erytroblasten telling: 0.27 10^{9}/L (0.00 - 0.07) ↑

Hemolytic anemia

Case report

- 2nd opinion UZL: laboratory test
  - **Hematological parameters**
    - Hb: 8.1 g/dL ↓
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    - Erytroblasten telling: 0.27 10^{9}/L (0.00 - 0.07) ↑
  - **Cytology PB**
    - RBC:
      - Hypochromia ++, no target cells
      - No sferocytosis
      - Polychromasia, erytroblasts
    - nl WBC, ↑ PLT
  - **Biochemical parameters:**
    - LHD: 2041 U/L (135 – 250) ↑
    - Haptoglobin: <0.10 g/L (0.30 - 2.00) ↓
    - Bilirubine total: 6 .33 mg/dL (≤ 1.18) ↑
    - Bilirubine direct: 0 .66 mg/dL (≤ 0.50) ↑
    - Iron: 23 µg/dL (65 – 175) ↓
    - Transferrine: 2 .79 g/L (2.00 - 3.60)
    - Transferrine saturation: 6 % (16 – 45) ↓
    - Ferritine: 42 µg/L (30 – 400) ↓
    - icteric sample
  - **DAT**: negative
  - **Urine analysis**:
    - RBC heme +
    - urobilinogen ++

Case report

- 2nd opinion UZL: Laboratory test
  - **Diagnostiek RBC enzymes**
    - Glucose-6-phosphatase: geen deficiëntie
    - Pyruvate kinase: geen deficiëntie
  - **Diagnostiek RBC membraanpathologie**
    - **Osmotische resistentie**: verhoogde osmotische resistentie, compatibel met ijzergebrek, thalassemie of verhoogde reticulocytose.

Case report

- 2nd opinion UZL: Laboratory test
  - **Diagnostiek RBC membraanpathologie** *(Uitgevoerd door LHUB-ULB Site Anderlecht (Hopital Erasme))*
    - Cryohemolyse niet uitgevoerd
    - EMA 2.0% (refw: < 19.0%)
    - Ektacytometrie Het profiel is abnormaal maar niet typisch voor erfelijke sferocytose.
    - Elektroforese niet uitgevoerd

  ⇒ BESLUIT: Screeningstests hebben de aanwezigheid van erfelijke sferocytose NIET aangetoond.

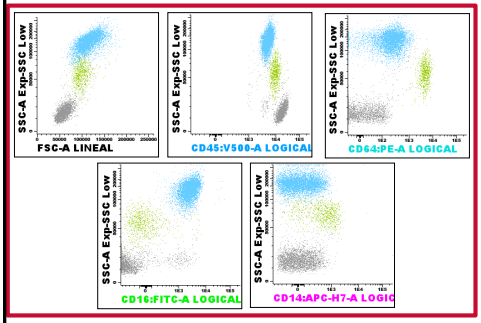
Case report

- 2nd opinion UZL: Labora
  - **Flow**:
    - PN

Mystery solved: PNH

Case report

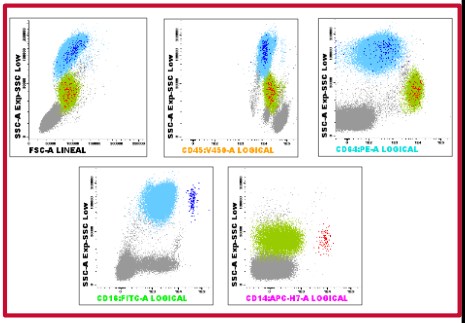
PNH results (Peru, 01/06/2016)



CONCLUSION:
- No observa expresión bimodal de los antígenos CD14 y CD16.
- Se observa déficit de expresión de CD14 en población monocítica.

No compatible con HPN.

PNH results (Peru, 08/08/2017)

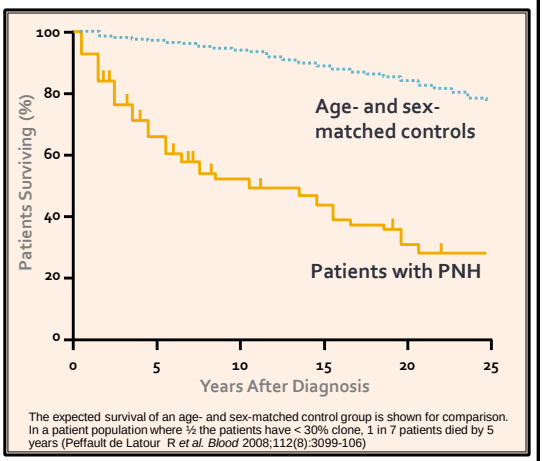


CONCLUSION:
- Se observa déficit de expresión de CD16 en población granulocitica.
- Se observa déficit de expresión de CD14 en población monocítica.

Compatible con HPN.

PNH: epidemiology

- uncommon (incidence of 1 per 10^5-10^6)
- 5 years mortality: ~35%
- diagnosed at all ages
- median age: early 30s
- affects men and women equally
- progressive disease

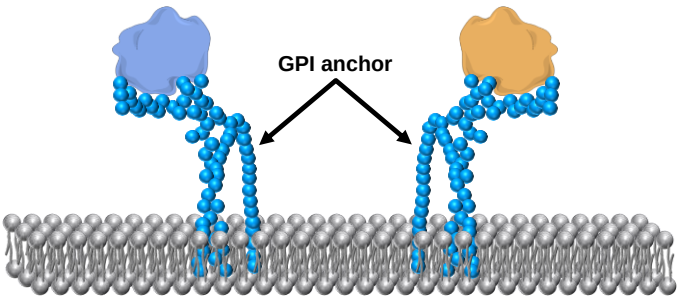


The expected survival of an age- and sex-matched control group is shown for comparison. In a patient population where ½ the patients have < 30% clone, 1 in 7 patients died by 5 years (Peffault de Latour R *et al. Blood* 2008;112(8):3099-106)

1. Hill A *et al. Blood* 2006;108: abstract 985. 2. Hillmen P *et al. NEJM* 1995;333:1253-58. 3. Socié G *et al. Lancet* 1996;348:573-7. 4. Hill A *Br J Haematol* 2007;137:181-92. 5. Lee JW *et al.* EHA 2010, abstract 506.

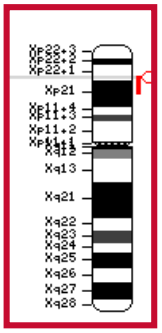
The defect in PNH

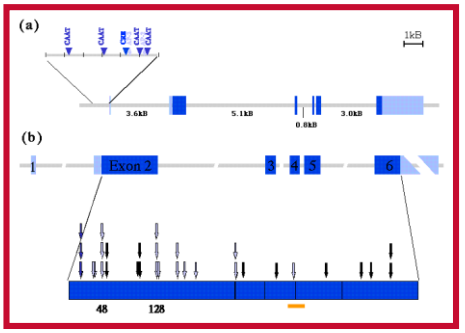
- an acquired hemolytic disorder
- somatic mutation of the *PIG-A* gene
- no binding of GPI-anchored proteins to the cell surface

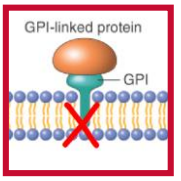


GPI, glycosylphosphatidylinositol;
PIG-A, phosphatidylinositol glycan class A;

Molecular genetic lesion: PIG-A

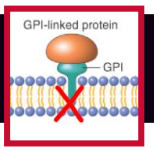






- Mutations throughout entire **p**hosphatidyl**i**nositol **g**lycan complementation class A **(PIG-A)** gene at Xp22.13 (no hot spot)
- ~100 mutations - frameshifts (most common), small deletions/insertions
  - Mostly: complete loss of function/protein (glycosylphosphatidylinositol (GPI) anchor)
  - Rare: protein is still partially functional

GPI anchored proteins



Hematopoietic Stem Cell

CD59, CD109, CD90

RBC
CD55
CD58
CD59
PrPc
AChE
JMH Ag
Dombroch
HG Ag

Platelets
CD55
CD58
CD59
CD109
PrPc
GP500
Gova/b

B Cells
CD24 CD55
CD58 CD59
CD48 PrPc
CD73 CD108

T Cells
CD55 CD58
CD59 CD48
CD52 CD87
CD108 PrPc
ADP-RT CD73
CD90 CD109
CD16

NK Cells
CD55
CD58
CD59
CD48
CD52
PrPc
CD16

Monocytes
CD14 CD55
CD58 CD59
CD48 CD52
CD87 CD109
CD157 Group-8
PrPc GPI-80
CD16

PMN
CD55 CD58
CD59 CD14
CD16 CD24
CD48 CD66b
CD66c CD87
CD109 CD157
LAPNB1 PrPc
p50-80 GPI-80
ADP-RT NA1/NA2

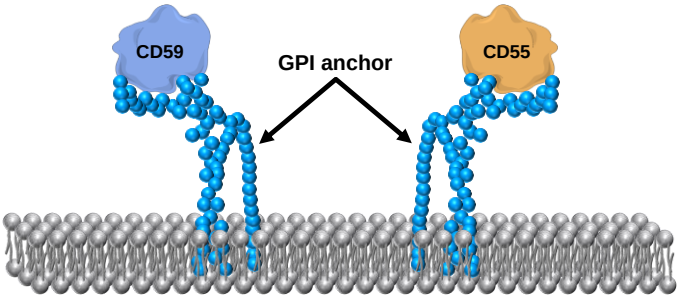
The defect in PNH

CD59

- Forms a defensive shield for RBCs from complement-mediated lysis
- Inhibits the assembly of the membrane attack complex

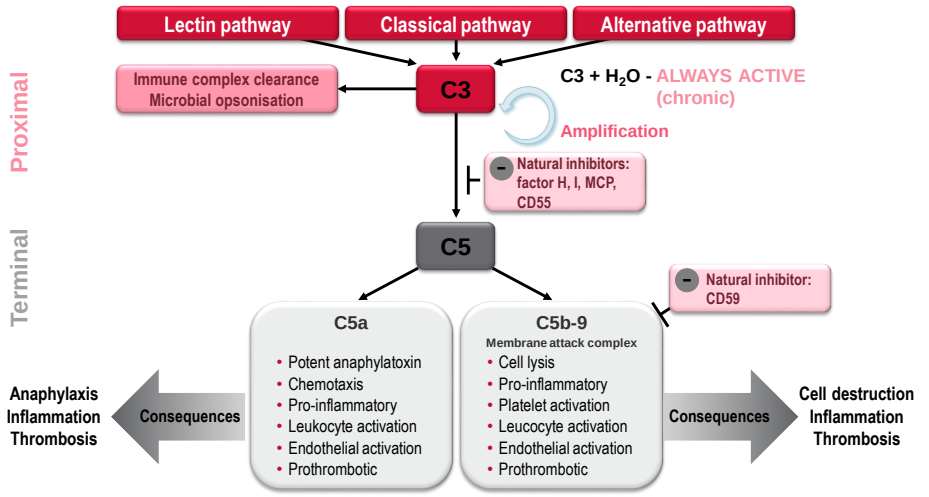
CD55

- Prevents formation and augments instability of the C3 convertases, attenuating the complement cascade



GPI, glycosylphosphatidylinositol;
PIG-A, phosphatidylinositol glycan class A;
RBC, red blood cell

PNH: chronic, uncontrolled complement activation

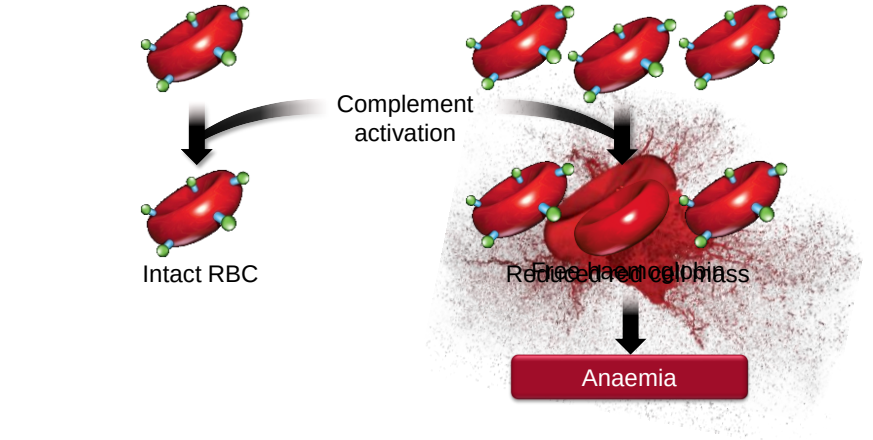


MCP, membrane cofactor protein

PNH: haemolytic anaemia

Normal RBCs are protected from complement attack by a shield of terminal complement inhibitors

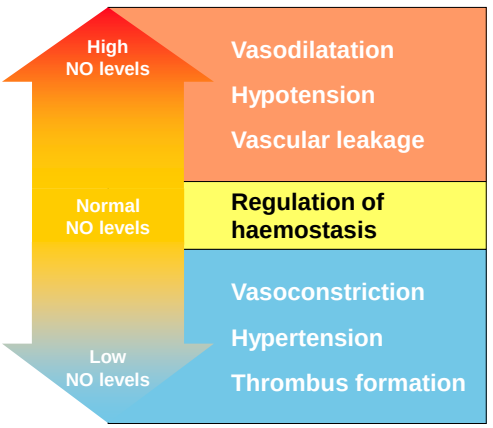
Without this protective complement inhibitor shield, PNH RBCs are destroyed



Hemolysis and Nitric Oxide

- RBC hemolysis => cell-free Hb => depletes nitric oxide (NO)
- NO depletion => smooth muscle dysfunction
  - vasoconstriction => pulmonary and systemic hypertension, erectile failure
  - gastro-intestinal contractions => abdominal pain, dysphagia, severe lethargy
- NO depletion => thrombotic events
  - Platelet activation and aggregation (hypercoagulability-platelet hyperreactivity)

The crucial role of NO

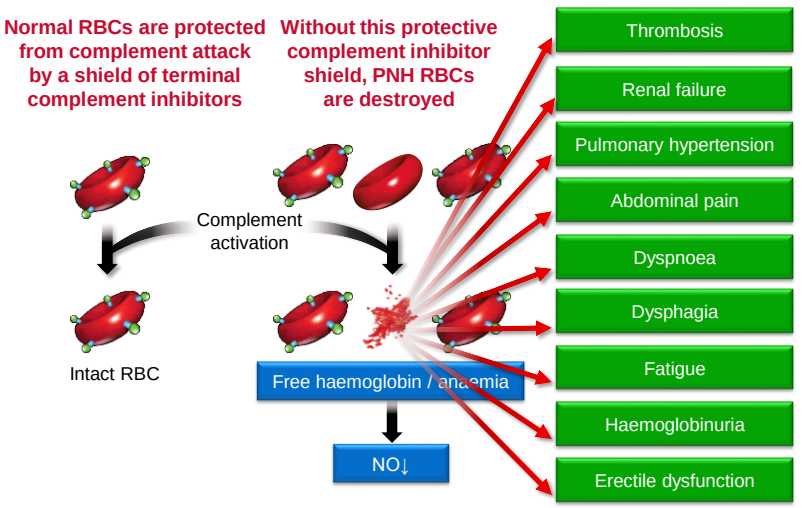


→ **Maintained smooth muscle tone**
→ **Controlled platelet activity**

→ **Impaired regulation of smooth muscle tone**
→ **Platelet activation and aggregation**

Rother RP et al. JAMA 2005;293:1653-62

Consequences of chronic complement activation



PNH: thrombosis

- All patients with PNH are at risk for thrombosis
- Occurs in typical and atypical sites
  - Hepatic veins (Budd–Chiari syndrome)
  - Other intra-abdominal veins (portal, splenic, splanchnic)
  - Cerebral sinuses (CVA, TIA)
  - Dermal veins
- 40-67% of deaths are due to thrombosis (first thrombotic event can be fatal)

Hillmen P et al. Blood 2007;110:4123-8; udebert HJ et al. J Neurol 2005;252:1379-86; Lee JW et al. Haematologica 2010;95(suppl 2):abs 506

Clinical indications for PNH testing

Intravascular haemolysis as evidenced by haemoglobinuria or elevated plasma haemoglobin

- Evidence of unexplained haemolysis with accompanying:
  - iron-deficiency, or
  - abdominal pain or oesophageal spasm, or
  - thrombosis (see below), or
  - granulocytopenia and / or thrombocytopenia
- Other acquired Coombs-negative, non-schistocytic, non-infectious haemolytic anaemia

Thrombosis with unusual features

- Unusual sites:
  - Hepatic veins (Budd–Chiari syndrome)
  - Other intra-abdominal veins (portal, splenic, splanchnic)
  - Cerebral sinuses
  - Dermal veins
- With signs of accompanying haemolytic anaemia
- With unexplained cytopenia

Evidence of bone marrow failure

- Suspected or proven aplastic or hypoplastic anaemia
- Refractory cytopenia with unilineage dysplasia (MDS)
- Other cytopenias of unknown aetiology after adequate work-up

Clinical indications for PNH testing

Table 1 Reasons for first-time PNH testing in 323 unique patients

Reason	*N* = 323 (% of total)
Hypoplastic/aplastic bone marrow	32 (10%)
Hemolytic anemia	82 (25%)
DAT negative	42 (13%)
DAT positive	30 (9.2%)
DAT not performed	10 (3.0%)
MDS	21 (7%)
Cytopenia	60 (19%)
Cytopenia with hemolysis	5 (1.5%)
Unexplained cytopenia	55 (17%)
Thrombosis	59 (18%)
Venous	51 (16%)
Arterial	6 (1.9%)
Mixed arterial/venous	2 (0.62%)
Other clinical reasons	13 (4%)
No clinical info	56 (17%)

DAT, Direct antiglobulin test; MDS, myelodysplastic syndrome.

Table 2. Reasons for PNH testing in 25 PNH patients. Distribution of positive first-time tests, and relative risk per reason for screening is displayed for 267 unique patients with sufficient clinical information

Reason	*N* = 25 (% of PNH patients)	Positive tests/ screening group (%)
Hypoplastic/aplastic bone marrow	15 (60%)	15/32 (47%)
Hemolytic anemia	6 (24%)	6/82 (7%)
DAT negative	4 (16%)	4/42 (10%)
DAT positive	1 (4%)	1/30 (3%)
DAT not performed	1 (4%)	1/10 (10%)
MDS	1 (4%)	1/21 (5%)
Cytopenia	2 (8%)	2/60 (3%)
Cytopenia with hemolysis	1 (4%)	1/5 (20%)
Unexplained cytopenia	1 (4%)	1/55 (2%)
Thrombosis	1 (4%)	1/59 (2%)
Venous	1 (4%)	1/51 (2%)
Arterial	0 (0%)	0/6 (0%)
Mixed arterial/venous	0 (0%)	0/2 (0%)
Other	0 (0%)	0/13 (0%)
All reasons (overall)	25 (100%)	25/267 (9%)

MDS, myelodysplastic syndrome; DAT, direct antiglobulin test.

Mercier A, *et al.* IJLH, 2017, 3:329-36.

Classification of clinical PNH

Category	Rate of vascular hemolysis	BM
Classic PNH	Florid (LDH, often episodic macroscopic hemoglobinuria)	Cellular BM with erythroid hyperplasia, +/-normal morphology
PNH in BM failure disorder	Mild (often minimal abnormal biochemical markers of hemolysis)	Evidence of concomitant BM failure syndrome (AA or low-risk MDS)
Subclinical PNH	No clinical or biochemical signs of hemolysis	Evidence of concomitant BM failure syndrome (AA or low-risk MDS)

Parker CJ., Hematology ASH Education Book, 2011:21-9.

Table 3. Clinical PNH classification

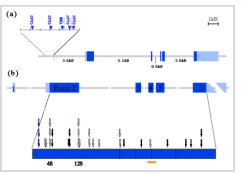
Classification	*N* = 25 (% of total patients)
Classical PNH	6 (24%)
PNH in bone marrow disorders	6 (24%)
Subclinical PNH	13 (52%)

PNH, Paroxysmal nocturnal hemoglo

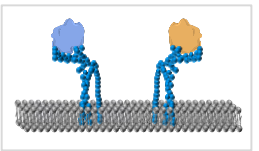
Mercier A, *et al.* IJLH, 2017, 3:329-36.

PNH diagnosis: What can we test?

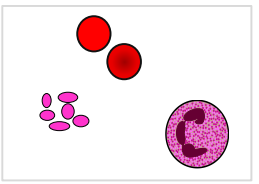
- Defects at DNA level (PIG-A mutations)



- Missing surface molecules



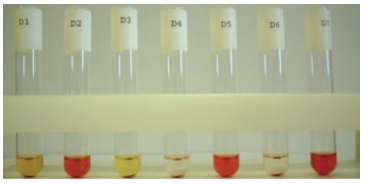
- Effect of the missing molecules on function of the various cell populations



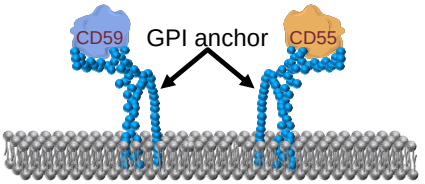
PNH testing: options

☒ **Molecular:** sequencing of *PIG-A* gene for mutations. Not applicable to routine testing as many mutations without clear association to PNH subtype and its severity

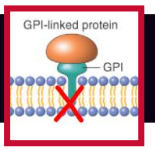
☒ **Ham's test:** based on the differential susceptibility of PNH RBCs to complement-mediated haemolysis. But time-consuming, with lack of standardisation and restricted sensitivity



☑ **Flow cytometry:** directly demonstrates the disease phenotype (GPI-linked antigen deficiency)



GPI anchored proteins



Hematopoietic Stem Cell

CD59, CD109, CD90

RBC: CD55, CD58, CD59, PrPc, AChE, JMH Ag, Dombroch, HG Ag

Platelets: CD55, CD58, CD59, CD109, PrPc, GP500, Gova/b

B Cells: CD24, CD55, CD58, CD59, CD48, PrPc, CD73, CD108

T Cells: CD55, CD58, CD59, CD48, CD52, CD87, CD108, PrPc, ADP-RT, CD73, CD90, CD109, CD16

NK Cells: CD55, CD58, CD59, CD48, CD52, PrPc, CD16

Monocytes: CD14, CD55, CD58, CD59, CD48, CD52, CD87, CD109, CD157, Group-8, PrPc, GPI-80, CD16

PMN: CD55, CD58, CD59, CD14, CD16, CD24, CD48, CD66b, CD66c, CD87, CD109, CD157, LAPNB1, PrPc, p50-80, GPI-80, ADP-RT, NA1/NA2

Considerations for flow cytometric PNH testing

Sample source	PB (BM is not optimal)
Anticoagulant	EDTA (preferred), heparine or ACD
Sample volume	Minimum 1 ml; 3 ml is adequate for most testing, though more might be needed if WBC is very low
Maximum sample age	Up to 7 days for RBC; <48h for WBC
High-sensitivity analysis	0,01%; at least 250,000 events of specific cell type collected

"Guidelines for diagnosis and monitoring of PNH and related disorders by flow cytometry". M. Borowitz *et al.*, Cytometry Part B (clinical cytometry), 2010

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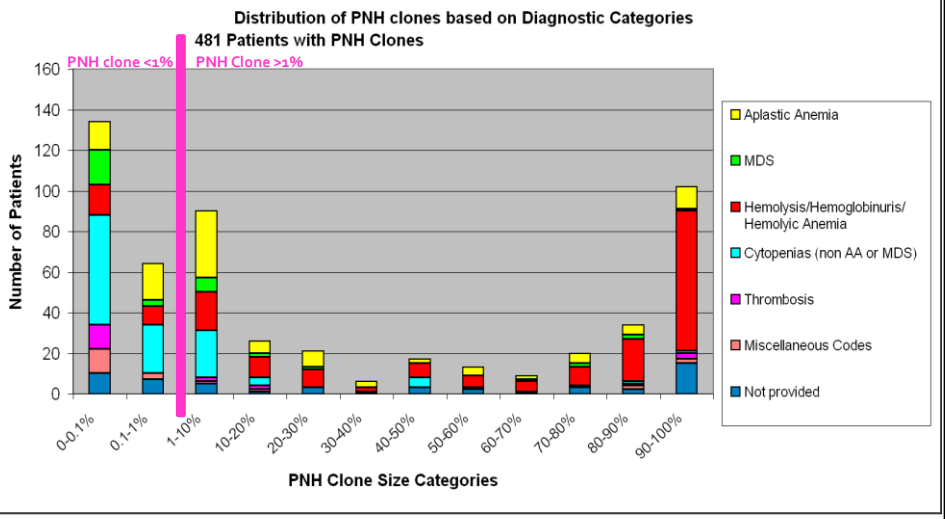
Distribution of PNH WBC clone sizes



M. Movalia, AJ. Illingworth, ESCCA 2012 abstract POS-IM-09, ESCCA course 2012.

Follow-up of PNH+ patients

113 patients with PNH clone

	<0.1%	0.1-1%	>1.0-10%	>10-100%
Increased	0	2 (16%)	9 (38%)	0
Decreased	0	2 (16%)	5 (21%)	1
No Change	33	8 (67%)	10 (41%)	43
Totals	33 (29%)	12 (11%)	24 (21%)	44 (39%)
Monitoring recommendations	6-12 m	3-6 m	3-6 m	as indicated

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⇒ Follow-up testing in patients with PNH clone sizes of 0,1-10% should be performed

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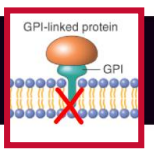
Considerations for flow cytometric PNH testing

Sample source	PB (BM is not optimal)
Anticoagulant	EDTA (preferred), heparine or ACD
Sample volume	Minimum 1 ml; 3 ml is adequate for most testing, though more might be needed if WBC is very low
Maximum sample age	Up to 7 days for RBC; <48h for WBC
Routine analysis	1%; at least 5,000 events of specific cell type collected
High-sensitivity analysis	0,01%; at least 250,000 events of specific cell type collected
Cell populations analyzed	

GPI anchored proteins



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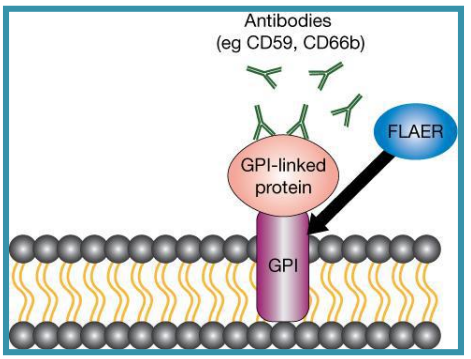
Cell populations analyzed	**Granulocytes** in all cases. **Monocytes** provide confirmatory information. No role for analysis of lymphocytes due to long life span. **RBC** in at least those cases with a WBC PNH clone detected by WBC analysis (or in all cases). RBC's alone is not recommended.

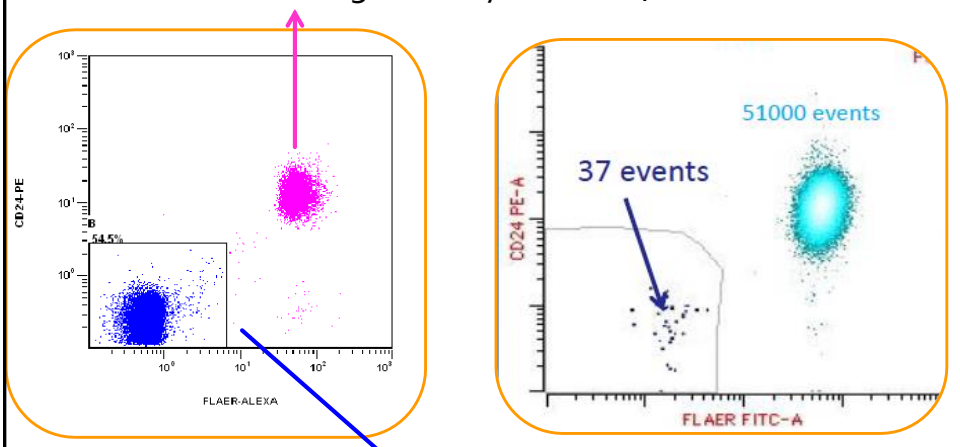
Detection of PNH clones in WBC

- **GPI-AP**
  - CD24 (granulocytes)
  - CD14 (monocytes)
- **FLAER = fluorescent aerolysin**
  - Fluorochrome-conjugated inactive variant of the bacterial derived protein aerolysin (52-kDA protein secreted by Aeromonas Hydrophilia)
  - Aerolysin is a molecule that directly binds to the GPI anchors on leucocytes (**not erythrocytes**)

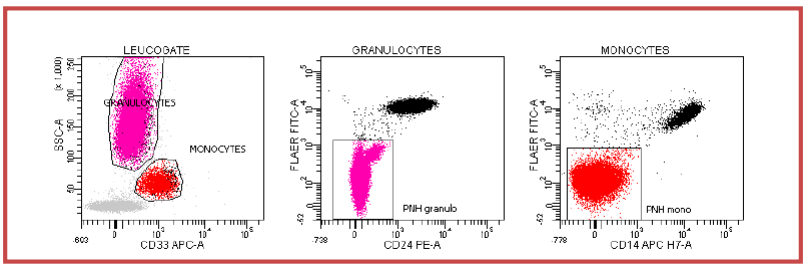


Detection of PNH clones in WBC

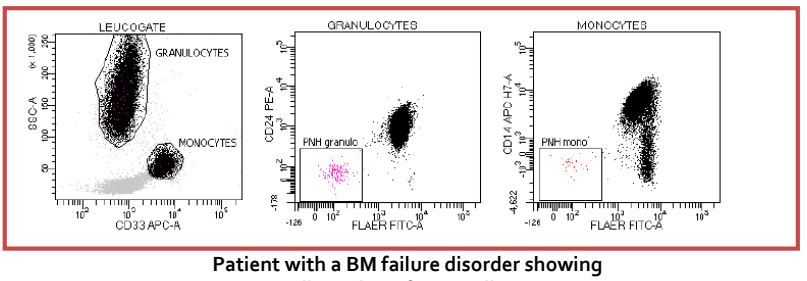
Normal granulocytes (CD24+/FLAER+)



PNH granulocytes (CD24-/FLAER-)



Patient with classic PNH showing a large clone (PNH granulocytes (86%) and PNH monocytes (83%))



Patient with a BM failure disorder showing a small number of PNH cells (0,3%)

Classification of clinical PNH

Category	Rate of vascular hemolysis	BM	Flow cytometry
Classic PNH			**Large population (>50%)** GPI-anchor deficient PMN
PNH in BM failure disorder			% of GPI-anchor deficient PMNs is usually **relatively small (<10%)**
Subclinical PNH			**Small (<1%) population** of GPI-anchor deficient PMN

Table 3. Clinical PNH classification at diagnosis and granulocyte clone sizes

Classification	*N* = 25 (% of total patients)	PNH clone size % Mean	Median	Range
Classical PNH	6 (24%)	55	65	1.7–95
PNH in bone marrow disorders	6 (24%)	44	34	0.3–98
Subclinical PNH	13 (52%)	12	3.2	<0.1–62

PNH, Paroxysmal nocturnal hemoglobinuria.

Mercier A, *et al.* IJLH, 2017, 3:329-36.

Parker CJ., Hematology ASH Education Book, 2011:21-9.

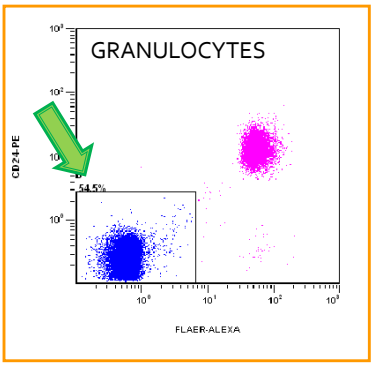
Types of PNH cells (RBC)

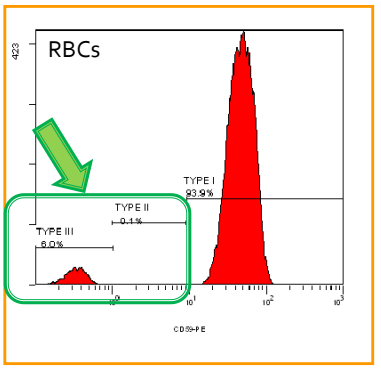
- **Type I cells** => normal cells
  - Life span RBC: 120 days
- **Type II cells** => partial deficiency of GPI-anchor
  - Life span RBC: ~45 days
- **Type III cells** => complete deficiency of GPI-anchor
  - Life span RBC: <20 days (10-15 days)

⇒ total PNH clone: type II and type III cells

Detection of PNH clones in RBC (1)

- RBC clone size often underestimated because of transfusion or hemolysis

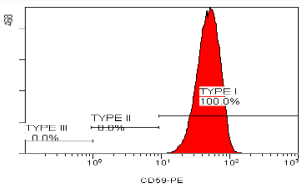




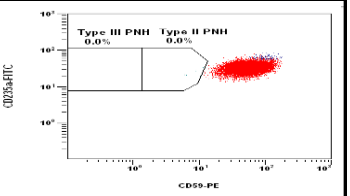
Detection of PNH clones in RBC (2)

- RBC clone size often underestimated because of transfusion or hemolysis
- Significant RBC clones are never seen without WBC clones
- CD59, CD55:
  - CD55 less abundant expressed: not recommended as a sole reagent
  - Rare cases of congenital CD55 or CD59 deficiency are reported
    - CD55 deficiency : no hemolysis
    - CD59 deficiency: clinic cfr. PNH with hemolysis and trombosis
  - Addition of CD235a
- RBC testing: best way to identify Type II cells

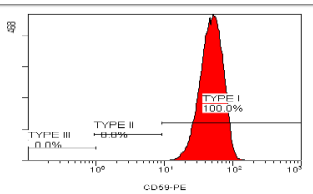
CD59 expression – Type I, II , III RBCs



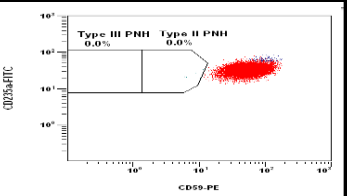
Normal RBC's: normal CD59 expression
Type I cells

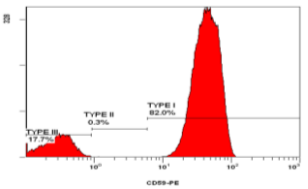


CD59 expression – Type I, II , III RBCs

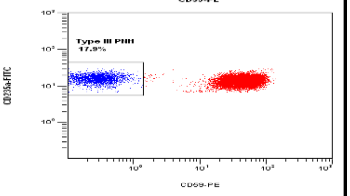


Normal RBC's: normal CD59 expression
Type I cells

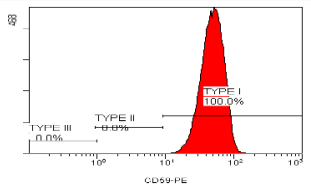




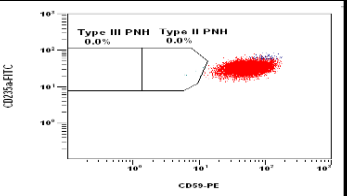
PNH clone: complete CD59 deficiency
Type III cells

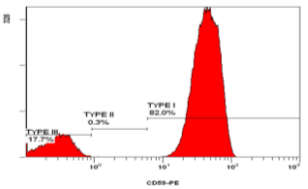


CD59 expression – Type I, II , III RBCs



Normal RBC's: normal CD59 expression
Type I cells





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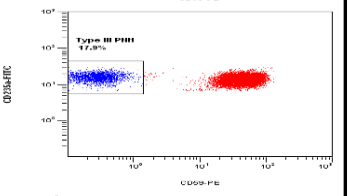
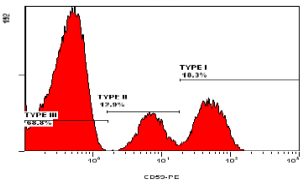
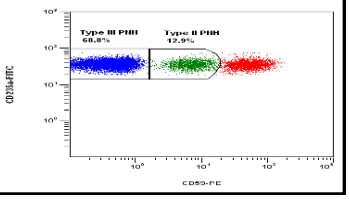


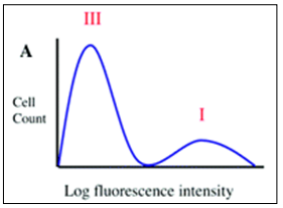


PNH clone: complete CD59 deficiency (Type III cells) and partial CD59 deficiency (**Type II cells**)

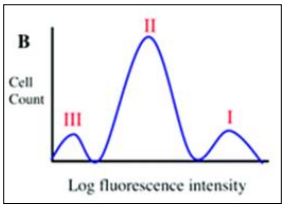


Detection of PNH clones in RBC (3)

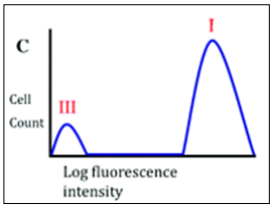
- RBC testing has clinical significance and should be done in patients with a PNH WBC clone
  - symptomatology is associated with size + type of RBC clone
  - proportion + type of cells: varies greatly among PNH patients
    - Type I and type III: most common phenotype
    - Type I, type II and type III: 2° most common phenotype
    - Type I and type II: least common phenotype



high % of type III RBCs: clinically apparent hemolysis



high % of type II RBCs but low % of type III cells: hemolysis may be modest



low % of type III cells: only biochemical evidence of hemolysis may be observed

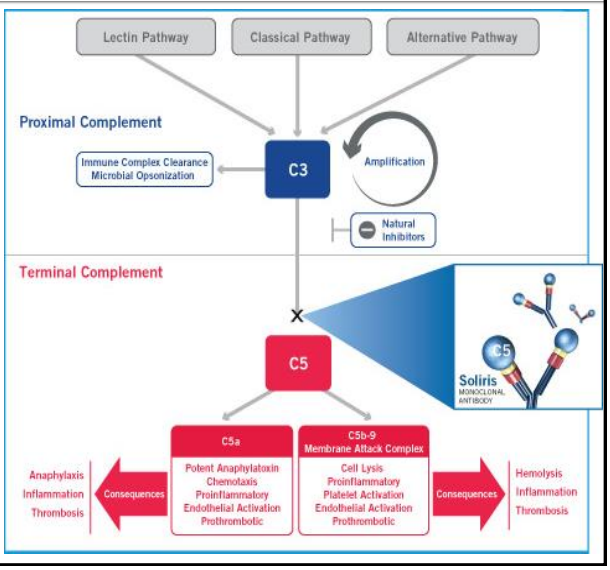
Parker CJ.Hematology Educ Book ASH. 2011: 21-29

Treatment of PNH

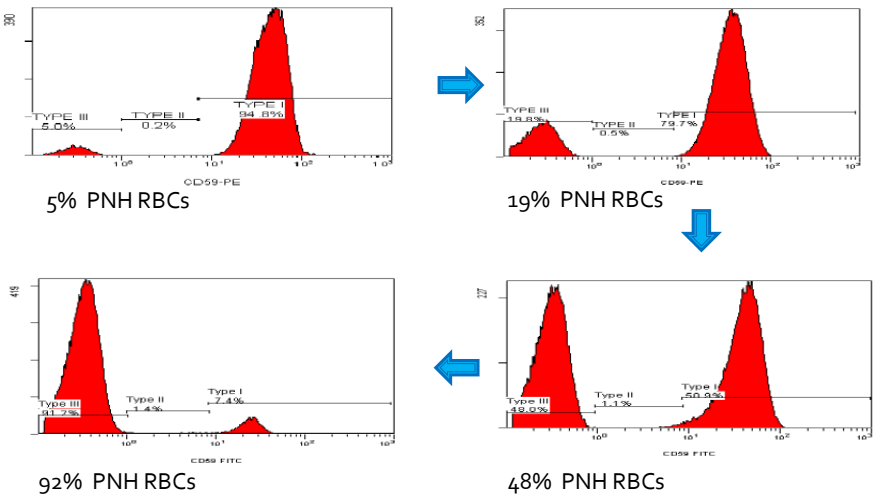
- Hematopoietic SCTx
- Anti-complement therapy:
  - eculizimab
    - stabilization / improved hemoglobin levels
    - reduced transfusion requirements
    - reduced measures of hemolysis (eg, normalization of LDH)
    - improved quality of life
- Novel complement inhibitors in development / under study

Eculizumab (Soliris®)

- Humanized anti-C5 antibody
- Complement inhibitor: binds to C5 => inhibits cleavage of C5
  - Terminal complement - C5a and C5b-9 activity blocked
  - Proximal functions of complement remain intact



Effects of eculizumab on RBCs of PNH patient with a 95% WBC PNH clone



Protection of PNH RBCs from complement-mediated lysis

Dahl-Chase Diagnostic Services; Hillmen P et al. N Engl J Med 2006;355:1233-43

Classification of clinical PNH

Category	Rate of vascular hemolysis	BM	Flow cytometry	Benefit from eculizumab
Classic PNH	Florid (LDH, often episodic macroscopic hemoglobinuria)	Cellular BM with erythroid hyperplasia, +/- normal morphology	**Large population (>50%)** GPI-anchor deficient PMN	**Yes**
PNH in BM failure disorder	Mild (often minimal abnormal biochemical markers of hemolysis)	Evidence of concomitant BM failure syndrome (AA or low-risk MDS)	% of GPI-anchor deficient PMNs is usually **relatively small (<10%)**	Typically **no**, but some patients (<10%) who have relatively large clones and clinically significant hemolysis, may benefit
Subclinical PNH	No clinical or biochemical signs of hemolysis	Evidence of concomitant BM failure syndrome (AA or low-risk MDS)	**Small (<1%) population** of GPI-anchor deficient PMN	**No**

Parker CJ., Hematology ASH Education Book, 2011:21-9.

PNH registry

- The aim of the PNH Registry is to collect data to characterise the progression of PNH as well as associated clinical outcomes, mortality and morbidity. Results from the PNH Registry will provide a better understanding of PNH and its real-world outcomes.
- Implemented in following countries: Argentina, Australia, Austria, Belgium, Canada, Colombia, Czech Republic, Denmark, Finland, France, …
- Type of data :
  - demographics (age, gender)
  - medical history
  - flow cytometry results
  - symptomatology
  - treatment
  - clinical outcomes
  - safety events of interest
  - pregnancy

Conclusions

PNH is a rare disease

⇒ PNH testing should only be done in selected patients
- BM failure / cytopenia
- Thrombosis (unusual anatomical locations)
- **Intravascular hemolysis, Coombs negative and related symptoms**

⇒ Flow cytometry is the method of choice for diagnosing/monitoring PNH clones