The Role of Flow Cytometry in Diagnosis and Monitoring of Patients with Paroxysmal Nocturnal Haemoglobinuria

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What is PNH?

Introduction

What is PNH?

An ‘acquired’ haemolytic anaemia

Clinical Triad of:

1) Bone marrow failure – cytopenias of varying severity
2) Thrombosis in unusual anatomical locations
3) Chronic haemolysis (increased LDH). Acute episodes of haemolysis (complement mediated) – Haemoglobinuria.

At least one of the symptoms is present all patients though underlying bone marrow failure is ubiquitous

What is PNH?

- Paroxysmal Nocturnal Haemoglobinuria (PNH) is characterised by failure of normal haematopoiesis and clonal expansion of haematopoietic stem cells lacking GPI-anchored proteins due to a *PIG-A* mutation.

- Deficiency of the GPI-linked complement regulators CD55 (DAF) and CD59 (MIRL) from PNH red cells renders them highly sensitive to lysis by terminal complement resulting in intravascular haemolysis and haemoglobinuria.

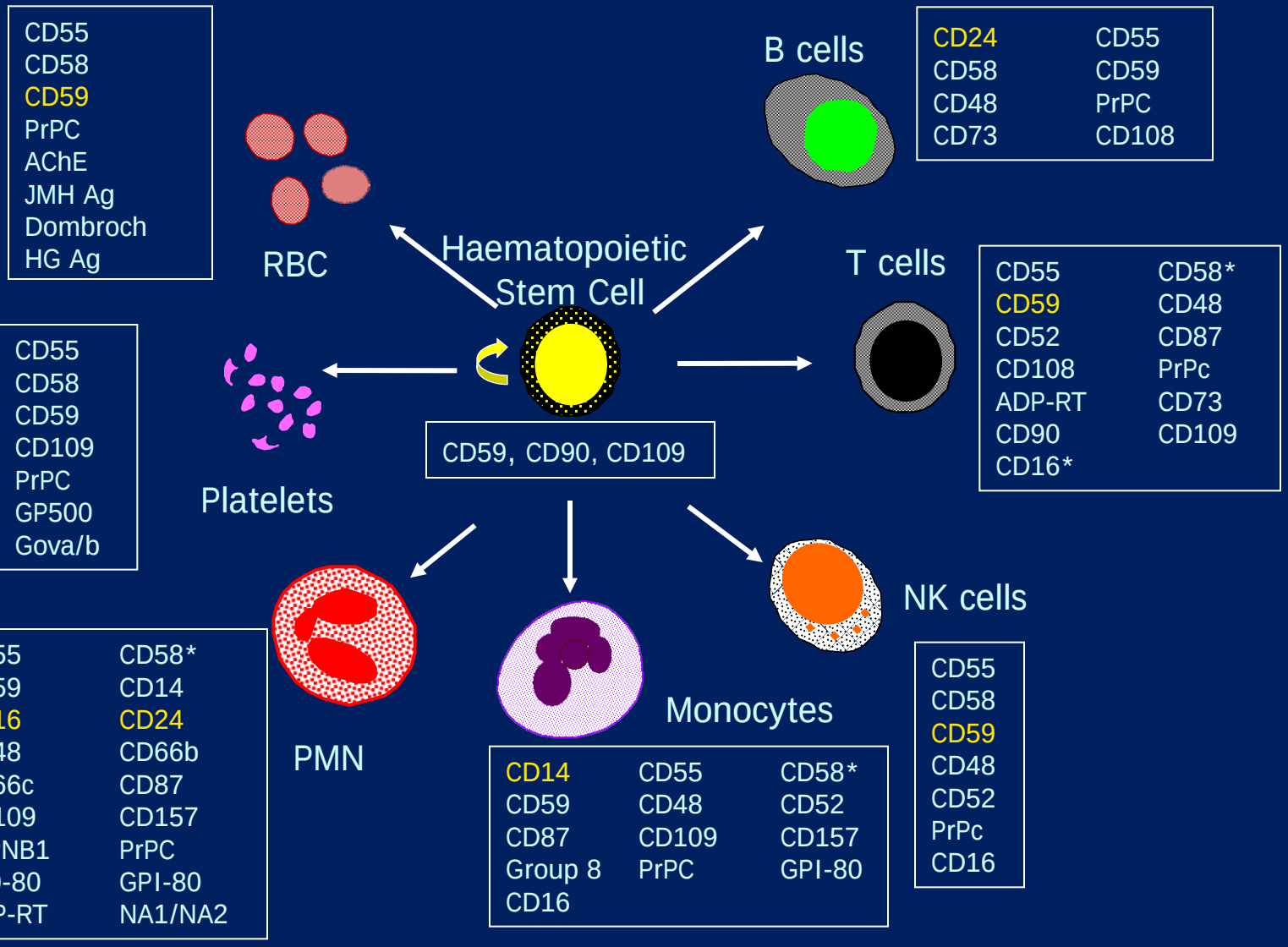
- Major long term transfusion requirement for haemolytic and aplastic PNH patients

Disease Pathogenesis

Requirements:

1) Haematopoietic stem cells with *PIG-A* mutation or mutations.
2) Selection process against normal stem cells in the form of immune-mediated bone marrow failure, i.e. aplastic anaemia.
3) Non-malignant , clonal expansion of the progeny of the PNH stem cell to give GPI-deficient haematopoiesis.

Proteins Deficient from PNH Blood Cells

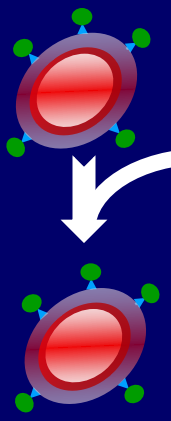


(Courtesy of Prof Lucio Luzzatto)

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

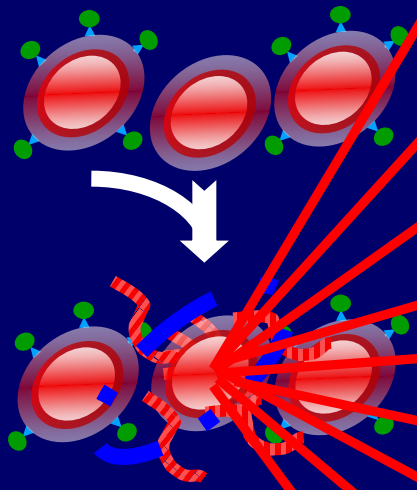
Normal red blood cells are protected from complement attack by a shield of terminal complement inhibitors [2,3]

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

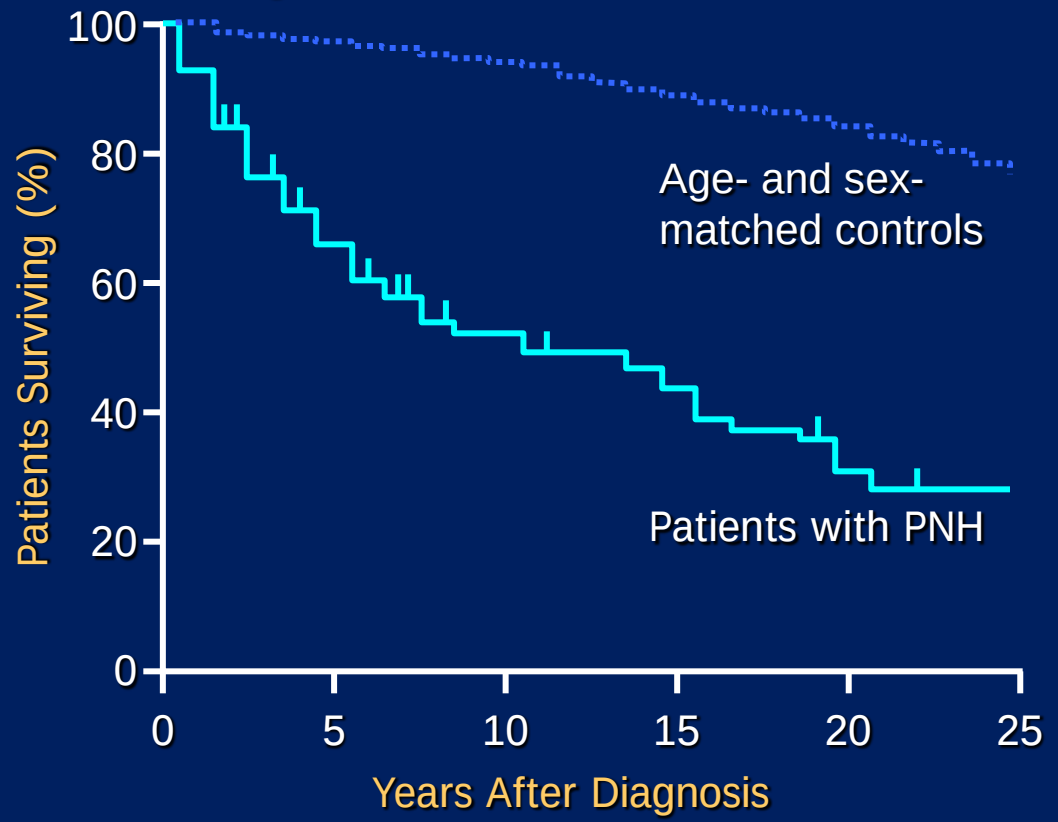
Significant Impact on Morbidity [3]

(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.

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.

Flow Cytometry

PNH Screening and Diagnosis

Laboratory Investigation of PNH

1) Flow cytometry immunophenotyping is the method of choice for PNH testing

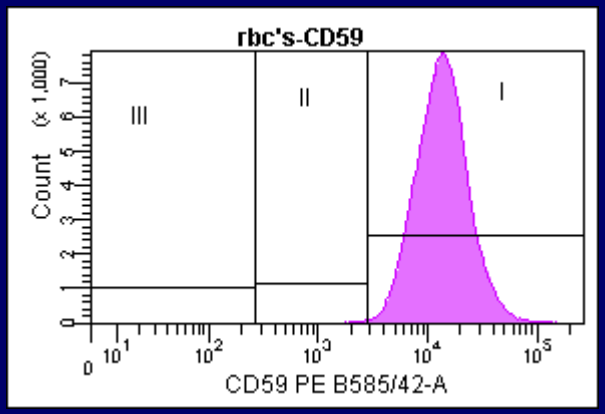
2) Diagnosis or identification of PNH cells by demonstrating deficiency of GPI-linked proteins from granulocytes/monocytes/red cells

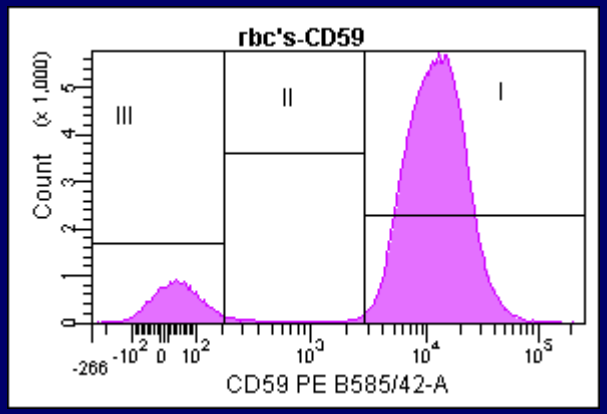
3) Guidance
    1. ICCS guideline document
    2. Detailed PNH testing method.

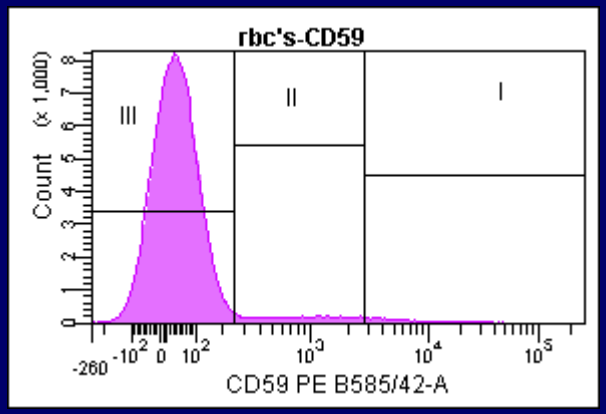
1. Guidelines for the diagnosis and monitoring of paroxysmal nocturnal hemoglobinuria and related disorders by flow cytometry. Borowitz MJ, et al. Cytometry B Clin Cytom. 2010 Jul;78(4):211-30

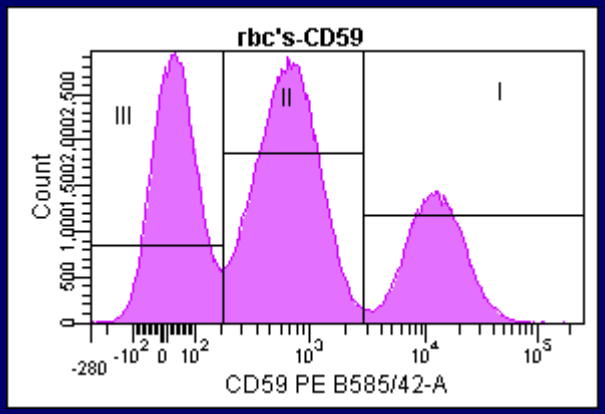
2. Practical guidelines for the high-sensitivity detection and monitoring of paroxysmal nocturnal hemoglobinuria clones by flow cytometry. Sutherland DR, Keeney M, Illingworth A. Cytometry B Clin Cytom. 2012

Optimised Assay for Red cells

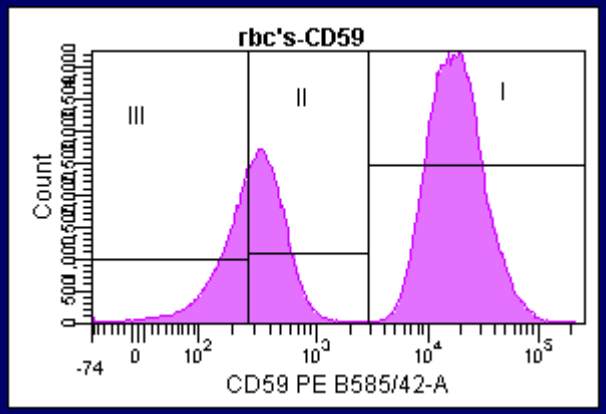


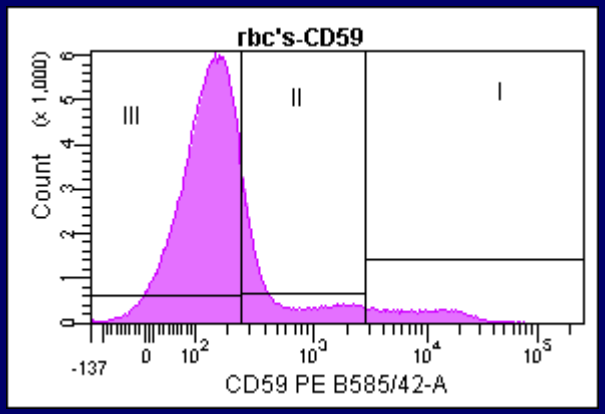


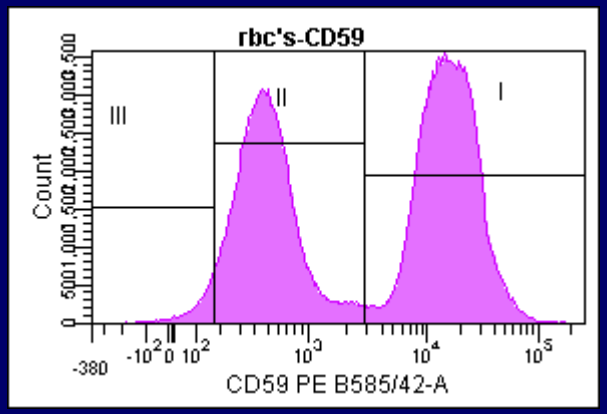


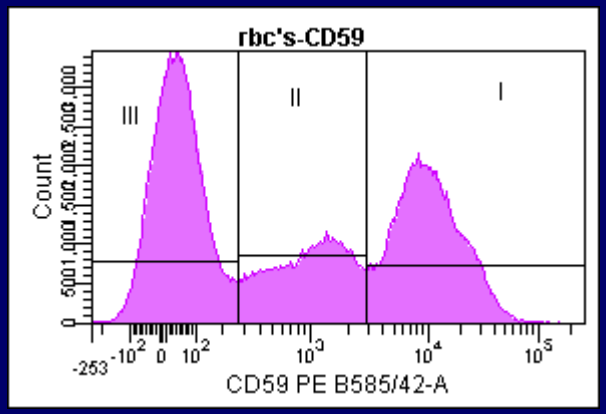


CD59PE
Clone MEM-43

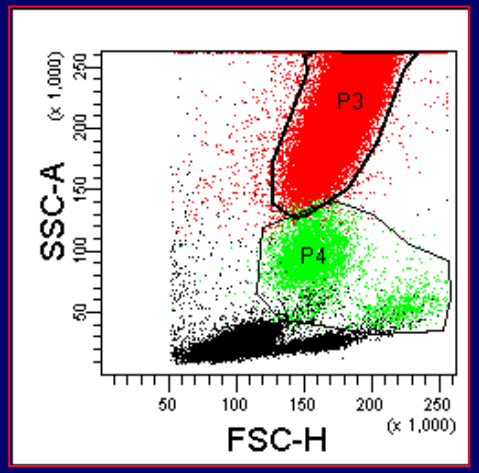


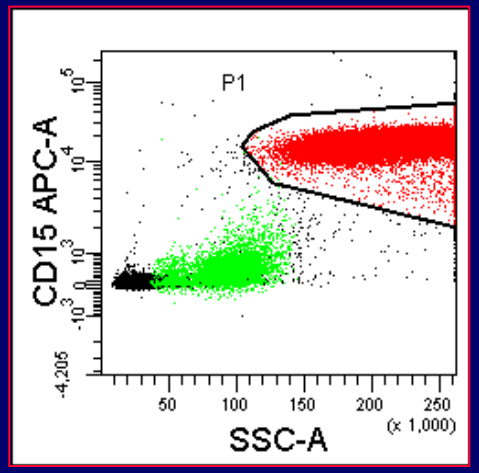


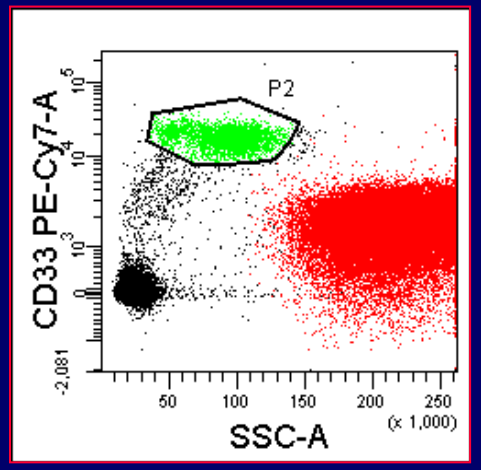




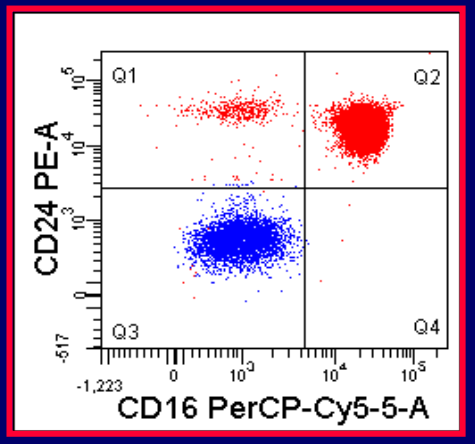
Optimised 6 colour assay for PNH Granulocytes and Monocytes

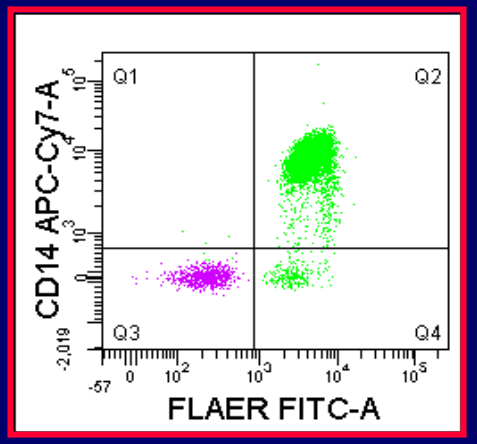






Preset regions to identify granulocytes (P1+P3) and monocytes (P2+P4)

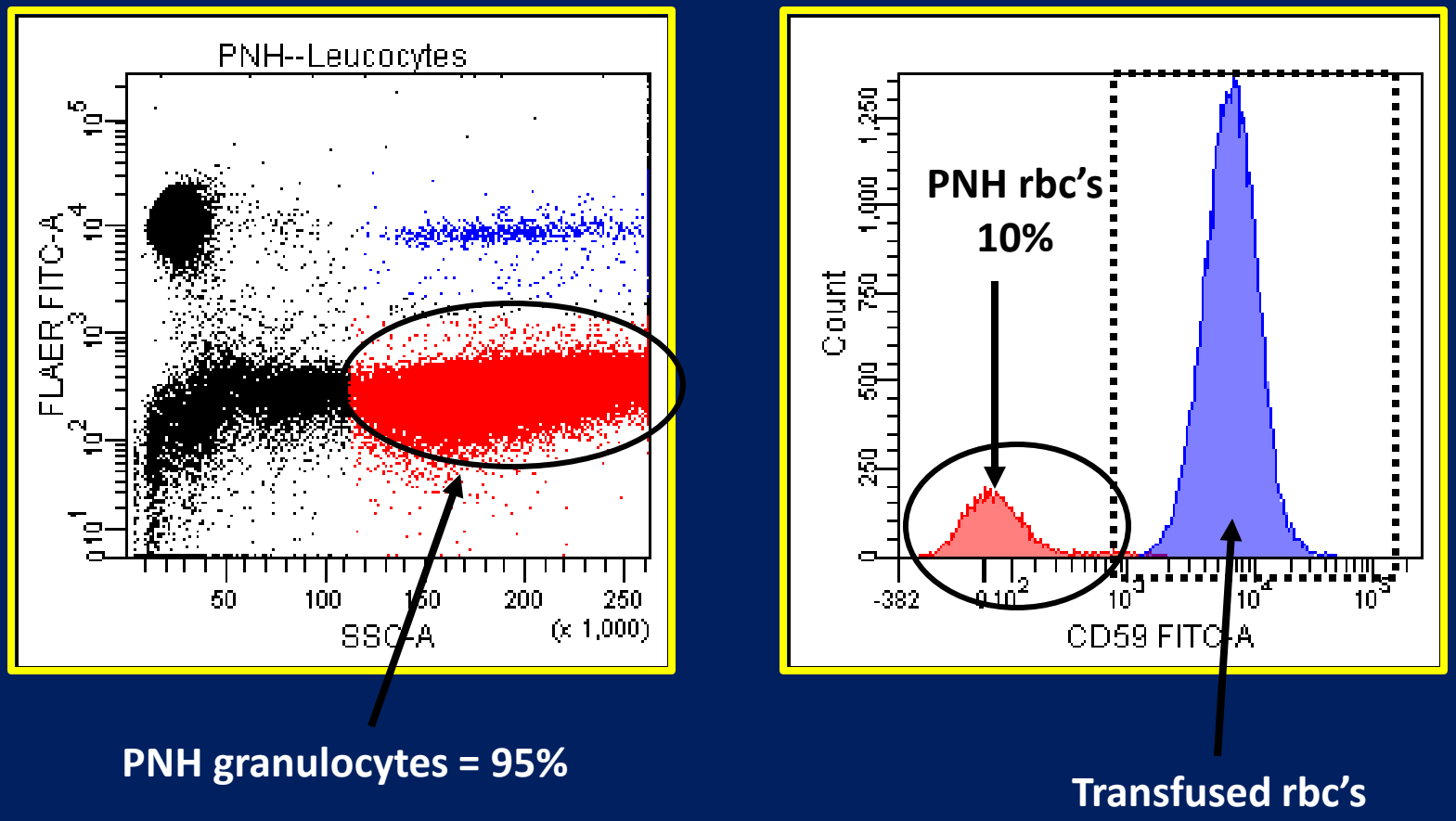




PNH granulocyte clone = 8.9%

PNH Monocyte clone = 8.1%

PNH Clone Sizes in a Typical Haemolytic PNH Patient



Disparity between granulocyte and red cell PNH clone size due to haemolysis and transfusion.

How big is my PNH clone this time?

Has it gone down again?

Patient Monitoring by Flow Cytometry

Monitoring of PNH Clones by Flow cytometry

- For the newly diagnosed patient with PNH there is the prospect of attending hospital in-patient and out-patient clinics for many years (>50).
- Clinical utility of PNH clone size measurements in identifying patients at risk of thrombosis.
  - Hall et al, Blood, 2003 - >50% PNH Granulocytes.
  - Moyo et al, BJH, 2004 - >61% PNH Granulocytes.
- Clinical utility of serial measurements of PNH clone sizes.
  - Can this predict clinical behaviour
  - Prospectively guide more effective patient management

Complement Blockade Therapy with Eculizumab

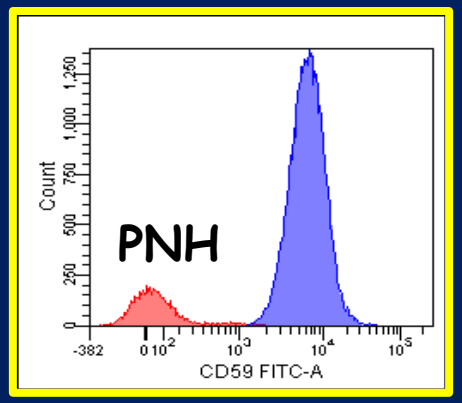
- Complement blockade therapy with Eculizumab has profound benefits for patients with haemolytic PNH [1,2]
  - Reducing or eliminating transfusion dependence
  - Improving other clinical symptoms related to the severe haemolysis in PNH
  - Improving quality of life

- Flow Cytometry has played a key role in monitoring the effectiveness of the therapy.

1 Hill A et al Blood 2005
2 Hillmen P et al N Engl J Med *2004*
3 Hillmen P et al N Engl J Med *1995*

Complement Blockade Therapy

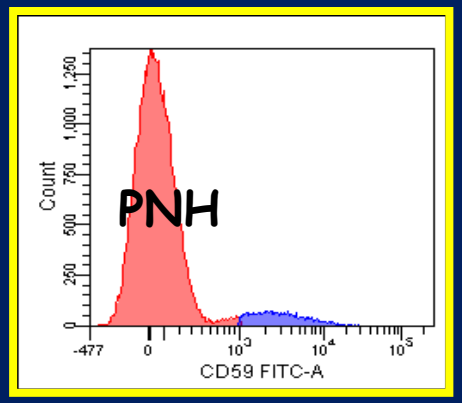
- For all patients with PNH we monitor the level of PNH red cell and granulocyte clones by flow cytometry on a regular basis.
- For patients on eculizumab this is done more systematically (3 monthly).
  - Red cell PNH clone - effectiveness of response to eculizumab
  - Granulocyte PNH clone reflects activity at the HSC (stable/increasing/decreasing)



Pre-treatment

Eculizumab Blockade

Red cells



On treatment

Terminal Complement Activation causes Intravascular Haemolysis

Classical pathway

Blocked by eculizumab

C5a

C3 → C3b → C5

Opsonisation

C5b → C5b, 6, 7, 8, 9

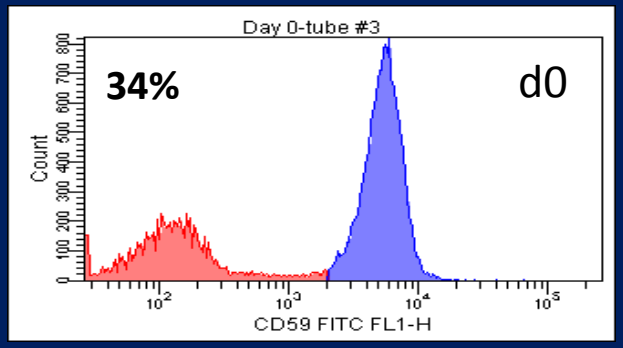
Membrane attack complex

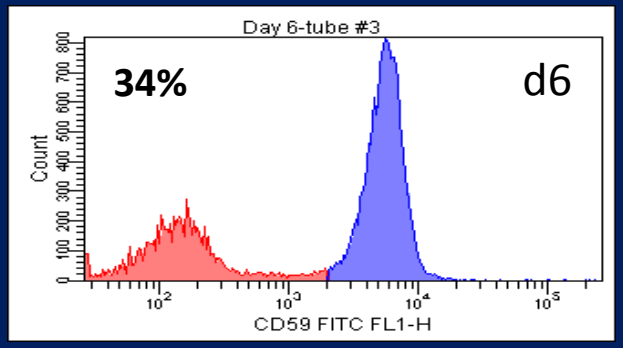
Alternative pathway

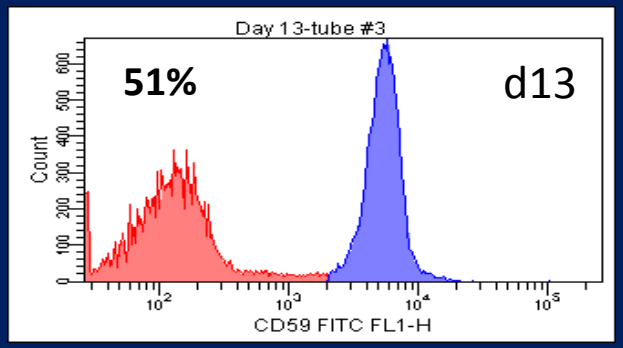
- CD55 (DAF) disrupts C3 covertase
- CD59 (MIRL) binds MAC preventing C9 binding
- CD55 & CD55 GPI-linked→deficient in PNH patients
- PNH rbc's undergo chronic haemolysis

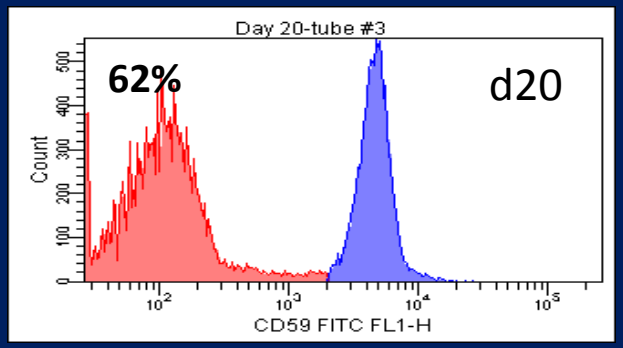
SOLIRIS® (eculizumab) [package insert]. Alexion Pharmaceuticals; 2007.

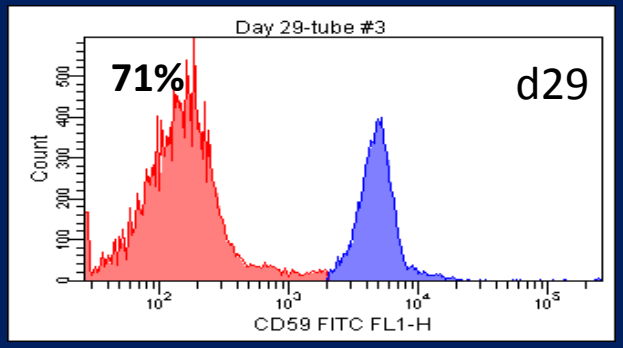
Patient 1: Granulocyte PNH clone >99%
Initial Phase: Day 0 to 29 of Eculizumab therapy
Changes in proportion of PNH red cells and residual normal red cells





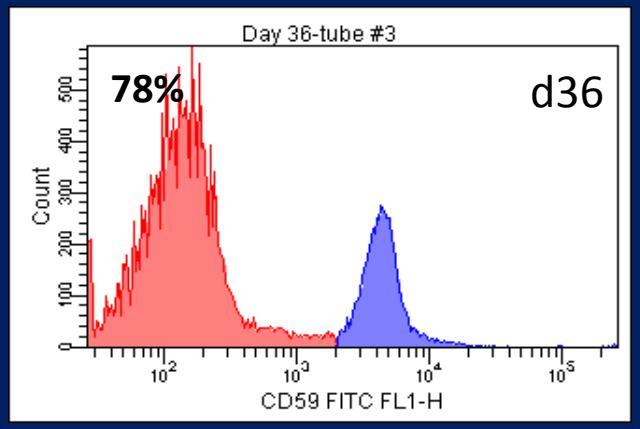


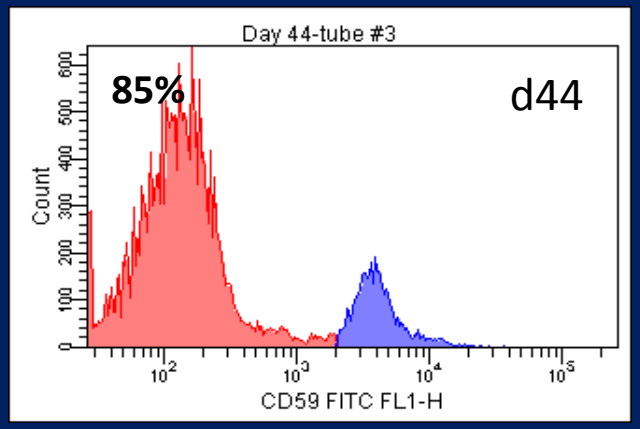


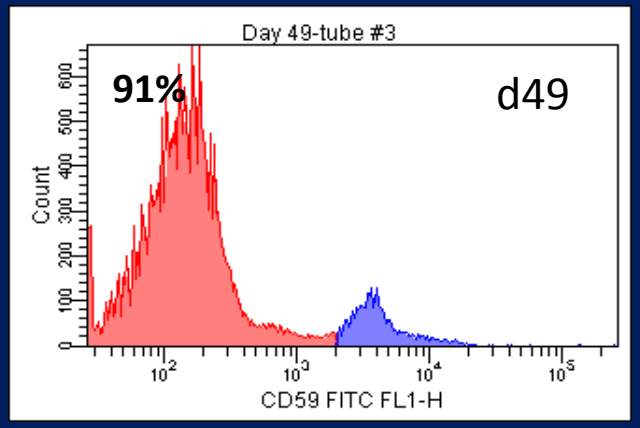


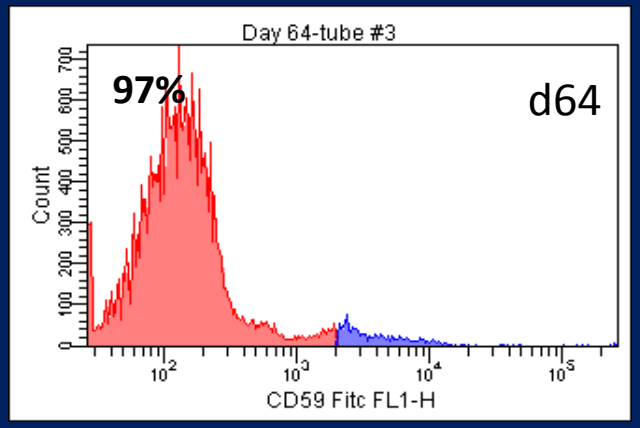
Index case

Patient 1: Granulocyte PNH clone >99%
Phase: Day 36 to 64 of Eculizumab therapy
Changes in proportion of PNH red cells and residual normal red cells



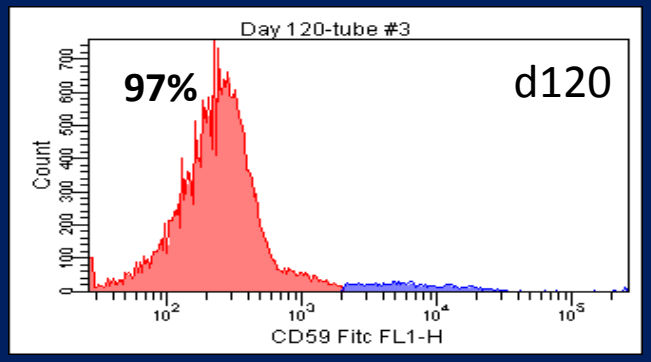


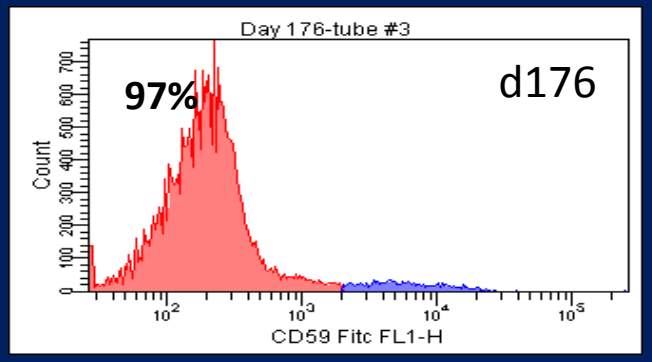


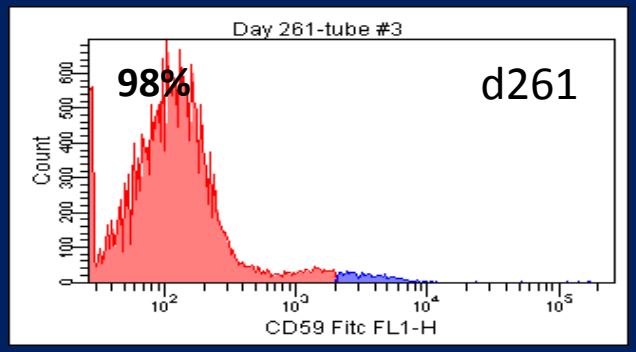


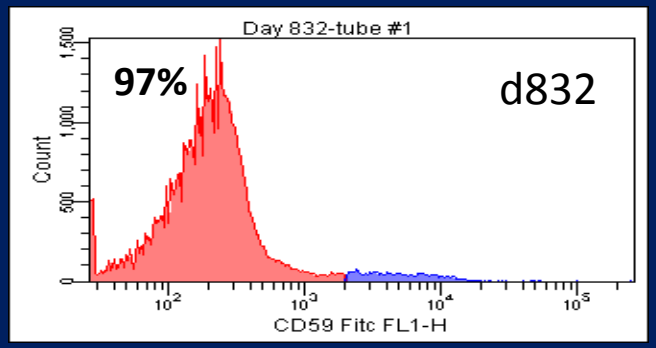
Index case

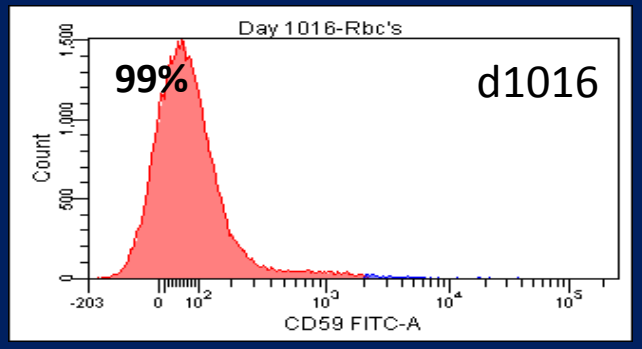
Patient 1: Granulocyte PNH clone >99%
Phase: Day 120 to 3 years of Eculizumab therapy
PNH red cells remain >97%





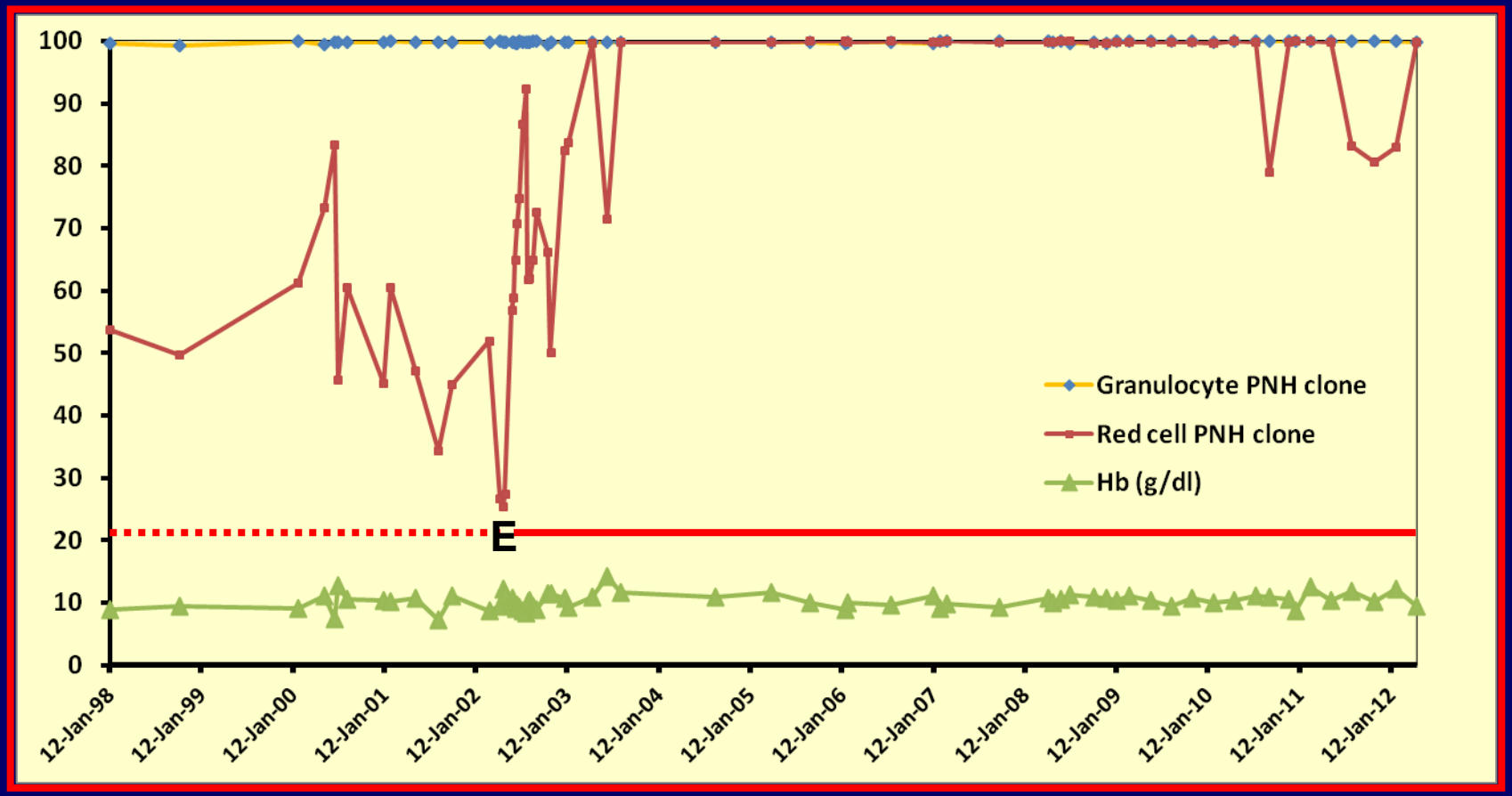






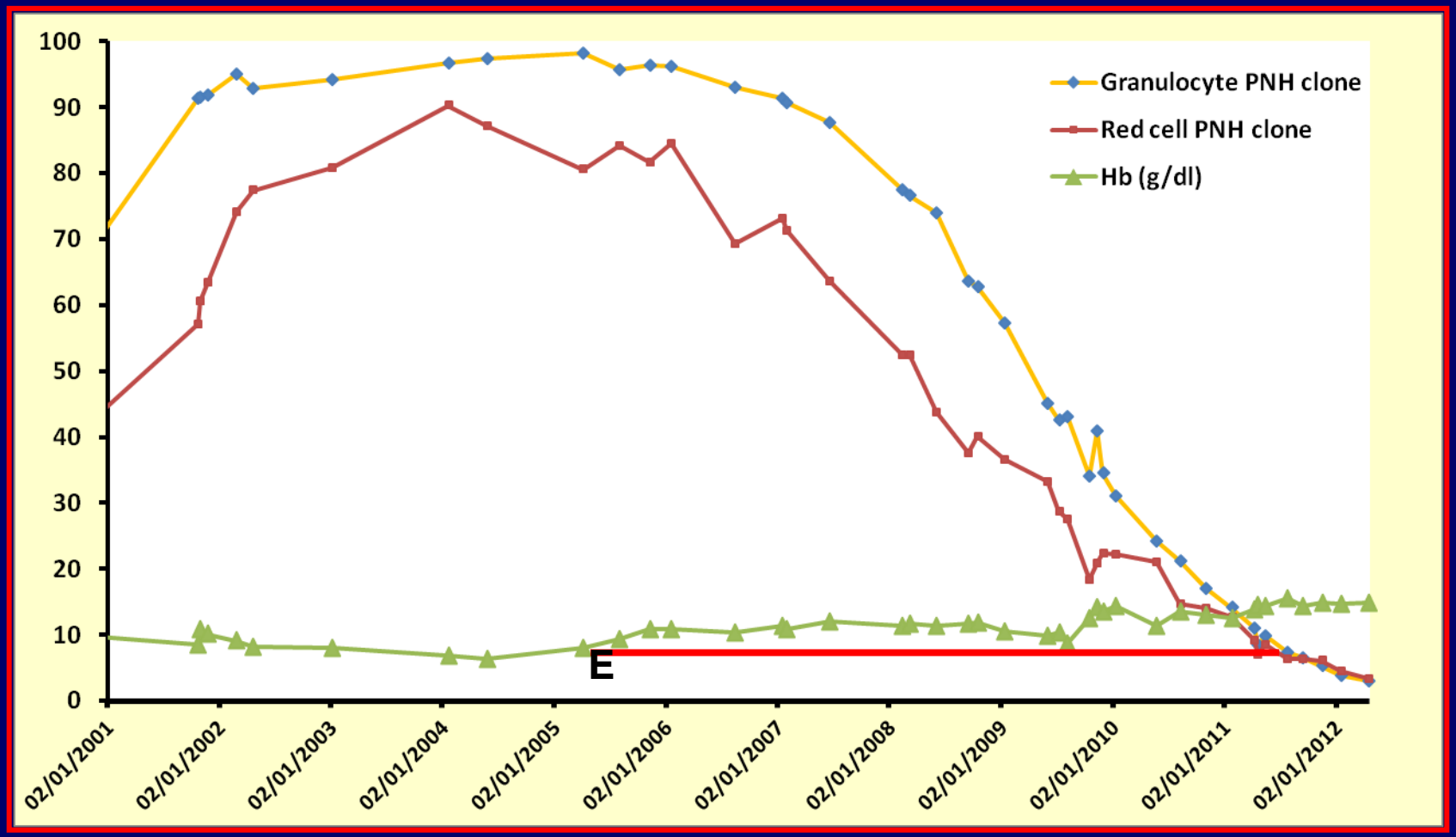
Index case

Long term follow up – Eculizumab therapy



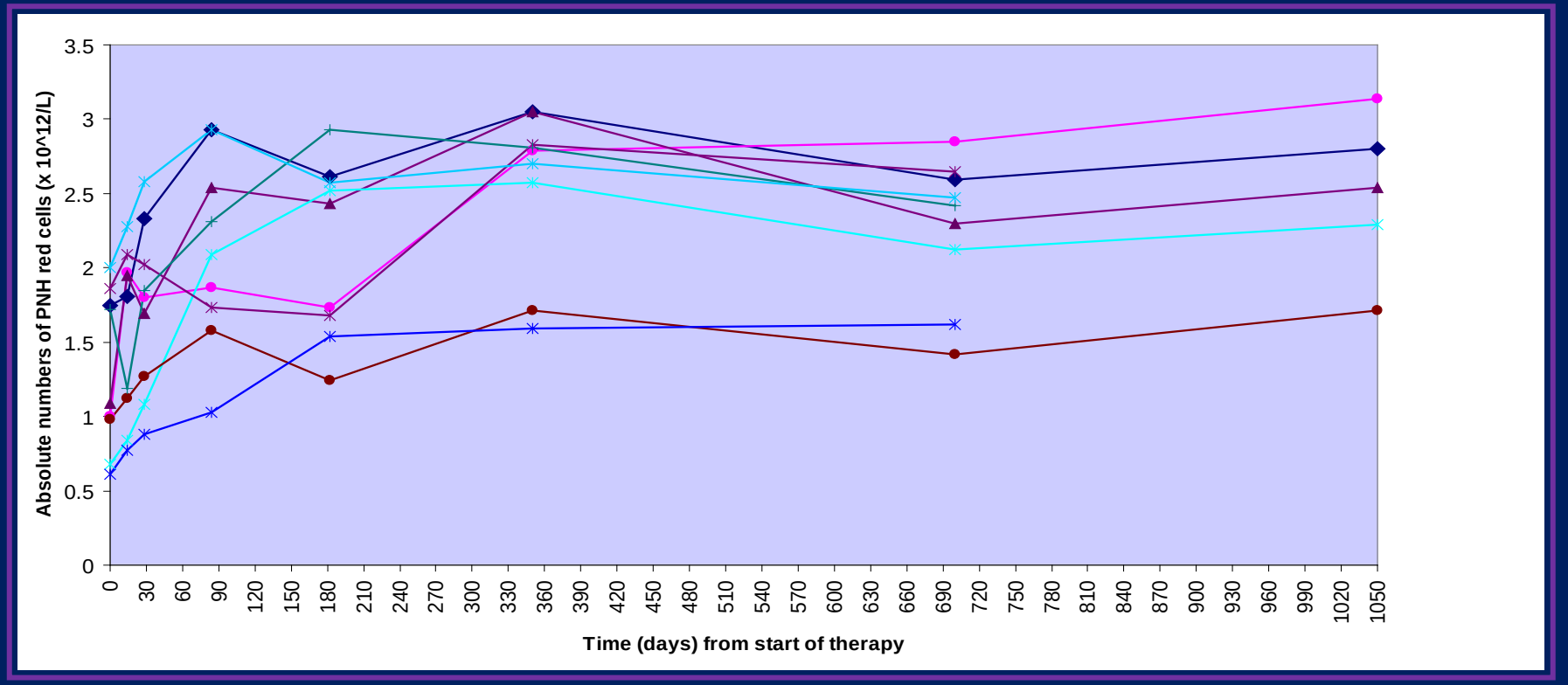
Index patient: started 22/5/2002

Long term follow up – Eculizumab therapy



Therapy from June 2005 – May 2011

Long Term Temporal Changes In Absolute Numbers PNH Red Cells & Sustained Improvements And Transfusion Independence



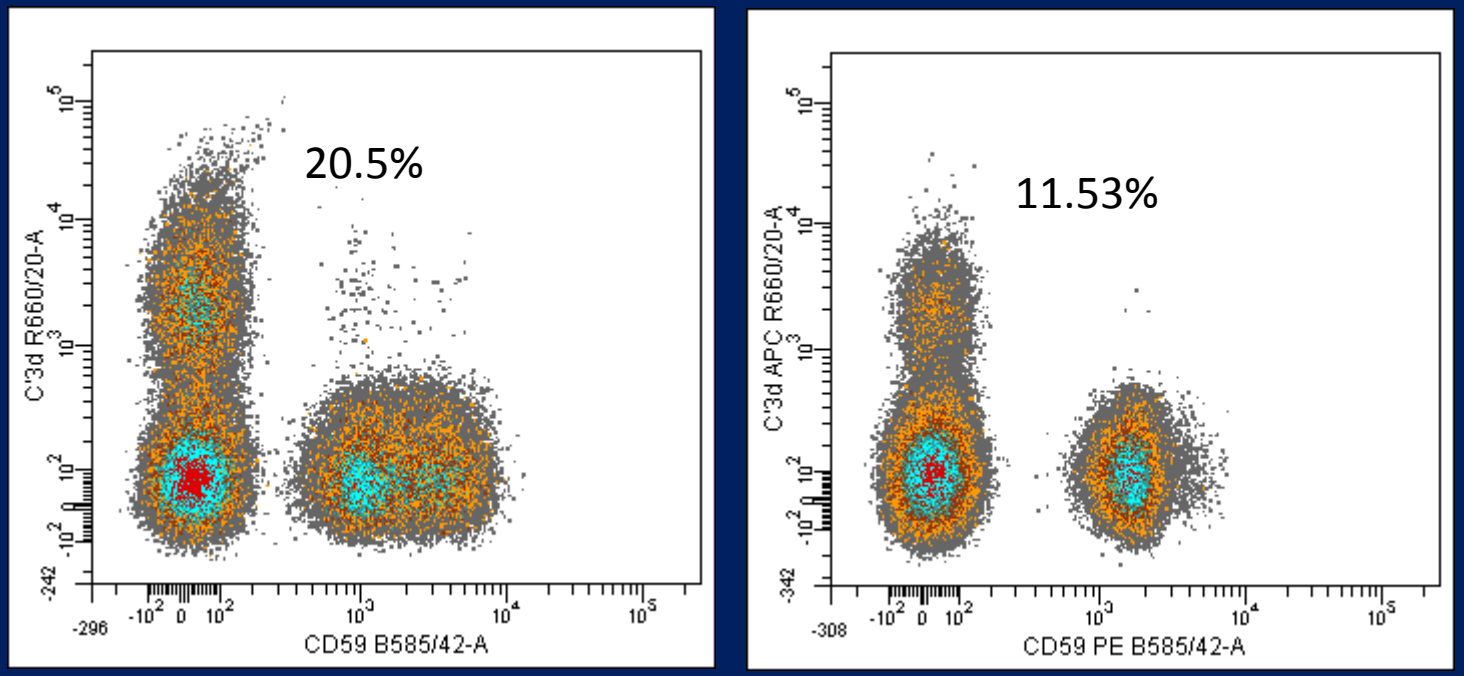
Impact of Eculizumab Therapy

- Eculizumab: positive clinical benefits
  - Rapid resolution of haemolysis
  - Long term stability in rbc PNH clone size
  - Transfusion independence
  - Markedly improved QoL

- Eculizumab: >100 patients now on therapy
  - Monitoring by flow cytometry of red cells and leucocytes
  - 10 years – changing the natural history/ clinical course of the disease

Long term Eculizumab treatment
Complement C’3 binding

C’3d APC



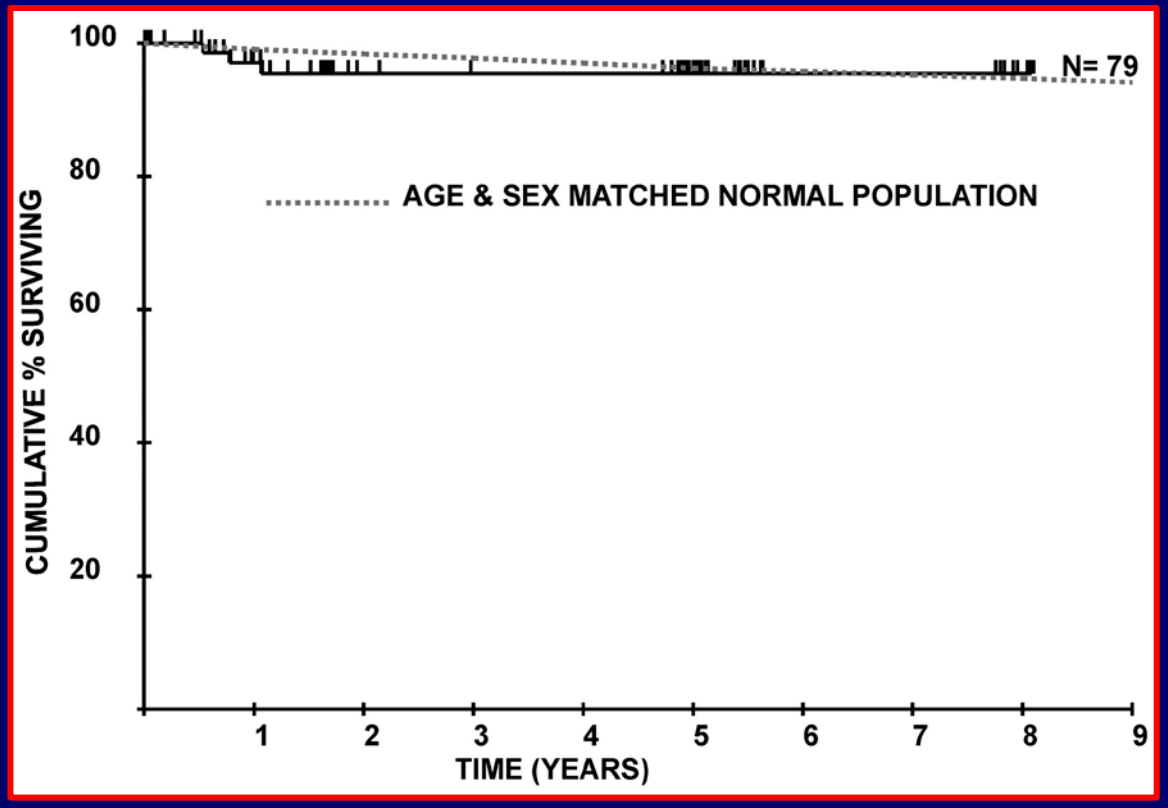
CD59 PE

- Accumulate C’3d on the cell membrane.
- Extra vascular removal of coated red cells by macrophages
- Positive DCT

Complement fraction 3 binding on erythrocytes as additional mechanism of disease in paroxysmal nocturnal hemoglobinuria patients treated by eculizumab. Risitano AM et al, Blood. 2009 Apr 23;113(17):4094-100.

Eculizumab prevents intravascular hemolysis in patients with paroxysmal nocturnal hemoglobinuria and unmasks low-level extravascular hemolysis occurring through C3 opsonization. Haematologica. 2010 Apr;95(4):567-73. Hill A, Rother RP, Arnold L, Kelly R, Cullen MJ, Richards SJ, Hillmen P.

Overall survival of 79 patients from initiation of eculizumab treatment compared with an age- and sex-matched normal population.



Kelly R J et al. Blood 2011;117:6786-6792

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Summary

1. Immunophenotyping/Flow Cytometry is the best method for screening, diagnosis and follow up of patients with PNH.

2. Follow-up/monitoring is important for clinical management of patients

    1. Risk of thrombosis
    2. Disease remission
    3. Response to therapy
    4. Disease progression

Acknowledgements

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Alexion

UKNEQAS LI

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CCS PNH Guideline team

British Blood Transfusion Society

Leeds NCG PNH Team