

## Flow cytometric analysis of CD55 and CD59 expression on blood cells in paroxysmal nocturnal haemoglobinuria

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**Abstract:** PNH is a rare clonal disorder of hematopoietic stem cells, therefore all blood cells lineages are involved. The main feature is an increased sensitivity of erythrocytes to complement-mediated cell lysis due to deficiency of membrane-bound GPI (glycosylphosphatidylinositol)-anchored proteins which normally function as inhibitors of reactive hemolysis. In the present study, we performed flow cytometric analysis using monoclonal antibodies against CD55 and CD59 for the detection of PNH-type clone in the blood of 50 patients (28 females and 22 males, age range 7-67 yrs). In one patient only we found a large population (95%) of granulocytes with decreased expression of both CD55 and CD59 molecules (type I PNH) and in two others with partial loss of CD55 expression (type II PNH). The expression was determined chiefly on granulocytes which in the control group showed reliable and high expression of CD55 and CD59.

**Key words:** Paroxysmal nocturnal haemoglobinuria - CD55 - CD59 - DAF - MIRL - Haemolytic anaemia - Flow cytometry

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### Introduction

Paroxysmal nocturnal haemoglobinuria (PNH) is a rare acquired hematological disorder characterized by anemia, intravascular hemolysis, bone marrow hypoplasia, as well as tendency to thrombosis and infection. Increased sensitivity of erythrocytes to complement-mediated cell lysis is due to deficiency of membrane-bound GPI (glycosylphosphatidylinositol)-anchored proteins which normally function as the inhibitors of reactive hemolysis [7, 17]. GPI-anchored proteins include: complement regulatory proteins like DAF (decay accelerating factor, CD55), MIRL (membrane inhibitor of reactive lysis, CD59), and other membrane proteins participating in immune function (CD14, CD16, CD24, CD48, CD58, CD66b, CD67) [4, 12, 13, 15]. PNH is a clonal disorder of hematopoietic stem cells and all blood cell lineages are involved. Disturbances of GPI-anchored proteins are commonly due to somatic mutation of the phosphatidylinositol glycan complementation class A (PIG-A) gene [9]. The mutation can be either spontaneous or facilitated by some conditions [7]. The coexi-

stence of multiple clones with different mutations of PIG-A gene in the same PNH patient has been also reported. Apart from mutation there is also a survival advantage of the PNH clone [1,10]. Dominance of PNH clone is probably due to negative selection against the non-mutated cells rather than positive selection of the PIG-A gene mutant cells [3, 10]. In PNH patients, usually coexist normal and deficient subpopulations of cells with regard to GPI-anchored protein expression. PNH-phenotype has been also described in various hematological disorders, such as aplastic anaemia, myelodysplastic syndromes, myeloproliferative and lymphoproliferative syndromes like acute non-lymphoblastic leukemias, non-Hodgkin's lymphomas, Hodgkin's disease and acute lymphoblastic leukemias [10, 12, 15, 18]. Various tests are used for the determination of PNH. Routine diagnostics involves: the Ham's test (acidified serum lysis test) and the sugar water yeast (sucrose hemolysis) test but both determine only the sensitivity of red blood cells to complement-mediated lysis. For the evaluation of reduced expression of GPI-anchored proteins on all blood cells, flow cytometric analysis is probably the best method to date [13,14]. In the present study, using monoclonal antibodies against CD55 and CD59 and flow cytometry, we searched for PNH-type clone in the blood of patients with clinical symptoms suggesting PNH.

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## Materials and methods

**Patients.** We studied 50 patients (28 females, 22 males, aged 7 to 67 years) clinically suspected for PNH, diagnosed at the Department of Haematological and Haemopoietic Proliferative Diseases and Department of Child Infectious Diseases of University of Medical Sciences, Poznan, Poland. This group clinically manifested hemolytic anaemia, in some patients accompanied by hematuria, abdominal pain and signs of persistent infection, most commonly of the urogenital tract. Five control subjects were healthy (blood donors).

**Detection of PNH-type cells.** Five milliliters of peripheral blood was collected from antecubital vein to a tube with EDTA anticoagulant. One-hundred microliter blood samples were immunostained in tubes with the following two-color combinations of monoclonal antibodies (Becton Dickinson): isotype control mAbs and anti-CD45 FITC/CD14 PE, anti-CD3 FITC/CD19 PE, anti-CD4 FITC/CD8 PE, anti-CD3 FITC/CD16+56 PE mAbs for determination of main subpopulations of leukocytes.

For the detection of PNH-type granulocytes, monocytes and lymphocytes we used fluorescein-isothiocyanate (FITC)-labeled anti-CD55 (clone IA10) and phycoerythrin (PE)-labeled anti-CD59 (clone p282, H19) monoclonal antibodies (Becton Dickinson).

The cells were incubated with antibodies at room temperature for 15 min in the dark, then a 2-mL volume of FACS-lysing solution (Becton Dickinson) diluted 1:10 was added to each tube, gently mixed, and incubated for further 10 min in the dark. The tubes were washed twice with PBS and centrifuged at  $1200 \times g$  for 4 min. The white cell pellets were resuspended in 0.5 mL PBS and analyzed by flow cytometry using a FACScan flow analyzer and CellQuest software (Becton Dickinson). At least  $20 \times 10^3$  events were collected. Expression of CD55 and CD59 molecules was quantified by the determination of mean fluorescence intensity (MFI).

## Results and discussion

In the group of patients with the suspicion of PNH, alterations of expression of CD55 and CD59 were found in 3 patients only. In two patients, partial depletion of the above-mentioned markers (so-called type II, Fig. 1) was established, while in one a total loss of both antigens was evident (so-called type I, Fig. 2) [14]. The criterion of recognition of cell clone showing absence of both markers was the detection of cell subset (above 5% of cells) in which there was a fall of expression of both antigens, at least 1 log MFI. Population of phagocytes (neutrophils and monocytes) was considered as the most suitable for the evaluation of CD55 and CD59 expression. For cytometric analysis only such cells were selected which in terms of FSC/SSC (size, cell granularity) showed features of living, undamaged and not apoptotic cells. In the control group (healthy blood donors), there was no alteration in the expression of CD55 and CD59 on phagocytic cells (Fig. 3).

The results of this study show that PNH is rather rare disorder in spite of clinical suspicions. We were able to confirm PNH diagnosis in only 3 patients out of 50 tested, what corresponded to 6% of cases.

We analyzed the expression of two GPI-anchored proteins CD55 and CD59. Both are regarded as the most valuable diagnostic markers of PNH [6, 12, 19]. CD59

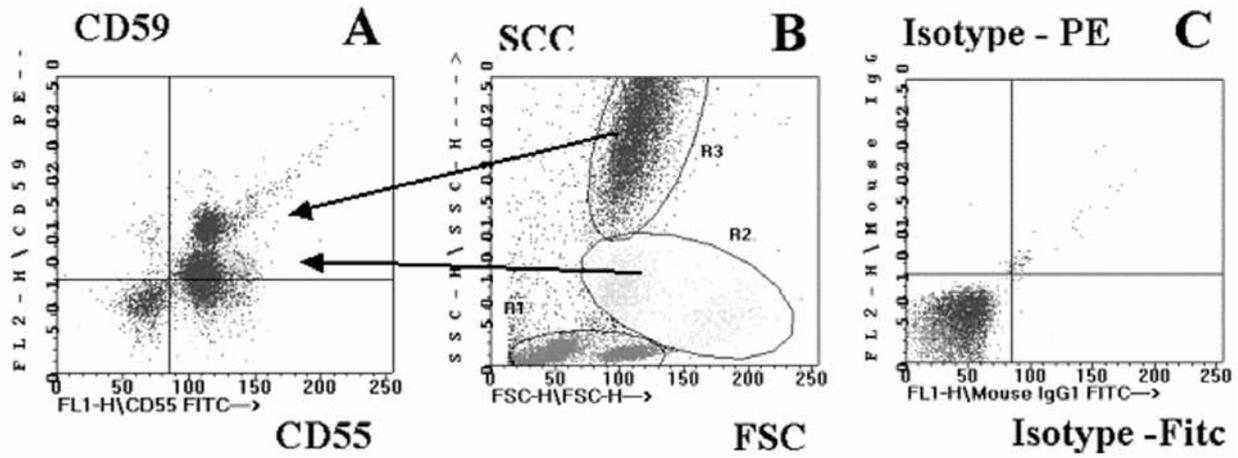
is probably the most important protein, protecting the cell from the complement-dependent lysis [17].

According to Hsi [8], it is necessary to evaluate both markers because the assessment of only one antigen (CD59) provides false positive results in blood donors. Other members of GPI complex such as CD67, CD48, CD24 and CD16 are also considered as markers of PNH [17], but their use for detection of the disease-specific cell clone is less common.

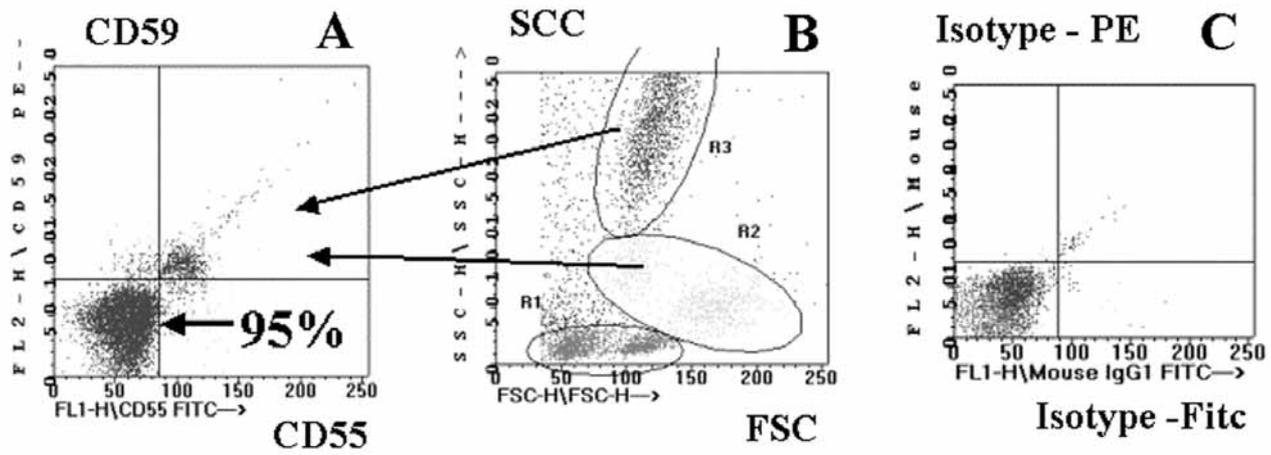
Expression of CD55 and CD59 may be determined on all blood cells such as leukocytes, erythrocytes and platelets [17]. We have chosen the population of phagocytes (both granulocytes and monocytes) as relatively durable during laboratory processing. Erythrocytes showing deficit of GPI proteins are known to be hemolyzed easily, what makes the detection of searched clone difficult [12, 13]. Alternatively one can assess reticulocytes, because this cell subset is usually increased in patients with PNH and shows higher resistance to hemolysis than mature red blood cells [4]. According to Oelschlagel *et al.* [12], the optimal approach appears to be a simultaneous evaluation of both erythrocytes and granulocytes for the expression of CD55 and CD59. This is possible with the use of commercial kits REDQUANT and CELLQUANT for assessment of the respective cell subsets.

Detection of the cell clone possessing PNH phenotype may be facilitated by the application of monoclonal antibodies against CD11b and glycophorin in concert with CD55 and CD59. This approach provides better gating of the appropriate subpopulations [18]. Another possibility for better definition of negative clones could be to determine separate cell subsets based on Laser Scanning Cytometry, new technology combining capabilities of flow cytometry and morphology [5] or to assess distribution and density of target molecules [16]. In this study, the presence of negative cell population above 5% was adopted as a criterion for recognition of the PNH cell clone. Cell acquisition was  $20 \times 10^3$ . According to Wang *et al.* [18], using sensitive flow cytometry and acquisition of  $10^5$  cells one can accept the presence of  $>0.003\%$  cells within CD11b+ cells (granulocytes) as a PNH clone. It may explain confirmation of PNH diagnosis in three patients only within the cohort of 50 patients. Such low number of positive cases may be also due to *in vivo* lysis of cells showing altered expression of GPI complex and problems with the detection of single cells.

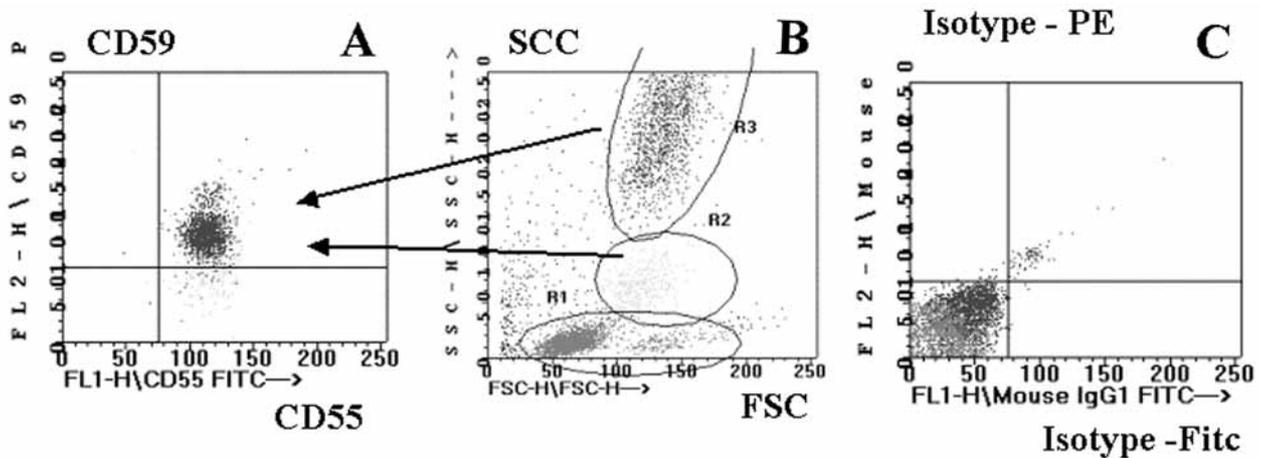
It is worth to remember that the clone with features of PNH can be found in the blood of patients with myeloid or lymphoproliferative disorders [7, 11]. Moreover, the presence of CD55-negative and CD59-negative cell clone (most commonly only one marker is lacking) is considered to be a good prognostic factor in myelodysplastic syndromes. This is manifested by lower tendency to progression towards acute leukemia, less karyotypic



**Fig. 1.** Scattergram A shows partial loss of CD59 expression on backgated (scattergram B) monocytes and granulocytes in patient with type II PNH. Scattergram C represents isotype control.



**Fig. 2.** Scattergram A shows almost absence of CD55 and CD59 expression on backgated (scattergram B) monocytes and granulocytes in patient with type I PNH. Scattergram C represents isotype control.



**Fig. 3.** In comparison to PNH, monocytes and granulocytes (scattergram B) shows uniform and full expression of CD55 and CD59 antigens on all cells in healthy individual. Scattergram C represents isotype control.

disturbances and better response for cyclosporin treatment. It is supposed that the presence of PNH clone in the course of myelodysplastic syndrome suggests milder damage of the bone marrow [7].

To summarize, cytometric evaluation of CD55 and CD59 antigens in the population of blood granulocytes/monocytes permits rapid confirmation of diagnosis of paroxysmal nocturnal hemoglobinuria, at least in those patients in which complement-dependent cell lysis is relatively low. Moreover, this assay may add to monitoring and prognosis in patients with myelo- and lymphoproliferative disorders.

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Received: December 3, 2003

Accepted after revision: December 15, 2004