An overview of PNH: Pathophysiology, New Diagnostic Guidelines and EQA

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Paroxysmal Nocturnal Haemoglobinuria

- Clinical aspects of PNH
- New ICCS Guidelines
- EQA and PNH testing

Incidence and Prevalence of PNH in Britain

Yorkshire population 3,742,835 (2001 census)

- Incidence 1.3/ million/ year
- Estimated prevalence 15.9/ million

Great Britain population 57,105,375 (2001 census)

- estimated 75 new cases of PNH per year
- predicted prevalence of 908 patients
- 25% had PNH neutrophil clone size of > 50%

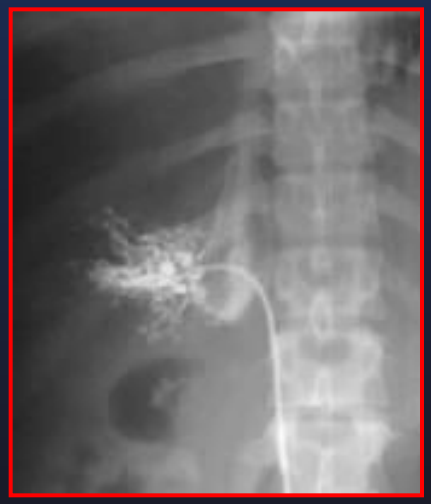
Hill *et al.*, Blood, November 2006, 294a

PNH – Triad of Clinical Features

Haemoglobinuria

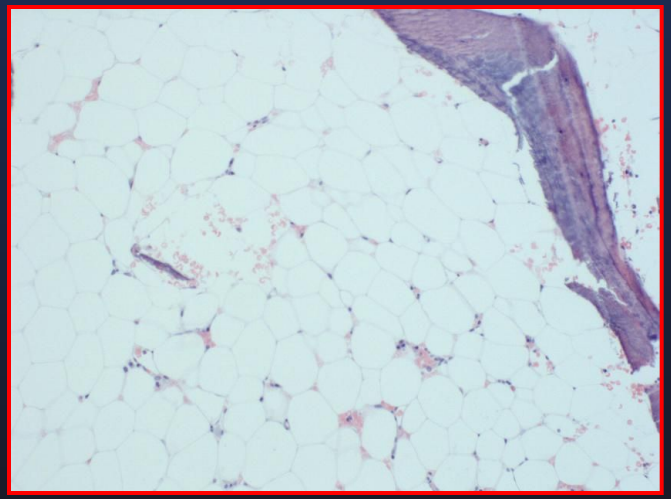
Intravascular haemolysis
- disabling symptoms
  - abdominal pain
  - dysphagia
  - erectile failure
  - severe lethargy

Budd-Chiari syndrome



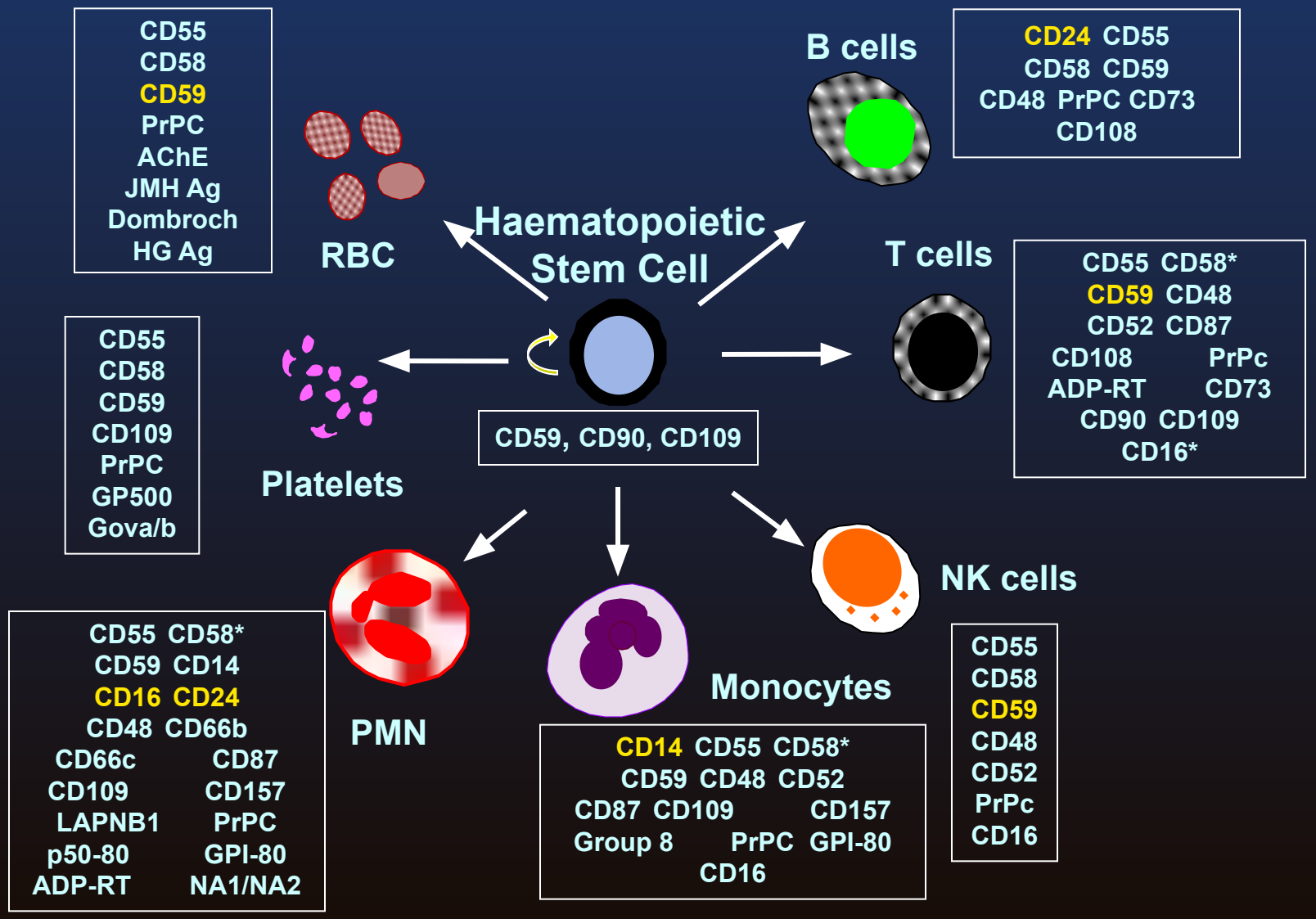
Thrombosis
- liver, cerebral
- 50% of patients
- 33% of patients is fatal

Aplastic anaemia



Bone Marrow Failure
- often precedes PNH
- selects for PNH clone

Proteins Deficient from PNH Blood Cells



(Courtesy of Lucio Luzzatto)

Why does PNH occur?

PNH clones

- Lack complement regulatory molecules and therefore probably “weakened”
- Have no malignant potential
- Occur at low levels in normal individuals

BUT:

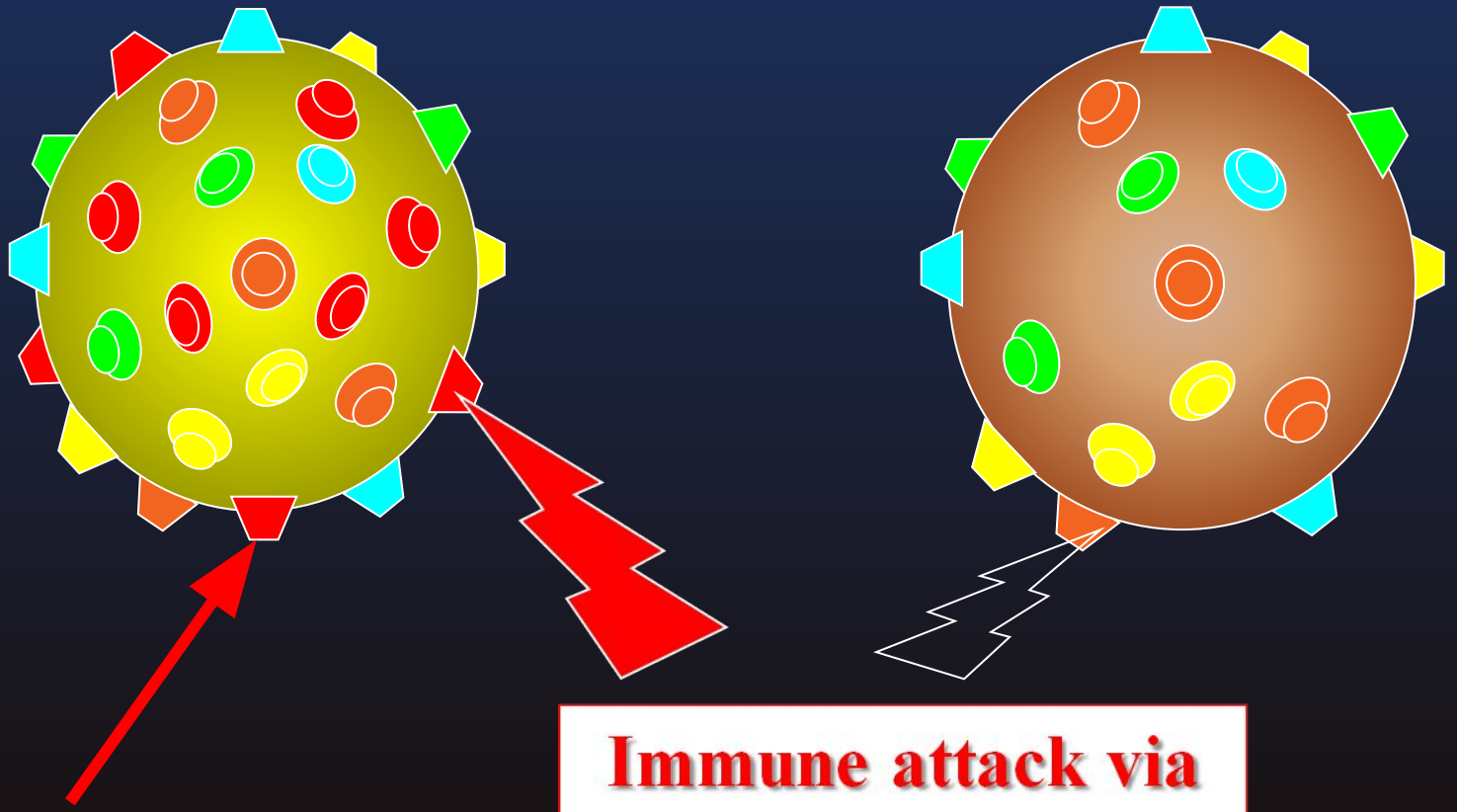
- PNH “always” occurs with aplastic anaemia
- Both rare disorders (1 in 100,000+) so unlikely to be chance

- **Dual pathogenesis theory**
  - Dacie, 1980; Rotoli & Luzzatto, 1989

Relative Growth Advantage in PNH

Normal stem cells

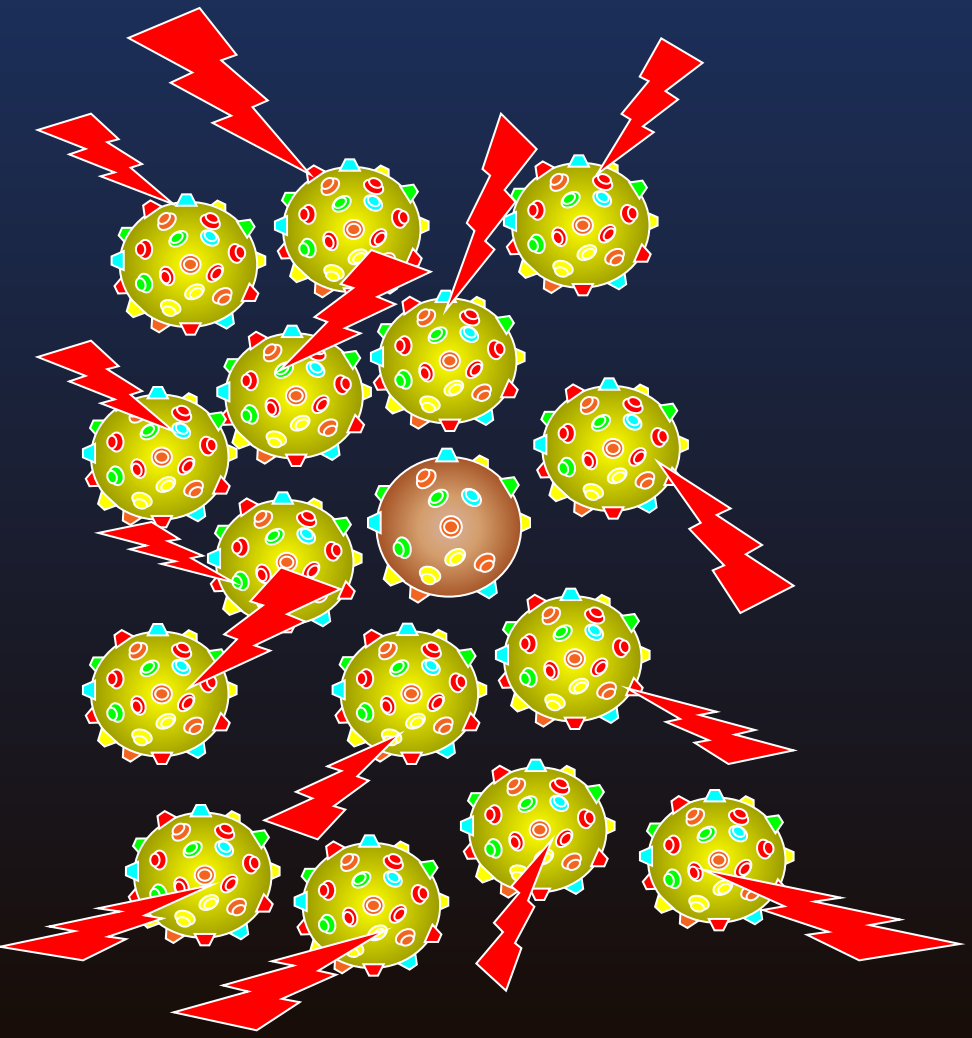
GPI-deficient (PNH) stem cells



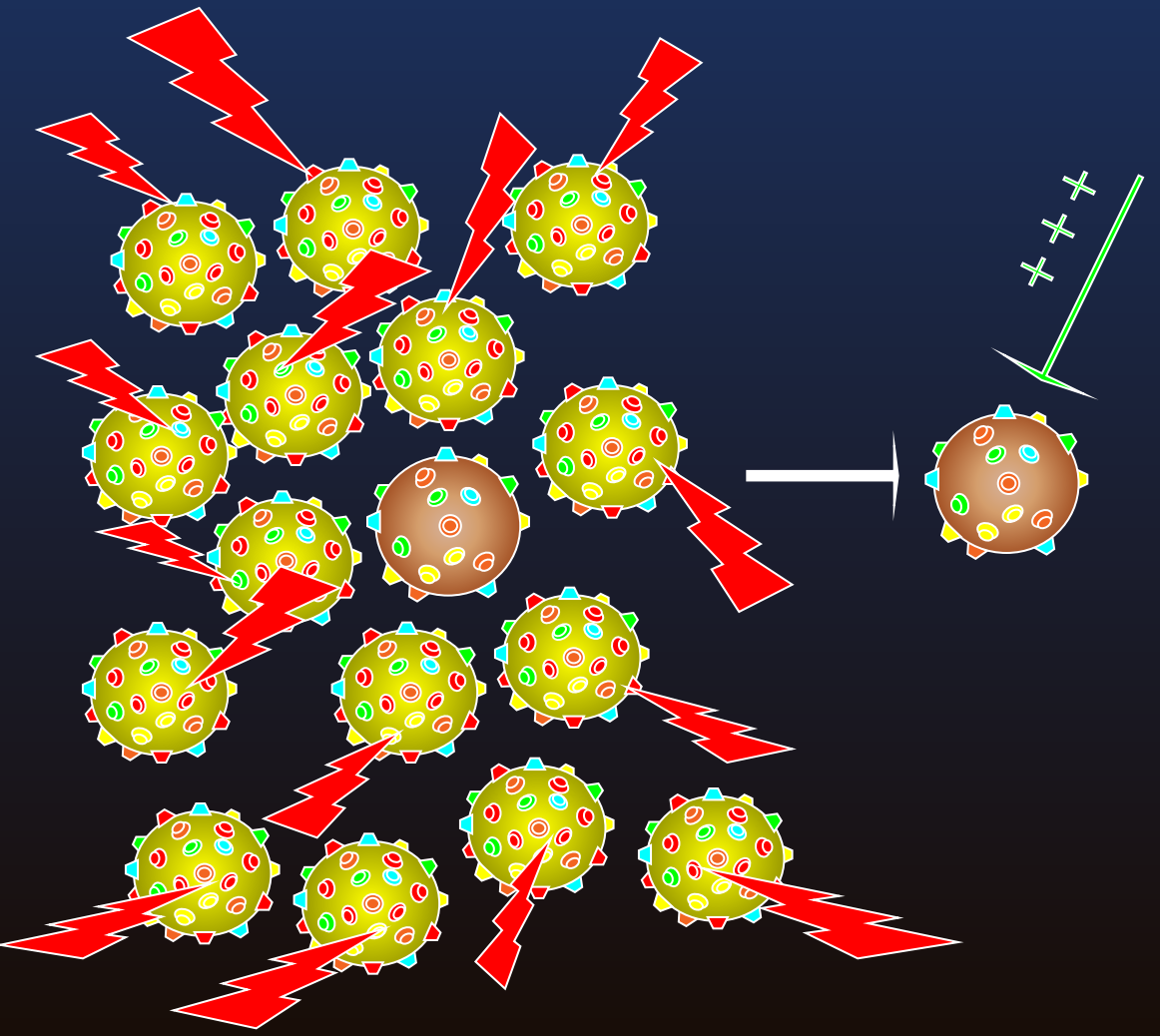
GPI-linked antigen

Immune attack via GPI-linked antigen (aplastic anaemia)

Relative Growth Advantage in PNH

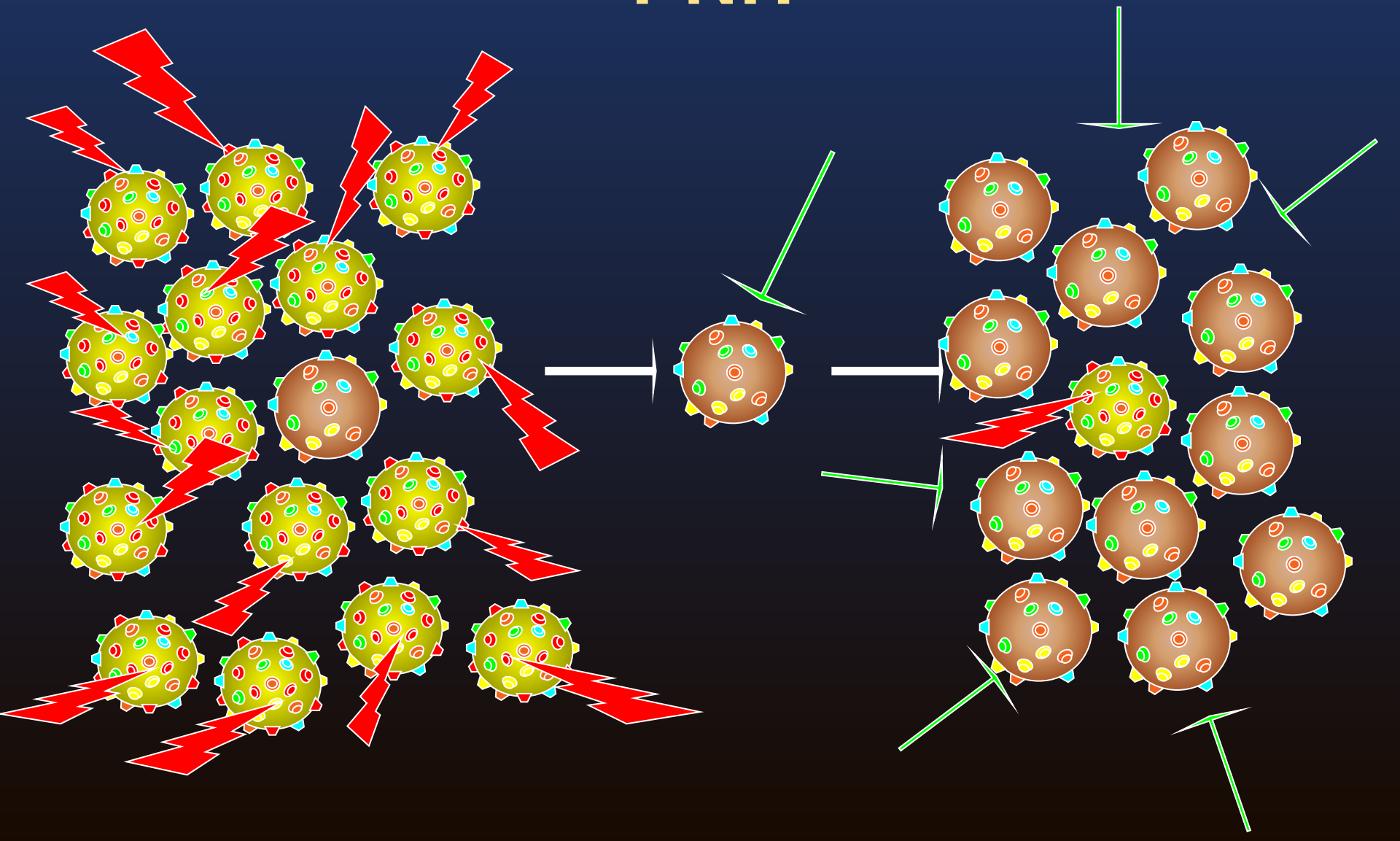


Relative Growth Advantage in PNH



Intense growth factor driven expansion

Relative Growth Advantage in PNH



Natural History of PNH

Four publications detailing four groups on the natural history of the disease:

1) England: 80 consecutive patients between 1940–1970[1]
2) USA and Japan: 176 (USA) and 209 (Japan) patients[2]
3) France, 2 reports:
   - 220 patients between 1950–1995[3]
   - 460 patients between 1950–2005[4]

1. Hillmen P, Lewis SM, Bessler M *et al*. New England Journal of Medicine 1995;333:1253-8
2. Nishimura J, Kanakura Y, Ware RE *et al*. Medicine 2004;83:193-207
3. Socie G, Mary JY, Gramont A *et al*. Lancet 1996;348:573-7
4. Peffault de Latour R, Mary JY, Salanoubat C *et al*. Blood 2008; Jun 5

Natural History of PNH

Country	**UK**[1]	**France**[2, 3]	**USA**[4]	**Japan**[4]
Median age at diagnosis	42 yrs	34.2 yrs	30 yrs	45 yrs
Median survival	10 yrs	22 yrs	23.3 yrs	25 yrs
Thrombosis	39%	30.7% (10yrs after diagnosis)	31.8%	4.3%
Prior AA	29%	30%	29%	37.8%
Transformation to leukaemia/MDS	0%	7.6% (10yr incidence)	1.7%	2.9%

1. Hillmen P, Lewis SM, Bessler M *et al*. New England Journal of Medicine 1995;333:1253-8

2. Socie G, Mary JY, Gramont A *et al*. Lancet 1996;348:573-7

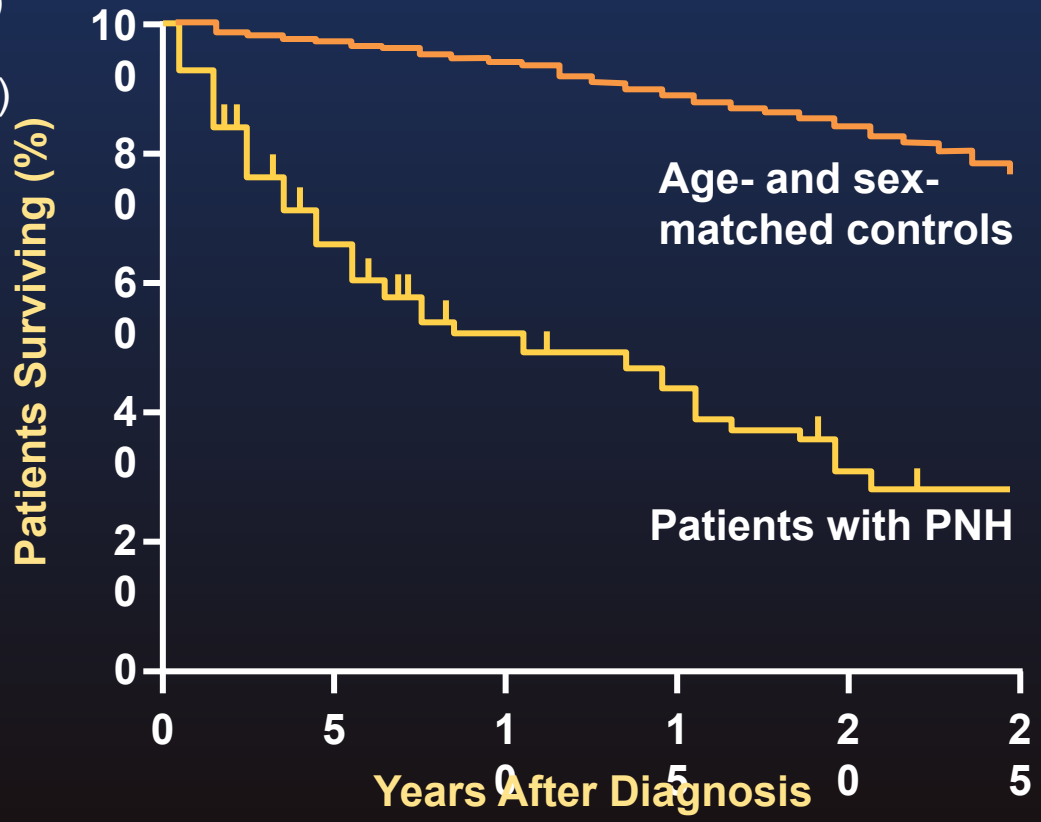
3. Peffault de Latour R, Mary JY, Salanoubat C *et al*. Blood 2008; Jun 5

4. Nishimura J, Kanakura Y, Ware RE *et al*. Medicine 2004;83:193-207

Paroxysmal Nocturnal Haemoglobinuria: A Chronic Disabling and Life-Threatening Disease [1,2]

- Estimated 4,000 – 6,000 patients in U.S [3]
- 5 year mortality: 35% [1]
- Diagnosed at all Ages – Median age early 30's [4,5]
- Quality of life diminished [1,6]
- Progressive disease [1,2]

Actuarial Survival From the Time of Diagnosis in 80 Patients With PNH [1]



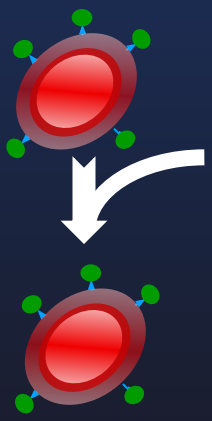
The expected survival of an age- and sex-matched control group is shown for comparison [1].
In a patient population where ½ the patients have <30% clone, **1 in 7 patients died by 5 years [7].**

(1) Hillmen P et al. NEJM 1995; 333:1253-8; (2) Parker C et al. Blood 2005;106(12):3699-709; (3) Hill A et al. Blood 2006;108:985; (4) Moyo VM et al. BJH 2004;126:133-38; (5) Nishimura J et al. Med 2004;83:193–207; (6) Socié G et al. Lancet 1996;348:573-7; (7) Peffault de Latour R et al. Blood 2008;112(8):3099-106.

PNH is a Progressive Disease of Chronic Haemolysis [1-4]

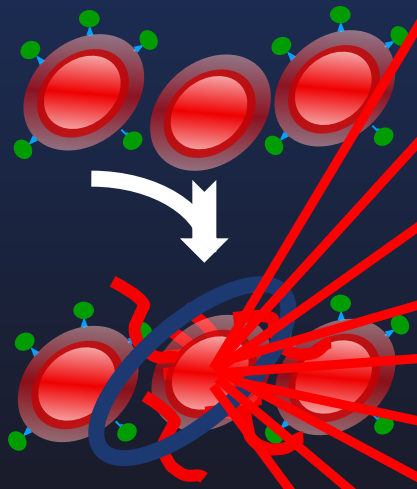
Normal red blood cells are protected from complement attack by a shield of terminal complement inhibitors [2,3]

Without this protective complement inhibitor shield, PNH red blood cells are destroyed [2,3]



Intact RBC

Complement Activation



Free Haemoglobin in the Blood from Destroyed PNH RBCs

Anaemia

Thrombosis

Renal Failure

Pulmonary Hypertension

Significant Impact on Survival [3]

Abdominal Pain

Dyspnoea

Fatigue

Dysphagia

Haemoglobinuria

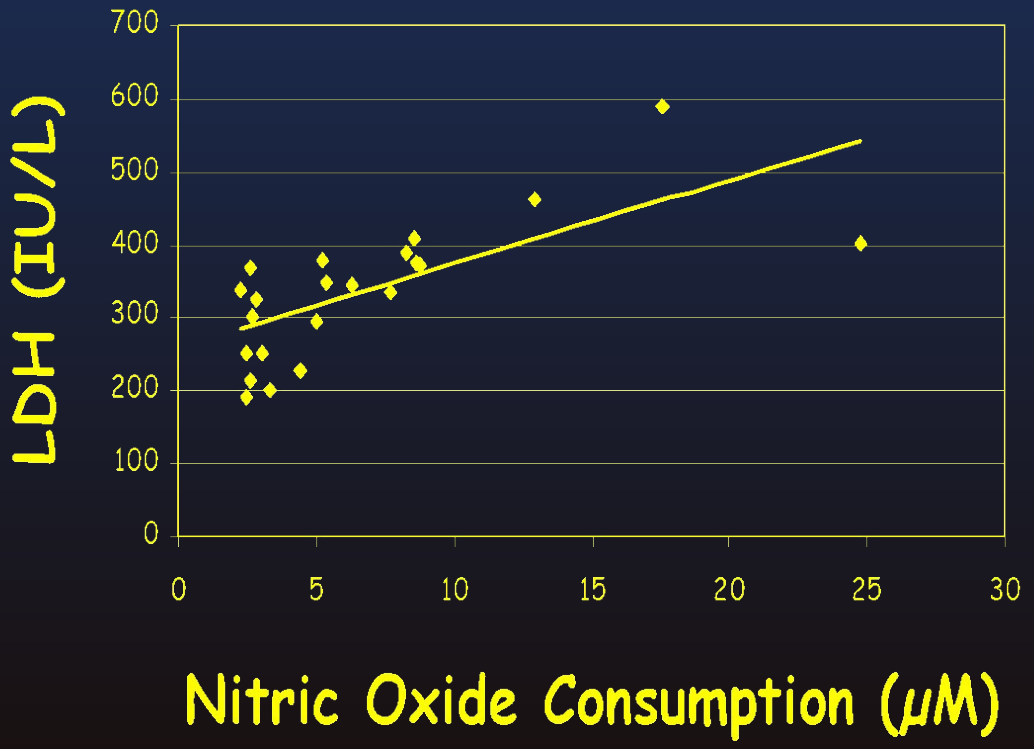
Erectile Dysfunction

Significant Impact on Morbidity [3]

(1) Rother R et al. JAMA 2005;293:1653-1662; (2) Brodsky RA. Blood Rev 2008;22:65-74;
(3) Rother R et al. Nat Biotech 2007;25:1256-1264; (4) Socie G et al. Lancet 1996;348:573-577.

Symptoms and relationship to nitric oxide scavenging

- **Dysphagia, abdominal pain** & **erectile failure** completely resolved during eculizumab treatment
- Attributed to smooth muscle dystonia due to the **scavenging of nitric oxide** by free plasma haemoglobin



From Sickle cell disease patients; Courtesy of Dr Mark Gladwin, NIH, Bethesda

Haemolysis and Nitric Oxide

- Red blood cell destruction during haemolysis releases cell-free haemoglobin [1]
- Cell-free haemoglobin scavenges NO [1]
- NO depletion results in smooth muscle dysfunction – abdominal pain, dysphagia, severe lethargy, erectile failure
- Reduced nitric oxide can cause pulmonary hypertension [2,3]:
  - Vasoconstriction [1]
  - Clotting [1]
    - Platelet hyperreactivity [4]
    - Impaired fibrinolysis [5]
    - Hypercoagulability [5]

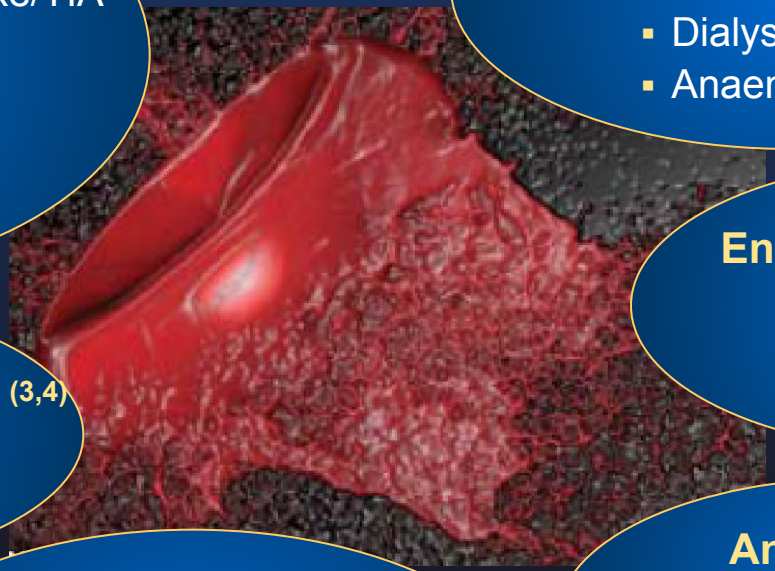
(1) Rother R et al. JAMA 2008;293:1653-1662; (2) Villagra J et al. Blood 2007;110(6):2166-72; (3) Hill A et al. Blood 2008;112(11):486; (4) Wiedmer T et al. Blood 1993;82(4):1192-6; (5) Grünewald M et al. Blood Coag Fibrinolysis 2003;14:685-95.

Chronic Haemolysis is the Underlying Cause of Progressive Morbidities and Mortality of PNH [1-5]

THROMBOSIS [2,4,5]

Venous	Arterial
▪ PE/DVT	▪ Stroke/TIA
▪ Cerebral	▪ MI
▪ Dermal	
▪ Hepatic/Portal	
▪ Abdominal ischemia	

Chronic Kidney Disease [3,4]
- Renal insufficiency
- Dialysis
- Anaemia



End Organ Damage [2,3,4]
- Brain
- Liver
- GI

Pulmonary Hypertension [3,4]
- Dyspnoea
- Cardiac Dysfunction

Anaemia [2,4,5]
- Transfusions
- Haemosiderosis

Fatigue / Impaired Quality of Life [3,4]
- Abdominal pain
- Dysphagia
- Poor physical functioning
- Erectile dysfunction

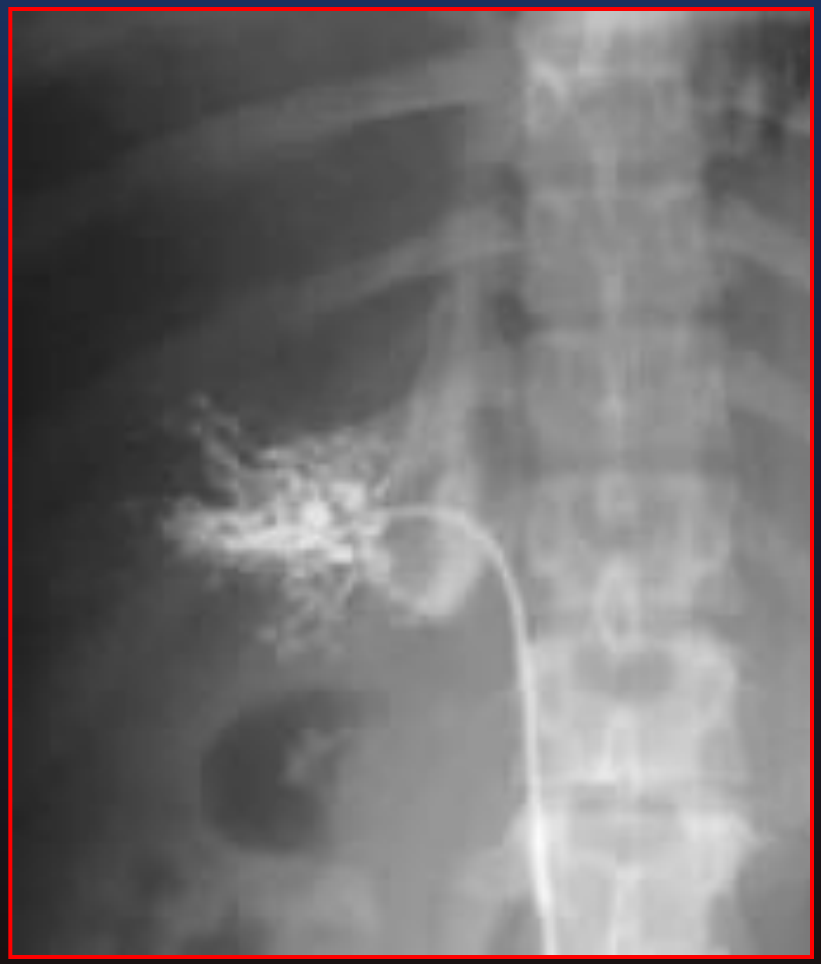
(1) Parker C et al. Blood 2005;106:3699-709; (2) Hillmen P et al. NEJM 1995;333:1253-58; (3) Rother R et al. JAMA 2005;293:1653-62; (4) Rother R et al. Nat Biotech 2007;25:1256-1264; (5) Socie G et al. Lancet 1996;348:573-577.

Renal Damage in PNH

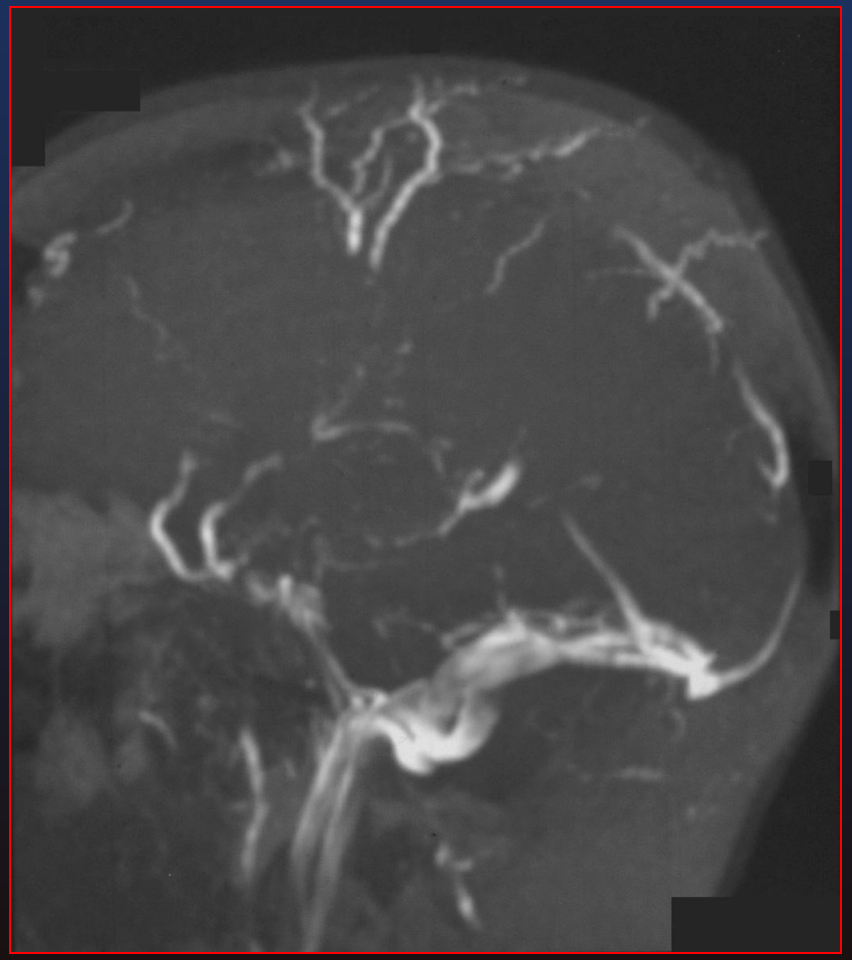
- Chronic haemolysis and cell-free plasma haemoglobin lead to chronic kidney disease in PNH [1,2]
- Renal damage in PNH may be due to repetitive exposure of tissue to cell-free haemoglobin [3,4]
- 64% of patients with PNH have stage 1-5 chronic kidney disease [5]
- Renal failure has been identified as the cause of death in approximately 8 – 18% of PNH patients [6,7]

(1) Parker C et al. Blood 2005;106:3699-3709; (2) Rother RP et al. JAMA 2005;293:1653-1662; (3) Clark DA et al. Blood 1981;57:83-9; (4) Hillmen P et al. NEJM 1995; 333:1253-8; (5) Hillmen P et al. Blood 2007;110(11):3678: Poster at American Society of Hematology 49th Annual Meeting; (6) Nishimura JI et al. Medicine 2004;83:193-207; (7) Rosse and Nishimura. Int J Hematol 2003;77:113–20.

Classical sites of venous thrombosis in PNH



Budd-Chiari syndrome

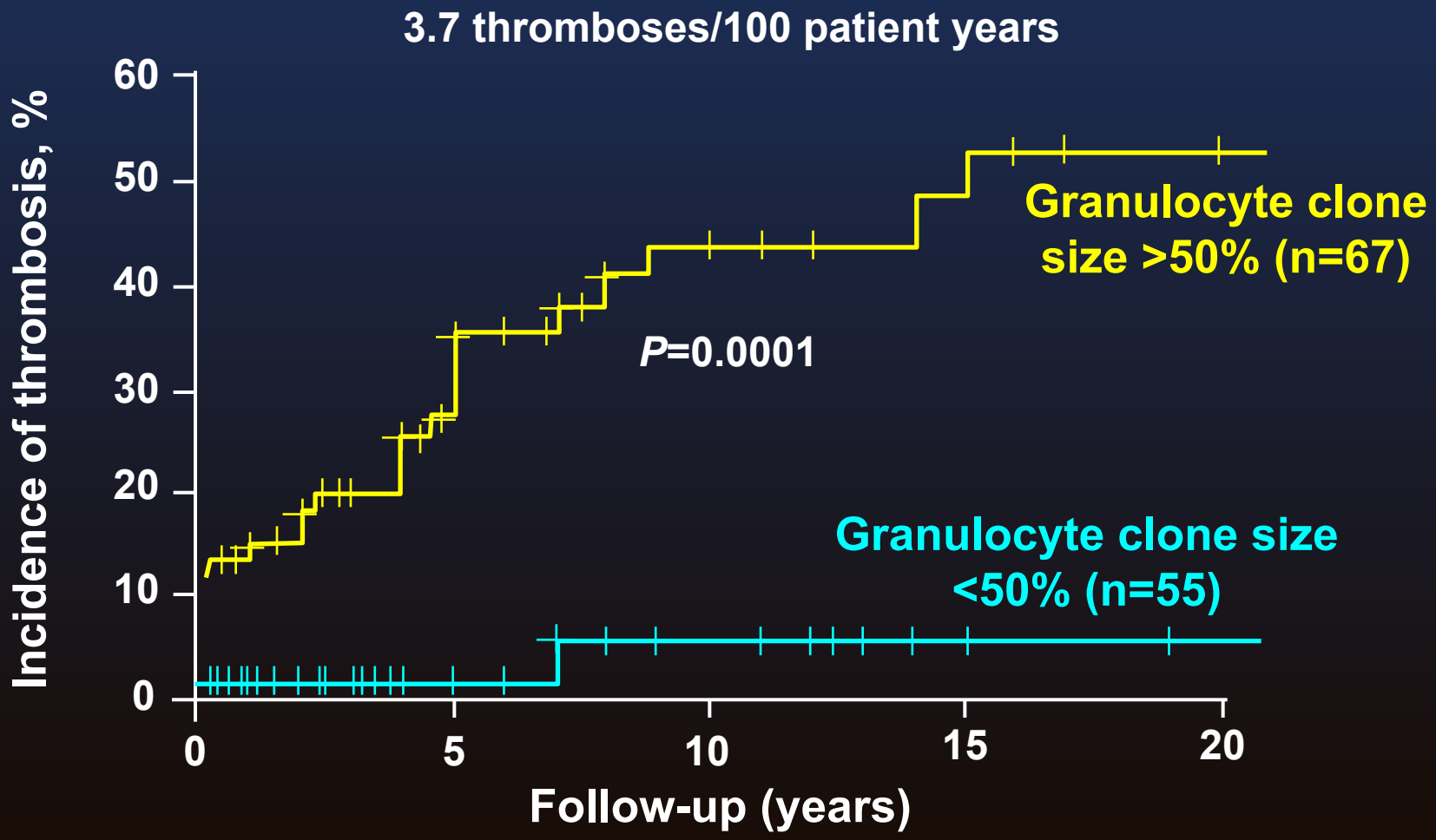


Superior Sagittal Sinus Thrombosis

PNH Clone Size and Thrombosis

(excluding warfarin prophylaxis patients)

Incidence of Thrombosis is Highest in Patients With a Large PNH Clone



Hall C et al. Blood

Laboratory Investigation of PNH

- Flow cytometry immunophenotyping is the method of choice for PNH testing
- Diagnosis or identification of PNH cells by demonstrating deficiency of GPI-linked proteins from granulocytes/monocytes/red cells
- There is little guidance or consensus on the best approach or for labs wanting to set up PNH testing

Laboratory Investigation of PNH

- In 2008 the Clinical Cytometry Society sponsored a workshop on PNH testing
- Approximately 100 attendees from flow cytometry community
- Out of this workshop came the desire to produce a consensus document that addressed many of the issues raised at this meeting

The need for a consensus guideline for PNH immunophenotyping

- The disease is rare and most labs have limited experience in PNH testing
- Clinical documents have recommended testing, including “high sensitivity” testing, without specifying how this should be done
- Flow cytometry is method of choice for PNH testing, but many different approaches exist
- Some external QA/proficiency testing data have shown a wide range in ability of labs to detect abnormal PNH populations

Parker et al, Blood 2005;106:3699, Sutherland et al, Am J Clin Pathol 132:564, 2009; Richards et al Cytometry B 76: 47 2009

Consensus Committee

Michael J Borowitz, MD, PhD
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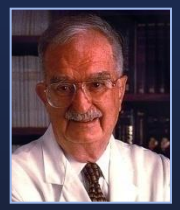

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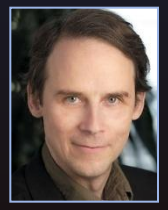

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ICCS PNH Testing Guidelines

Cytometry Part B (Clinical Cytometry) 78B:211–230 (2010)

Original Articles

Guidelines for the Diagnosis and Monitoring of Paroxysmal Nocturnal Hemoglobinuria and Related Disorders by Flow Cytometry

Michael J. Borowitz,[1]* Fiona E. Craig,[2] Joseph A. DiGiuseppe,[3] Andrea J. Illingworth,[4] Wendell Rosse,[5] D. Robert Sutherland,[6] Carl T. Wittwer,[7] and Stephen J. Richards[8]; On behalf of the Clinical Cytometry Society

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Background: Paroxysmal nocturnal hemoglobinuria (PNH) is a rare hematopoietic stem cell disorder characterized by a somatic mutation in the *PIGA* gene, leading to a deficiency of proteins linked to the cell membrane via glycophosphatidylinositol (GPI) anchors. While flow cytometry is the method of choice for identifying cells deficient in GPI-linked proteins and is, therefore, necessary for the diagnosis of PNH, to date there has not been an attempt to standardize the methodology used to identify these cells.

Methods: In this document, we present a consensus effort that describes flow cytometric procedures for detecting PNH cells.

Results: We discuss clinical indications and offer recommendations on data interpretation and reporting but mostly focus on analytical procedures important for analysis. We distinguish between routine analysis (defined as identifying an abnormal population of 1% or more) and high-sensitivity analysis (in which as few as 0.01% PNH cells are detected). Antibody panels and gating strategies necessary for both procedures are presented in detail. We discuss methods for assessing PNH populations in both white blood cells and red blood cells and the relative advantages of measuring each. We present steps needed to validate the more elaborate high-sensitivity techniques, including the need for careful titration of reagents and determination of background rates in normal populations, and discuss technical pitfalls that might affect interpretation.

Conclusions: This document should both enable laboratories interested in beginning PNH testing to establish a valid procedure and allow experienced laboratories to improve their techniques. © 2010 Clinical Cytometry Society

Key terms: flow cytometry; paroxysmal nocturnal hemoglobinuria; practice guidelines

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Disclosures: MJB, AJI, WR and SJR have consulting agreements with Alexion Pharmaceuticals; MJB and SJR have research support from BD Biosciences.
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DOI: 10.1002/cyto.b.20525

Borowitz M, Craig F, DiGiuseppe J, Illingworth A, Rosse W, Sutherland R, Wittwer, C and Richards S *Cytometry Part B (Clinical Cytometry). 2010:78B:211-230*

Recommendations in the ICCS PNH Testing Guidelines Document

- Recommendations tried to strike a balance between the virtues of standardization and the fact that there are limited data comparing methods; many approaches can be shown to work
- Many of the recommendations are based on the authors' experiences of 'what works' rather than systematic evaluation.

Contents Of The Document

- Rationale and History
- Clinical Indications
- Methodology
  - Routine testing
  - High sensitivity testing
  - RBC vs WBC analysis
- Interpretation of results
- Reporting
- Recommendations and future directions

Methodology

- Sample issues
- Comparison of RBC and WBC testing
  - Reagents
  - Analytical approaches
- Routine vs high sensitivity analysis
- Quality control issues

Red Cell Analysis: Routine testing

To detect clone sizes of at least 1%

ADVANTAGES

- Relatively straightforward
- Best way to identify Type II cells
- RBC clone size associated with symptoms

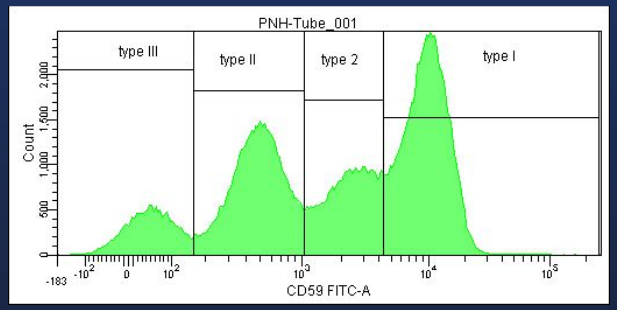
DISADVANTAGES

- Often underestimates clone size because of transfusion or haemolysis
- False negatives common

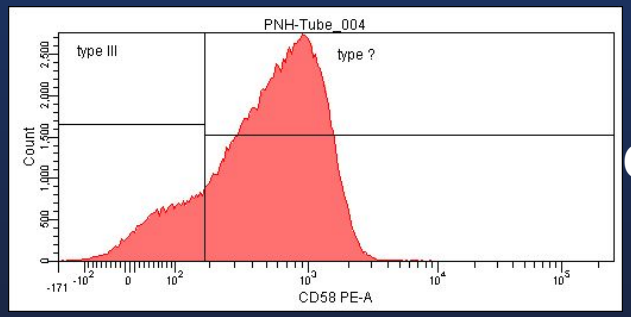
Routine Red Cell Analysis: Reagents

- For historical reasons, CD55 and CD59 are most commonly used
- CD59 is strongly expressed, while CD55 is weak
  - CD55 may not be necessary
  - Rare congenital CD59 deficiency cases
  - Some variation in CD59 clones
- Other GPI-anchored reagents (CD58) exist, but limited experience
- Anti-glycophorin (CD235a) may be used to identify red cells, but this may not be necessary for routine analysis
  - Can guard against failure of antibody to contact cells

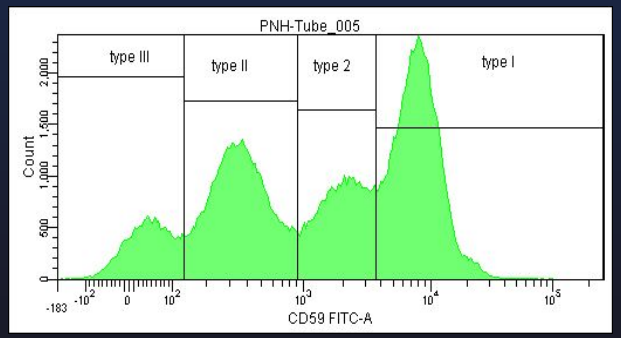
Red cell testing



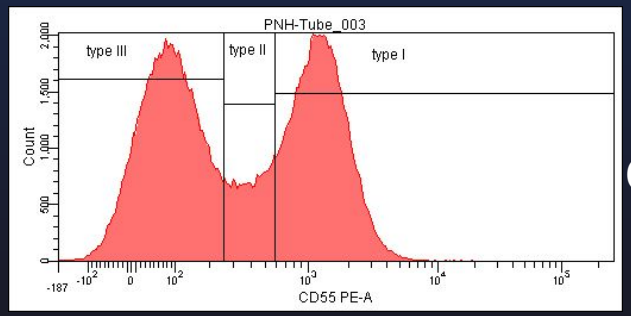
CD59 Fitc



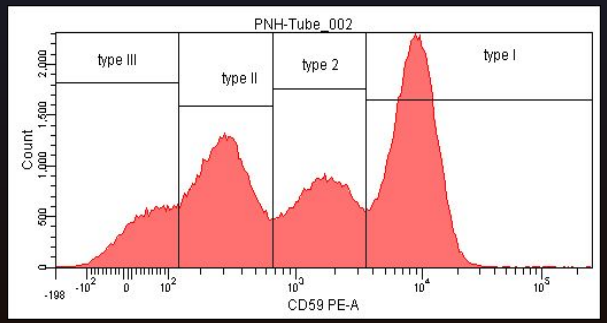
CD58PE



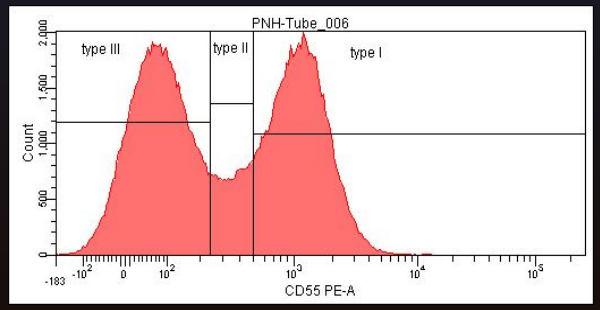
CD59 Fitc



CD55 PE



CD59 PE



CD55 PE

Leucocyte Analysis: Routine testing

- Granulocyte PNH clone probably gives most accurate estimate of PNH clone size
- Monocyte clones can usually be determined in same tube and confirms granulocyte result, though because monocytes are less numerous, precision is lower
- Type II granulocytes can occasionally be recognized but red cells are typically better for this purpose
- Lymphocytes are not a suitable target for testing

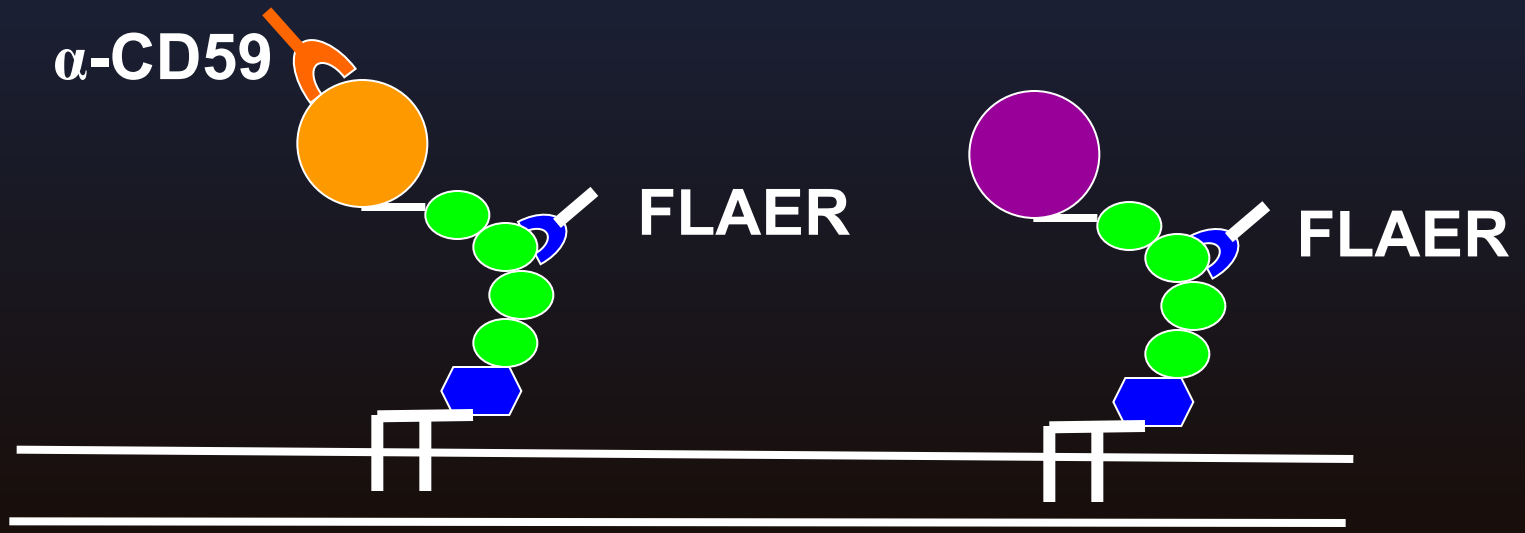
Leucocyte Analysis: Reagents

- CD55 and CD59 were used historically but these are not optimal
- CD16, CD66b, CD24 are most commonly used GPI-linked markers for granulocytes
- CD14 is often used for monocytes but some normal dendritic cells are CD14-negative and gate like monocytes
- FLAER is the most versatile reagent for detecting PNH white cells

WHAT IS FLAER?
FLuorescent AERolysin

- Aerolysin is a pore-forming toxin secreted by *Aeromonas hydrophila* - GPI-anchor serves as receptor

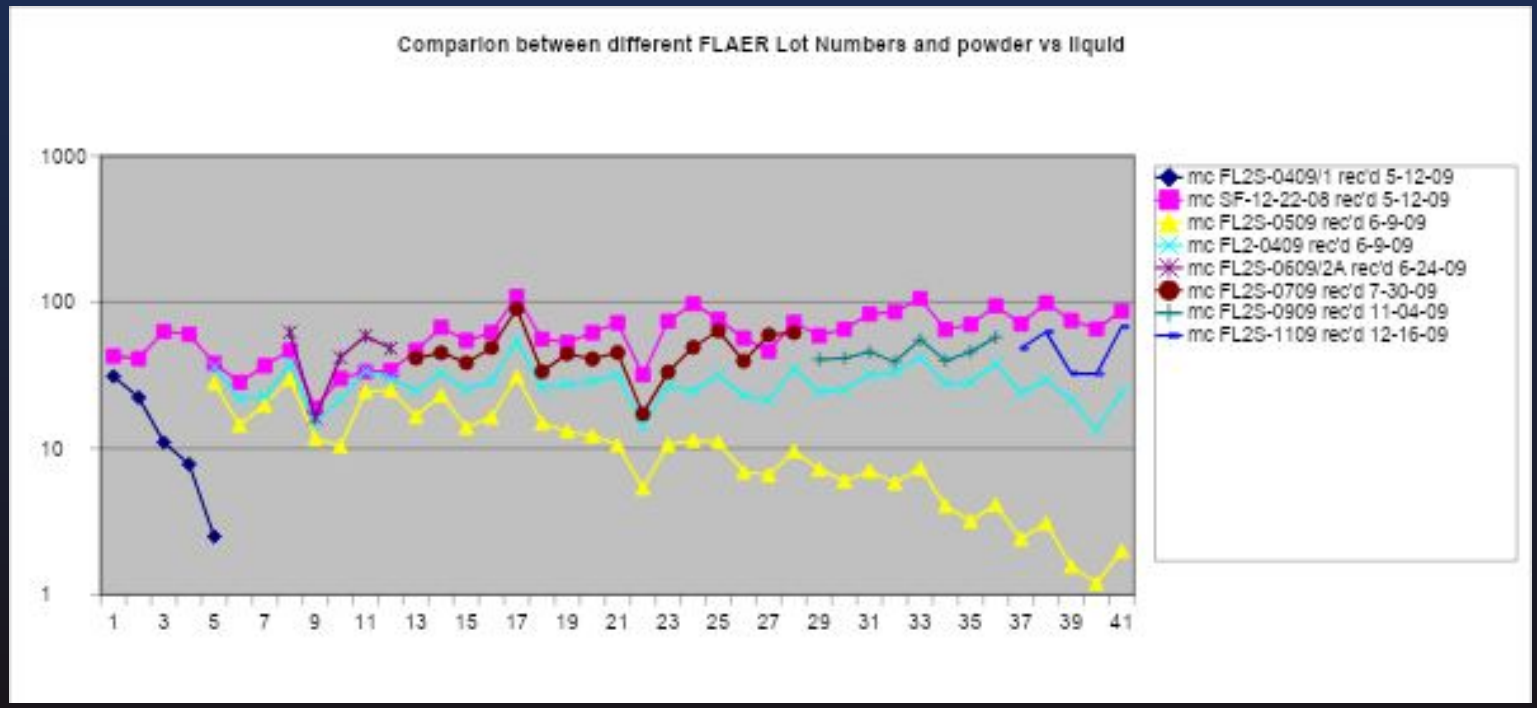
- FLAER – A488-conjugated mutant aerolysin binds to GPI -anchor rather than surrogate protein and is inactive so doesn't form channels



FLAER STABILITY

- Original formulation was lyophilized, requiring aliquoting and freezing
- Reconstituted FLAER was unstable
- Stability problems better with more recent lots
- New liquid formulation exists which is also stable, and can be treated more or less like any other monoclonal antibody
  - Sensitive to light and temperature

STABILITY OF FLAER



Courtesy Andrea Illingworth

Routine Analysis: Summary

- Adequate for detection of all cases of hemolytic PNH
- White cell analysis necessary as screen as too many false negatives with red cell screening assay alone
- Preferred granulocyte reagents are CD24, CD66b, CD16, FLAER
- Gating usually not critical
- Can obtain reasonable results with as few as 5-10K cells of interest

High Sensitivity Assays: Special concerns

- Need to collect more events
- Requirement for an extensive study of normals to determine background rates
- Essential to use multiparameter gating to ensure purity of the population used for the denominator
- Need to combine two GPI-linked WBC markers to maximize sensitivity
- FLAER particularly useful; because it is absent from both grans and monos an impure gate will not lead to interpretation of a small PNH clone when none is present

Guideline Summary I

- Broad agreement on the need for a consensus guideline
- Document reviews and clarifies clinical recommendations
- Blood identified as preferred sample
- Approach to routine and high sensitivity analysis addressed separately

Guideline Summary II

- Granulocyte analysis provides better estimate of size of PNH clone than RBC analysis
- Thus, routine red cell analysis not recommended without white cell analysis, though a granulocyte screening assay may be viable, especially in labs with low prevalence of PNH
- Lymphocyte analysis not recommended because of lifespan of lymphocytes

Guideline Summary III

- For high sensitivity WBC analysis, essential to use an antibody for gating, and to assess two different GPI-anchored markers, though in routine analysis this may not be necessary
- FLAER and CD24 are recommended as preferred granulocyte reagents, and CD59 is the best single RBC reagent; CD55 is not acceptable by itself
- Further research with other markers may result in revisions to these recommendations

EQA For PNH testing

- What kind of scheme?
- Screening vs high sensitivity (MRD) testing
- What material?
- What methodology?
- Educational aspects
- Scoring/performance issues
- Molecular testing

EQA For PNH testing

- What kind of scheme?
- ‘rare disease’ testing
- What cells to test?
- Single sample sent out to participating laboratories
- Exchange fresh material between small number of laboratories
- List mode data

EQA For PNH testing

- Screening vs high sensitivity (MRD) testing
  - Screening (~1%)
  - MRD 0.01%

- Methodology
  - Standardised procedure
  - Instrument set-up
  - Antibodies/reagents
  - Fluorochromes
  - Target populations

EQA For PNH testing

- What material?
- Small groups: exchange of known fresh patient samples
- Large International schemes: stabilized material.
  - Good statistical data but may perform differently compared to fresh material
- Large volume of material required from patients with low counts
- Any role for molecular screening for *PIG-A* mutations
  - Deep sequencing techniques

EQA For PNH testing

- Educational aspects?
- Scoring/performance issues?

  How to assess performance?
- Poor performance – educational aspects
- Educational aspects – good performance

- Is a standard method the way forward?

  How should this be determined?

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