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Paroxysmal nocturnal haemoglobinuria. Experience over a 10 years period

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SUMMARY

Introduction: Paroxysmal nocturnal hemoglobinuria (PNH) is a hemolytic, clonal and acquired disorder of the hematopoietic stem cell with a deficiency of all glycophosphatidyl-inositol (GPI) linked proteins. The aim of this retrospective study was to analyse haematological and biochemical data from 152 patients referred to our laboratory for diagnosis of PNH by flow cytometry (FC).
Methods: Patients and healthy donor (152 and 99 respectively) were studied. Ham, sucrose, lactate dehydrogenase (LDH), Iron, haptoglobin (Hp), blood cell morphology and Kaplow cytochemical stain for leukocyte alkaline phosphatase (LAP) were carried out. GPI-proteins anti-CD55 and CD59 in erythrocytes and the former, plus anti CD16b and CD66b on neutrophils were evaluated by FC.
Results: Anemia and/or leukopenia and/or thrombocytopenia, increased reticulocyte count and LDH were observed in patients with PNH clone. Some of them had dacriocytes, schistocytes. LAP was low. On average, we detected 50% CD59 (−) erythrocytes and 29, 83, 78% CD55/59 (−), CD16b (−), CD66b (−) neutrophils, respectively.
Conclusion: Paroxysmal nocturnal hemoglobinuria clone was detected in 20/152 patients. Negative population's percentages were high in patients with classic PNH, Hematimetry, LAP and adequate use of CF contribute to PNH clone detection in the laboratory.

INTRODUCTION

Paroxysmal nocturnal haemoglobinuria (PNH) or Marchiafava-Micheli syndrome is a haemolytic, clonal and acquired disorder of the haematopoietic stem cell with associated production of affected blood cells [32]. PNH is the consequence of the somatic mutation in an X-linked gene (PIG-A gene) involved in the synthesis of glycosyl phosphatidyl inositol (GPI). GPI anchors a number of proteins of the cellular membrane [21], and their lack may be responsible for most of the PNH symptoms [6, 13, 30]. Among these symptoms, cytopenias, iron deficiency, infections, altered kidney functions and thromboembolic events can be found in PNH patients [24]. Vein thromboembolism is one of the most severe conditions that occurs in

12–40% of cases, the most frequent being localization in the hepatic vein. On the other hand, intravascular haemolysis has been related to the presence of free haeme, which is able to interact with nitric acid metabolism and oxidative damage production, although it may be also associated to the occurrence of thrombosis through the activation of endothelial cells [9, 25].

As it has been reported, there is a diminished expression of proteins such as CD55 (DAF- decay accelerating factor), CD59 (membrane inhibitor of reactive lysis), CD16b (FcγRIIIb), CD66b (member of carcinoembryonic antigen), CD14 (membrane receptor) and CD24 (signal transducer CD24) as well as leucocyte alkaline phosphatase (LAP) [10].

PNH has been classified into three groups by International PNH Interest Group as follows: (i) CLASSIC: patients with haemolysis and thrombosis, (ii) SUBCLINICAL: patients with small PNH clone, without clinical or laboratory haemolysis and (iii) PNH in the setting of other bone marrow failure syndromes such as aplastic anaemia (AA) and myelodysplastic syndromes (MDS) [32]. It is currently considered that immune-mediated injury of hematopoietic cells is implicated in PNH marrow failure as well as in AA. A possible explanation being that some GPI-anchored proteins may be a critical target recognized by immune effector cells. PNH clones not possessing these critical GPI-anchored proteins will survive because they are selectively resistant to the autoimmune assault that eliminates most normal clones. MDS, another clonal condition, shares physiopathological characteristics with PNH, such as bone marrow failure, response to immunosuppressants and the presence of protein deficient cells anchored to GPI [5, 18]. The deficiencies in CD55 and CD59 have also been reported in other haematological disorders such as lymphoproliferative syndromes, particularly in acute lymphoblastic leukaemia and Hodgkin lymphoma [19, 20].

The oldest and most traditional laboratory tests employed for the diagnosis of PNH are Ham and sucrose which evaluate the susceptibility of erythrocytes to haemolysis due to complement activation [3, 7, 8]. More recently flow cytometry (FC) assays have also been introduced for the identification and quantification of GPI-anchored proteins (GPI-APs) [11, 15]. FC assays use labelled antibodies directed against GPI-anchored proteins (AP). At least two GPI-APs in two different blood cell populations have been recommended, which allows the exclusion of congenital absence of antigen expression and polymorphism, among others [2, 31, 35]. Although CD55 and CD59 were used on red cells for FC analysis, CD59 allowed for a more accurate expression and the identification of subpopulations: PNH I are erythrocytes with normal expression of CD59, while PHN II and PNH III have partial or complete deficiency of CD59 [2, 21, 24].

The aim of this retrospective study was to analyse clinical, haematological, and biochemical data from 152 patients referred to our laboratory for diagnosis of PNH by FC assays. This study followed guidelines outlined by [26]. Due to the unavailability of FLAER in our country, we were unable to follow the guidelines proposed by [2].

MATERIALS AND METHODS

Patients

One hundred and 52 patients, ages ranging between 2 and 86, 99 females and 53 males, referred to our laboratory for PNH diagnosis from 2002 to 2012, and 99 healthy donors were evaluated. Both the patients and the control group gave their informed consent according to the ethical standards for medical research on humans of the Helsinki Declaration of the World Medical Association and the Ethics Committee of Academia Nacional de Medicina.

Blood samples

Fifteen millilitre of peripheral blood (PB) was obtained from patients and from healthy controls. PB was collected in K3EDTA. Ham and sucrose tests were carried out as previously described [7, 8]. Lactate dehydrogenase (LDH) [14], iron and haptoglobin (Hp) were determined in serum samples. PB smears were employed to blood cell morphology and Kaplow cytochemical stain for LAP.

Monoclonal antibodies

The following monoclonal antibodies to GPI-linked protein CD55 PE (clone 67 N -L063, mouse IgG1),

CD59 FITC (clone MEM-43, mouse IgG2a), CD16b PE (3G8) all from Caltag Invitrogen (California, CA, USA), $CD66_b$ FITC (G10F5, BD Pharmingen, San Diego, CA, USA) as well as CD45- PE-Cy5 (HI30; BD Pharmingen) and CD49d (44H6; Chemicon International, Inc., Temecula, CA, USA) a transmembrane form were employed.

PNH clone detection

In erythrocytes, PB was centrifuge at 180 ***g*** for 10 min, and an aliquot of the globular package was washed three times with saline solution (PBS) and diluted 1/400 in PBS for identification of red cells. After that, 0.1 ml of the suspension was stained with antibodies anti-CD55 or anti-CD59 for 30 min at 4 °C, washed and suspended in isoflow Becton Dickinson (BD), and 25 000 red blood cells were acquired in FACScan-BD and the software used was CELLQUEST (BD San José, CA, USA). In neutrophils, two protocols were followed: (i) erythrocytes were lysed with NH4CL, washed with PBS and stained with both antibodies CD59 and CD55 for 30 min at 4 °C, washed and resuspended in isoflow: (ii) whole blood cells were stained with CD16b, CD66b and CD45 antibodies, and then, red blood cells were lysed with BD lysing solution and processed as indicated above. Thereafter, 5000 events were acquired in the neutrophils gate and the total of white cells in the sample ranging between 25 000 and 50 000. In addition, given that eosinophil does not express CD16, CD49d, staining was employed as strategy to corroborate the negative expression of CD16b on neutrophils [27].

FC analysis

The marking was done on erythrocytes and on neutrophils determining CD55 and CD59 expression on the former and the expression of CD55, CD59, CD16b and CD66b on the latter cells. To analyse these markers, the cut-off values were determined in erythrocytes and neutrophils from healthy controls. Taking into account the CD55 expression is decreased on erythrocytes, it was found convenient to set a ratio of the mean fluorescence intensity (MFI) detected between each patient and healthy control.

Besides, the cut-off values for the other GPI-AP were determined on the basis of percentage of negative cells in healthy controls expressing those markers. When GPI-AP was >1% of negative cells, PNH clone presence was considered. Representative flow cytometry analyses are shown in Figure 1.

RESULTS

Patients

They were classified according to the clinical background and the laboratory results in four groups. Group I, $n = 20$ (13.2%): patients with haemolytic anaemia and/or AA with PNH clone. Group II $n = 41$ (27.0%): bone marrow failure (AA, SMD) patients. Group III $n = 39$ (25.6%): anaemia and/or thrombosis (with or without haemolysis) patients. Some of them had unknown iron deficiency origin. Group IV $n = 52$ (34.2%): low counts of leucocytes and/or neutrophils of unknown origin patients.

As it is shown in Table 1A, the erythrocytes and haemoglobin values were decreased in all groups compared with healthy controls. Groups I, II and IV show a decrease in the absolute number of leucocytes. It is interesting to note that Group II, with AA-MDS, shows the lowest leucocyte counts. In Group I, on the other hand, there was a 'dismissed' platelets counts. ANOVA test (one-way analysis of variance) was used for the statistical analysis. Besides, Ham and sucrose tests gave negative results in Groups II, III, IV and in healthy subjects, while 18 PNH patients (Group I) tested positive for both tests (data not shown).

As can be observed in Table 1B, only patients from Group I (i.e. those with PNH clone) showed a significant decrease in MFI CD55 ratio on erythrocytes, as well as significant increase in low expression of CD59 on erythrocytes or CD55/CD59, CD16b and CD66b on neutrophils (Figure 1a,b). Different phenotypes were detected using anti-CD59 on erythrocytes.

Among the results obtained from Groups II, III and IV, there were patients showing a higher percentage of negative cells than the cut-off value in one GPI-AP marker on erythrocytes or on granulocytes, which were considered as *'inconclusive'* data. Besides, a small number of patients showed increased percentages of negative cells in different GPI-AP on the two cell populations that were named as *'suspect'*. The proportion

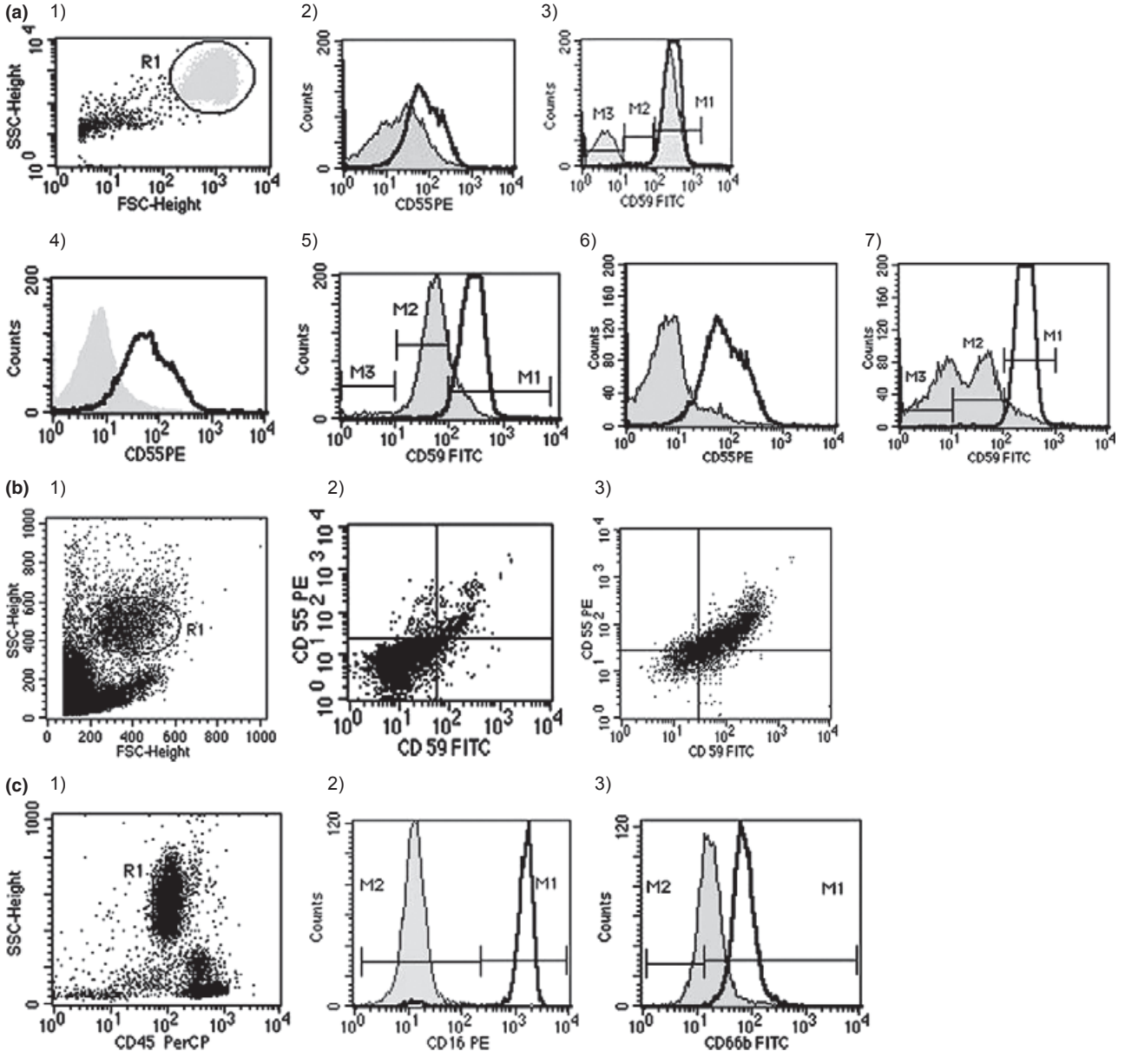

Figure 1. Representative flow cytometry histograms of GPI-AP expression in some patients with PNH clone. Dark lines correspond to normal control. a-1 Red blood cells (RBCs). Examples of the CD55 and CD59 expression are shown with different erythrocyte phenotypes. (a-2 and a-3) Phenotype I, (a-4 and a-5) Phenotype II, a-6 and a-7) Phenotype III. M, marker; M1, normal expression of CD59; M2, partial expression of CD59; M3, negative expression of CD59. B neutrophils (N). (b-1) R1 of N was drawn on forward and side Scatter. Two examples of CD59FITC and CD55PE are shown. (b-2) 86% and (b-3) 16% of negative N expression. C: N. (c-1) R1 of N was draw CD45PerCP and side scatter. c-2 and c-3, respectively, show low CD16b and CD66b expression. Overlay histograms: bold line shows normal control samples; grey shading for PNH patient samples. M1, normal expression of CD16 and/or CD66b; M2, low expression of CD16 and/or CD66b.

Table 1. Patients groups' characteristics: Group I: Patients with haemolytic anaemia and/or aplastic anaemia with PNH clone. Group II: Patients with bone marrow failure (aplasic anaemia, myelodysplastic syndromes). Group III: Patients with anaemia and/or thrombosis (with or without haemolysis). Some of them presented unknown iron deficiency origin. Group IV: Patients with low counts of leucocytes and/or neutrophils of unknown origin

Characteristics	Group I PNH	Group II AA-MDS	Group III Anaemia Thrombosis	Group IV Leucopenia	Healthy controls	*P* value
Number of patients	20 (13.2%)	41 (27%)	39 (25.6%)	52 (34.2%)	99	
(A) Haematological parameters (mean ± SD)						
Erythrocytes ($\times 10^{12}$/L)	3.0 ± 0.7	3.2 ± 0.8	3.9 ± 1.0	4.5 ± 0.6	4.7 ± 0.3	<0.01*
Haemoglobin (g/dL)	8.8 ± 2.2	9.5 ± 2.2	11.0 ± 2.5	12.0 ± 1.3	14.6 ± 0.9	<0.01*
Leucocytes ($\times 10^{9}$/L)	4.6 ± 2.1	3.2 ± 1.4	7.7 ± 3.2	3.5 ± 1.5	6.3 ± 1.4	<0.01†
Platelets ($\times 10^{9}$/L)	208 ± 240	81 ± 86	286 ± 149	245 ± 84	245 ± 55	<0.01‡
(B) Flow cytometry: GPI-anchored proteins expressions (%) (mean ± SD)						
CD55 (MFI pac/ MFI control)(E)§	0.50 ± 0.23	0.92 ± 0.21	0.96 ± 0.26	1 ± 0.28	1	
	25 ± 11	0.53 ± 0.76	0.34 ± 0.52	0.22 ± 0.21	0.22 ± 0.15	
CD59 (−) (E)§						
	29 ± 21	0.32 ± 0.44	0.16 ± 0.39	0.52 ± 1.10	0.17 ± 0.17	
CD55/59 (−) (N)¶						
	83 ± 16	3.3 ± 4.0	2.3 ± 3.6	1.7 ± 2.6	0.52 ± 0.59	
CD16b (−) (N)¶						
	78 ± 22	ND	ND	ND	0.43 ± 0.41	
CD66b (−) (N)¶						

ND, not done.
Statistical analysis: ANOVA (one-way analysis of variance).
*Between healthy controls and groups I, II, III,IV.
†Between Healthy controls and groups I, II, IV.
‡Between healthy controls and groups I, II.
§Erythrocytes. ¶Neutrophils.

of *'inconclusive'* results was found in 12%, 15% and 13% of patients and the proportion of *'suspect'* results in 3%, 2%, 5% of patients. Examples of data from five patients are shown (Table 2).

Characteristics of PNH group

Paroxysmal nocturnal haemoglobinuria (PNH) clone was found in 20 patients: 13 (65%) showed classic PNH, 6 (30%) had AA, and 1 (5%) had chronic myeloid leukaemia (CML). Eight patients (40%) were male and 12 (60%) female. Two patients were under eighteen, and the adults average age was 44 years old (range 18–75 years).

As can be observed in Figure 2, in PNH patients, the smear examination of erythrocyte morphology showed that the main alteration was the presence of elliptocytes followed by dacryocytes or tear-drop cells, target cells, basophilic stippling, schistocytes and spherocytes. In addition, the evaluation of haematological and biochemical parameters showed that 100% of the patients presented an increase in assays of the haemolytic state such as LDH and reticulocytes (Table 3), while the other parameters evaluated such as haptoglobin, LAP, Hb, iron, red cell distribution width (RDW) and indirect bilirrubin were variable among the samples. We also detected low neutrophils and, although as a whole, platelet counts were in the normal range; in 12/20 patients, decreased numbers were observed. Regarding the two patients with high numbers of platelets, one of them had CML diagnosis.

The FC analysis in PNH patients showed that the percentage of negative cells was high (>20%) for all

Table 2. Examples of Inconclusive and suspect PNH clone results

Patient group	Diagnosis	Result type	CD55 ratio E	CD59 (−) E	CD55-59 (−) N	CD16 (−) N
II	MSD	Inconclusive	0.8	0.11	0.07	**20.72**
III	Anaemia	Inconclusive	0.9	0.08	**1.17**	0.55
II	AA	Inconclusive	0.7	0.44	0.02	**7.45**
IV	Leucopenia	Suspect	0.6	0.25	**1.58**	4
II	AA	Suspect	1	**2.24**	0.07	**7.88**

Increased cells negative expression that the cut-off point in bold, MSD, myelodysplastic syndrome; AA, aplastic anaemia; E, erythrocytes; N, neutrophils; PNH, Paroxysmal nocturnal haemoglobinuria.

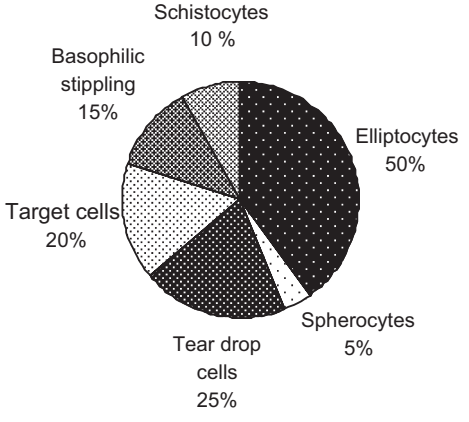

Figure 2. PNH patients: Erythrocyte morphology. Percentage of PNH patients (n = 20) with morphology alterations.

Table 3. Haematological and biochemical parameters in PNH patients

Parameter	Value	Patients (n/%)
LDH	High	20 (100%)
Reticulocytes	High	20 (100%)
Haptoglobin	Low	19 (95%)
Iron	Normal	19 (95%)
LAP	Low	19 (95%)
Hb	Low	19 (95%)
RDW	High	15 (75%)
Indirect bilirrubin	High	14 (70%)
Neutrophils	Low	13 (63%)
Platelets	Low	12 (60%)
MCV	High	9 (45%)

LDH, lactate dehydrogenase; LAP, leucocyte alkaline phosphatase; Hb, haemoglobin; RDW, red blood cell distribution width; MCV, mean cell volume; PNH, Paroxysmal nocturnal haemoglobinuria.

the markers determined in erythrocytes and neutrophils (Table 1B). Erythrocytes type I and III; 8/20 (40%) type I, II and III y 2/20 (10%) type I and II were found in 10/20 (50%) of patients.

When we analysed neutrophils, we found a different expression among the markers used: the percentage of negative expression of CD55/CD59 was smaller than that of CD16b and CD66b.

DISCUSSION

Paroxysmal nocturnal haemoglobinuria (PNH) is the consequence of PIG-A gene mutation. This product is involved in an early step in the synthesis of GPI. The clonal disorder affects the haematopoietic stem cell, which may, in turn, affect the production and function of erythrocytes, neutrophils and platelets. Investigating PNH clone in other clinical conditions was suggested as such disorders of bone marrow failure or where normal haematopoiesis failed [2, 24].

This study includes data from 152 patients referred to our laboratory, in the last 10 years, for the PNH diagnosis. According to their clinical background and laboratory results, we distributed our patients in four groups: patients with PNH clone, patients with AA and MDS, haemolytic anaemia or iron deficiency of unknown origin and patients with neutropenia. FC assays allowed for the diagnosis of 20 PNH cases.

Although PNH is rarely found in children or teenagers, we detected two patients with PNH clone in those age groups.

In agreement with other authors [22, 23, 33], we believe the PNH clone should be researched particularly

in the context of bone marrow failure, haemoglobinuria and thrombosis without apparent cause.

When we confronted FC data with the traditional HAM and sucrose tests, we found coincidences except in only two patients. One of them tested HAM positive, and the other only on sucrose. This patient showed a low percentage of erythrocyte type III; we might consider the Ham test to be less sensitive than sucrose in the detection of the PNH clone. The other patient, HAM positive but sucrose negative, showed an elevated score of LAP (data not shown), the highest level of haemoglobin and a high count of erythrocytes might have compensated for the lack of GPI (and anchored proteins) resulting in negative sucrose result.

In the erythrocyte morphology study of the PNH patients, the dacryocytes (tear-drop cells) were detected in 25% of the samples, which are frequently associated with medullar failure [1, 4]. The presence of schistocytes was observed in 5% of the patients, which often associated with thrombotic events [16, 36].

Results of PNH patients (Group I) show low haemoglobin and/or leucocytes count. Even though the average platelet count was normal, the range of values was wide. There were 12/20 patients with lower platelet and 2/20 patients with higher platelet counts (one of them with LMC diagnosis) (Table 1A). A higher reticulocyte count and LDH activity confirm the haemolytic condition and were the consequences of increased erythrocyte susceptibility to complement mediated lysis. LDH activity is a parameter of haemolysis which is associated with free haeme, the metabolism of nitric oxide and the oxidative stress. Increased thrombosis and platelet activation are other consequences [25, 35]. The unsettling of any of these systems causes serious damage to different organs. Current treatments with eculizumab prevent intravascular haemolysis in patients with PNH. The response to this is obtained by monitoring LDH levels [12, 17].

CD55 and CD59 expressions were used to analyse red cells, and with normal individuals, the absence of CD59 marking was found to be 0.22% of erythrocytes [2]; therefore, we think that the technique used has adequate sensibility. This should prove useful in PNH clone detection as regards early occurrence, identification of the subclinical type of disease or in the context of other clinical entities.

CD59 marking was not only more sensitive than that with CD55, it also enable the identification of different erythrocyte types (phenotype) in the light of fluorescence intensity [10], (Figure 1). Then, we concluded that, as stated in 2009 Consensus, CD59 is the marker of choice for erythrocytes.

Low expression of CD16 on neutrophils might be detected in myelodysplastic syndromes or in the context of apoptotic neutrophils in neutropenia. Besides less than 1% of people have null phenotype or CD16b absence on neutrophils [28, 29]; to confirm HNP clone, we evaluated CD66b expression as an additional control in our study.

Then, in concordance with other authors, we evaluated at least two GPI-linked antigens on two different blood cell populations. This would the exclusion of congenital absence of antigen expression, polymorphism, haemolytic event, transfused cells among others [2, 35]. Antigen deficiency may be present even in some normal individuals (data not shown) [34]. Furthermore, the proportion of affected cells differs among the cell populations.

The expression of CD14, a GPI-anchored protein, on monocytes was researched only in nine patients by triple dialling: CD66bFITC/CD16bPE/CD14 PerCP-Cy-5.5 (BD Pharmingen). The number of cells obtained in the monocytes region was between 2500 and 5000 cells (total white cells 25 000–50 000). The evaluation of patient sample was always matched to normal voluntary donors. We found low expression of CD14 in normal monocytes <1% and >70% on PNH patients (n = 3). LAP score was added to our protocol to evaluate more GPI-APs and resort to the use a different methodology.

The present retrospective study through FC has enabled us to make a reliable diagnosis of PNH clone presence in 20/152 patients in the context of haematological parameters. 'Suspect' results have alerted us to the need for further exploration including other GPI-AP.

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REFERENCES

1. Bando S, Niiya T, Murase M, Makino H. A feature of hematological findings in myelodysplastic syndromes. Rinsho Byori 1998;46:790–5.
2. Borowitz M, Craig F, DiGiuseppe J, Illingworth A, Rosse W, Sutherland R, Wittwer C, Richards S. Guidelines for the diagnosis and monitoring of paroxysmal nocturnal hemoglobinuria and related disorders by flow cytometry. Cytometry B Clin Cytom 2010;78:211–30.
3. Brodsky RA. Advances in the diagnosis and therapy of paroxysmal nocturnal hemoglobinuria. Blood 2008;22:65–74.
4. Domingo-Claros A, Larriba I, Rozman M, Irriguible D, Vallespi T, Aventin A, Ayats R, Milla F, Sole F, Florensa L, Gallart M, Tuset E, Lopez C, Woessner S. Acute erythroid neoplastic proliferations. A biological study based on 62 patients. Haematologica 2002;87:148–53.
5. Forés R, Alcocer M, Cabrera R, Sanjuán I, Briz M, Lago C, Fernández MN. Detection of PNH clones using flow cytometry in aplastic anemia and paroxysmal nocturnal hemoglobinuria. Sangre 1999;44:199–203.
6. Galassi N, Canalejo K, Riera N, Bengió R, Aixalá M. Flow cytometric analysis of an expansion paroxysmal nocturnal hemoglobinuria (PNH) clone in a patient with bone marrow failure. Am J Hematol 2001;67:277–8.
7. Ham TH, Dingle JH. Studies on destruction of red blood cells. II. Chronic hemolytic anemia with paroxysmal nocturnal hemoglobinuria: certain immunological aspects of the hemolytic mechanism with special reference to serum complement. J Clin Invest 1939;18:657–72.
8. Hartmann R, Jenkins D, Arnold A. Diagnostic specificity of sucrose hemolysis test for paroxysmal nocturnal haemoglobinuria. Blood 1970;35:462–75.
9. Helley D, Peffault de Latour R, Porcher R, Arrais Rodrigues C, Galy-Fauroux I, Matheron J, Schved J, Fischer A, Socié G. Evaluation of hemostasis and endothelial function in patients with paroxysmal nocturnal hemoglobinuria receiving eculizumab. Haematologica 2010;95:574–81.
10. Hernandez-Campo P, Almeida J, Sanchez M, Malvezzi M, Orfao A. Normal patterns of expression of glycosylphosphtidylinositol-anchored proteins on different subsets of peripheral blood: a frame of reference for the diagnosis of paroxysmal nocturnal hemoglobinuria. ytometry B Clin Cytom 2006;70:71–81.
11. Hall SE, Rosse WF. The use of monoclonal antibodies and flow cytometry in the diagnosis of paroxysmal nocturnal hemoglobinuria. Blood 1996;87:5332–40.
12. Hill A, Rother RP, Arnold I, Kelly R, Cullen MJ, Richards SJ, Hillmen P. Eculizumab prevents intravascular hemolysis in patients with paroxysmal nocturnal hemoglobinuria and unmasks low-level extravascular hemolysis occurring through C3 opsonization. Haematologica 2010;95:567–73.
13. Jankowska AM, Szpurka H, Calabro M, Mohan S, Schade A, Clemente M, Silverstein R, Maciejewski J. Loss of expression of neutrophil proteinase-3: a factor contributing to thrombotic risk in paroxysmal nocturnal hemoglobinuria. Haematologica 2011;96:954–62.
14. Kaplow I. Cytochemistry of leukocyte alkaline phosphatase. Am J Clin Pathol 1963;39:439.
15. Krauss JS. Laboratory diagnosis of paroxysmal nocturnal hemoglobinuria. Ann Clin Lab Sci 2003;33:401–6.
16. Lesesve JF, Crepin O, Siest JP, Régnier F, Zeltner S. Evaluation of schistocytes measurement guidelines]. Annales de biologie clinique (Paris) 2012;70:605–13.
17. Lindorfer MA, Pawluczkowycz AW, Peek EM, Hickman K, Taylor RP, Parker CJ. A novel approach to preventing the hemolysis of paroxysmal nocturnal hemoglobinuria: both complement-mediated cytolysis and C3 deposition are blocked by a monoclonal antibody specific for the alternative pathway of complement. Blood 2010;115:2283–391.
18. Maciejewski JP, Rivera C, Kook H, Dunn D, Young NS. Relationship between bone marrow failure syndromes and the presence of glycophosphatidyl inositol-anchored protein-deficient clones. Br J Haematol 2001;115:1015–22.
19. Meletis J, Terpos E, Samarkos M, Meletis C, Apostolidou E, Komninaka V, Korovesis K, Mavrogianni D, Boutsis D, Variami E, Viniou N, Konstantopoulos K, Loukopoulos D. Detection of CD55- and/or CD59-deficient red cell populations in patients with lymphoproliferative syndromes. Hematol J 2001;2:33–7.
20. Meletis J, Terpos E, Samarkos M, Meletis C, Apostolidou E, Komninaka V, Anargyrou K, Korovesis K, Mavrogianni D, Variami E, Viniou N, Konstantopoulos K. Red cells with paroxysmal nocturnal hemoglobinuria-phenotype in patients with acute leukemia. Hematology 2002;7:69–74.
21. Milanés Roldán MT, Fernández Delgado N, Fundora Sarraff T, Jaime Facundo JC, Hernández Ramírez P. Hemoglobinuria paroxística nocturna. Revista Cubana de Hematología, Inmunología y Hemoterapia 2003;19:1. [online],0-0.ISSN1561-2996.
22. Naithani R, Mahapatra M, Dutta P, Kumar R, Pati HP, Choudhry VP. Paroxysmal nocturnal hemoglobinuria in childhood and adolescence–a retrospective analysis of 18 cases. Indian J Pediatr 2008;75:575–8.
23. Naseem S, Varma N, Trehan A. Primary/de novo paroxysmal nocturnal hemoglobinuria in a child from north India: a case report with review of literature. J Pediatr Hematol Oncol 2009;31:274–6.
24. Parker C, Omine M, Richards S, Nishimura J, Bessler M, Ware R, Hillmen P, Luzzatto L, Young N, Kinoshita T, Rosse W, Socié G. Diagnosis and management of paroxysmal nocturnal hemoglobinuria. Blood 2005;106:3699–709.
25. Qin X, Hu W, Song W, Blair P, Wu G, Hu X, Song Y, Bauer S, Feelisch M, Leopold JA, Loscalzo J, Halperin JA. Balancing role of nitric oxide in complement-mediated activation of platelets from mCd59a and mCd59b double-knockout mice. Am J Hematol 2009;84:221–7.
26. Richards SJ, Hillmen P. Immunophenotypic analysis of PNH. Current Protocols in Cytometry, 2002; Vol. 1. 6.11.1–16.
27. Riera N, Canalejo K, Aixalá M, Rosso M, Gaddi E, de Bracco MM, Galassi N. Detection of CD16 low Neutrophil Subpopulations. Cytometry B Clin Cytom 2003;51B:45–6.
28. Riera NE, Saltó MR, Canalejo K, Felippo ME, Arrossagaray G, Aixalá M, de Bracco MM. Neutrophils without CD16b receptors. Medicina (B Aires) 2009;69:442–6.
29. Riera NE, Rosso Saltó M, Galán V, Canalejo K, Khoury M, Aixalá M, Kantor GL, Vermeulen M, Bengió R, de Bracco MM. Anti-polymorphonuclear neutrophil antibodies in patients with leukopenia or neutropenia. Int J Lab Hematol 2010;32:e96–105.
30. Rosse WF. New insights into paroxysmal nocturnal hemoglobinuria. Curr Opin Hematol 2001;8:61–7.
31. Ruiz-Delgado GJ, Vázquez-Garza E, Méndez-Ramírez N, Gómez-Almaguer D. Abnormalities in the expression of CD55 and CD59 surface molecules on peripheral blood cells are not specific to paroxysmal nocturnal hemoglobinuria. Hematology 2009;14:33–7.
32. Smith LJ. Paroxysmal nocturnal hemoglobinuria. Clin Lab Sci 2004;17:172–7.
33. Van den Heuvel-Eibrink MM. Paroxysmal nocturnal hemoglobinuria in children. Pediatr Drugs 2007;9:11–6.
34. Wanachiwanawin W, Siripanyaphinyo U, Piyawattanasakul N, Kinoshita T. A cohort study of the nature of paroxysmal noctur-

nal hemoglobinuria clones and PIG-A mutations in patients with aplastic anemia. Eur J Haematol 2006;76:502–9.

35. Young N, Meyers G, Schrezenmeier H, Hillmen P, Hill A. The management of paroxysmal nocturnal hemoglobinuria: recent advances in diagnosis and treatment and new hope for patients. Semin Hematol 2009;46(1 Suppl 1):S1–16.
36. Zini G, d'Onofrio G, Briggs C, Erber W, Jou JM, Lee SH, McFadden S, Vives-Corrons JL, Yutaka N, Lesesve JF; International Council for Standardization in Haematology (ICSH) ICSH recommendations for identification, diagnostic value, and quantitation of schistocytes. Int J Lab Hematol 2012;34:107–16.